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Correlation Between [^{3}H]Thymidine and Proliferating Cell Nuclear Antigen (PCNA)/Cyclin Indices in Archival, Formaldehyde-fixed Human Colorectal Tissues

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Sections were obtained from archival colorectal tissue samples preserved in paraffin since 1974, after an *in vitro* incubation with [^{3}H]thymidine and fixation in formaldehyde. These sections were submitted to immunohistochemical staining with the 19A2 monoclonal antibody against proliferating cell nuclear antigen (PCNA) (i.e. the PCNA identified as the auxiliary protein to DNA polymerase δ), followed by autoradiography. Analysis of this double-labelled material revealed an excess of PCNA-labelled over ^{3}H-labelled nuclei, as expected from our previous studies with this fixative. On the other hand, PCNA positive nuclei showed the same overall topographical distribution as the [^{3}H]thymidine-labelled ones, eventually revealing the same heterogeneity or abnormality in the spatial distribution of proliferative cells. Finally, there was a highly significant correlation ($r = 0.898$; $P < 0.0001$) between the [^{3}H]thymidine labelling index (TLI) and the proportion of PCNA-positive nuclei ($PCNA_F$-LI). PCNA immunostaining after formaldehyde fixation thus appears as a valid approach for mapping the proliferative compartment and demonstrating tumour heterogeneity or abnormalities in the distribution of proliferative cells. The excellent correlation between the $PCNA_F$-LI and the TLI also makes PCNA immunostaining a simple tool for retrospective or prospective studies on pathological material aimed at evaluating the potential relevance of proliferative indices to clinical prognosis or prediction of cancer risk.
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INTRODUCTION

ALTERATION OF the cell cycle parameters contributes to the loss of steady-state growth equilibrium of a tissue and to the histogenesis of benign and malignant pathological lesions. It was shown that the [^{3}H]thymidine labelling index (TLI) was higher and the duration of S-phase longer in colorectal tumours than in the normal mucosa of patients without gastrointestinal disease [1, 2]. Intermediate values for these parameters were obtained in the histologically normal mucosa next to a cancer, as well as in the precancerous mucosa of patients with inflammatory bowel disease [2]. On the other hand there are indications that cell kinetic parameters, especially the S-phase fraction, might have prognostic significance in colon cancer [2–7]. Measurement of the TLI using [^{3}H]thymidine and autoradiography is limited by ethical constraints and, like immunostaining of incorporated bromodeoxyuridine and (BrdU) requires prior administration (*in vivo* or *in vitro*) of a DNA precursor, which might pose technical problems, so that substitutes to these methods would be welcome. This prompted a series of studies on endogenous proliferation-associated markers, the most widely used being the Ki-67 antigen [8]. The present work deals with another potentially useful cell cycle marker, namely the proliferating cell nuclear antigen (PCNA/cyclin).

PCNA is a highly conserved protein that, as auxiliary protein of DNA polymerase δ, is associated to DNA replication [9, 10, review in ref. 11]. Two populations of PCNA molecules exist in the nucleus; both are preserved by formaldehyde fixation but only one—which represents PCNA tightly associated with DNA replication sites—appears to be maintained in cells or in animal tissues after methanol fixation [9, 11]. In the present work we used archival paraffin-embedded samples from colorectal mucosa that had been incubated with [^{3}H]thymidine prior to formaldehyde fixation and histological processing. We compared the topography and incidence of ^{3}H-labelled and/or PCNA positive cells in single sections by immunostaining with a monoclonal antibody [12, 13] and subsequent autoradiography. The data show a highly significant correlation between the proportion of PCNA positive nuclei and TLI.

MATERIALS AND METHODS

Preparation of the tissue sections for immunocytochemistry

The material used in this study was obtained in our previously reported work in which tissue slices from biopsy specimens had been incubated *in vitro* with [^{3}H]thymidine, fixed in neutral formaldehyde and embedded in paraffin as described previously [1, 2].

The biopsy samples were taken from the tumour and the adjacent normal mucosa of patients with colon ($n = 4$) or rectal ($n = 1$) cancer, and from the mucosa of 1 patient with Crohn's disease and another one presenting with ulcerative colitis.

Sections 5–6 μm thick were prepared from the corresponding archival paraffin blocks; they were dried at 37°C and stored at

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20°C up to the time of immunocytochemical processing. Before the latter, they were deparaffinised, rehydrated and treated for 1 h with 3% H_2O_2 in absolute methanol, in order to block endogenous peroxidase activity. The slides were then processed through the following steps: 2 min wash in running tap water; rinse in distilled water; 15 min immersion in 0.05 mol/l NH_4Cl in phosphate-buffered saline (PBS) at pH 7.2; 2 min in tap water; rinse in distilled water then in PBS; 30 min incubation at 20°C in 2 N HCl followed by washing in two successive baths (3 min each) 0.1 mol/l borax at pH 8.5.

All the slides were then incubated for 10 min in 0.5% Tween 20 in PBS, 2 × 10 min in PBS containing 0.1% bovine serum albumin (BSA), and 30 min in PBS–BSA, supplemented with normal sheep serum at a 1:20 final dilution, before being washed with PBS–BSA (2 × 10 min).

Immunocytochemical staining of PCNA

We used the 19A2-IgM mouse monoclonal antibody against purified PCNA from rabbit thymus, isolated and characterised by Ogata *et al.* [12, 13]. This was purchased in ascites fluid form from American Biotech Inc. (Plantation, Florida, 33313, U.S.A.). The ascites fluid was diluted 1:50 in reconstituted defatted milk, (i.e., a 10% solution of powdered milk from Gloria-Nestlé) in PBS–BSA. Previous experiments showed that this markedly reduces non specific binding of the antibody. Contact with the primary antibody was maintained for 18 h at 4°C. After two washes of 10 min each in PBS–BSA and one in Tris–BSA, pH 7.6, binding of the PCNA monoclonal antibody was detected by indirect staining with streptavidin–biotin–peroxidase (SBP) preformed complex (Amersham), bridged to the immune complex by a biotinylated, affinity purified sheep antibody to mouse Ig, (from Amersham). Exposure to this secondary antibody (diluted 1:150) was maintained for 2 h and was followed by a 30 min incubation with a 1:250 dilution of the SBP complex. Peroxidase activity was finally revealed using 0.06% 3-3′ diaminobenzidine (DAB) −0.01% H_2O_2 in Tris-buffered saline pH 7.6, as the substrate and a reaction time of 3 min.

Before autoradiography, the immunohistological preparations were rinsed for at least 4 h in running tap water (a precaution needed to reduce photographic background engendered by DAB products), then dipped in Ilford K5 emulsion diluted with one volume of distilled water. Exposure time was 15 days. Dektol Kodak was used for development of the autoradiographs, which were fixed with sodium thiosulphate, washed for 1 h in running tap water. Light (15 s of contact) counterstaining with Mayer's haematoxylin was then performed. The preparations were then dehydrated and mounted in DPX-mounting medium (Gurr).

Based on background labelling, a nucleus was scorable as [^{3}H]thymidine-labelled when presenting more than three silver grains. The PCNA labelling index ($PCNA_F$-LI) and TLI, were established in double-labelled regions of the tissue sections, scoring at least 200 epithelial cells in the crypt proliferative compartment (defined as the portion of the crypt including all mitotic or ^{3}H-labelled cells) of well orientated crypts, or, for tumors, all epithelial cells seen in the observed zone.

RESULTS

As previously reported [1, 2], [^{3}H]thymidine does not label the whole thickness of the incubated tissue fragments. Therefore, scoring for PCNA staining was restricted to the regions of the sections exhibiting marked ^{3}H-labelling, which corresponded to the first three crypts located near the edges of the section or, for tumours, up to a depth of about four to five cell diameters, starting from the edge of the section.

Immunostaining was readily identified over nuclei. In some preparations a faint background staining of the intercellular space was observed, but this always left the definition of the nuclear positivity easy to ascertain. Some cells in interphase or mitosis, also expressed cytoplasmic staining. This was more frequently observed near the edges of the tissue specimen, where the analysis was restricted to allow comparison with [^{3}H]thymidine labelling. It could correspond with free (and hence, more labile) PCNA, maintained only in regions of the sample that were rapidly reached by the fixative. However, only nuclear labelling was taken into account in determination of the $PCNA_F$-LI. Nuclei presenting [^{3}H]thymidine labelling also exhibited PCNA positivity with very few exceptions. By contrast, a variable proportion of nuclei clearly showed single PCNA positivity (Fig. 1). Selecting the autoradiographically positive regions defined above, the number of silver grains over labelled nuclei was at least 15–20 (i.e. > back-ground level). We scored all the cells in these zones and classified them as unlabelled or labelled with either ^{3}H alone, PCNA alone, or with both labels. This showed that the $PCNA_F$-LI exceeded the TLI, by about 27% on average, the ratio between the two indices varying between 1.02 and 1.7.

The mean value and the range of individual values for the TLI and the $PCNA_F$-LI were, respectively, as follows: 21.5% (range 6.0–39.0) and 29.9% (range 8.0–55.7) for tumours; 23.8% (range 19.9–29.3) and 26.4 (range 24.3–30) for adjacent apparently normal mucosa; 24.5% (range 19.9–29.5) and 31.5% (range 21–42) for inflammatory bowel disease (IBD). This indicates fair agreement between the two indices as criteria of the relative rate of proliferative activity.

On the other hand, an expansion of the proliferative compartment from the lower two thirds to the upper third of the crypt was observed with both labels in patients with IBD (Fig. 2). Similarly, in 2 patients, the tumour showed two zones with markedly different proliferative activities, which were revealed by the radioautographic labelling as well as by the PCNA immunopositivity (Table 1, cases 138a and 140b). Since we are dealing here with double-labelled tissue sections, one may safely

Fig. 1. Autoradiographic detection of [^{3}H]thymidine incorporation and immunocytochemical demonstration of nuclear PCNA in a colon adenocarcinoma (case n°132). Light counterstaining with haematoxylin was performed after autoradiography. Note superimposition of the two labels in most but not all nuclei, some PCNA positive nuclei being devoid of photographic silver grains. (The arrow point to such a nucleus.)

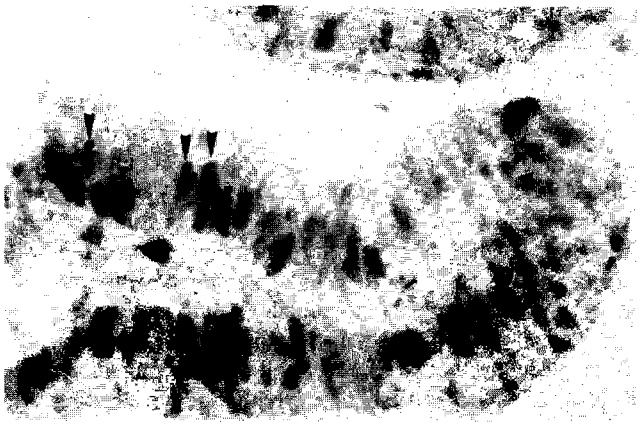

Fig. 2. Immunocytochemical demonstration of nuclear PCNA in a section of the uninvolved colon mucosa of a patient with a colon cancer (case n° 134). Note extension of the proliferative compartment towards the surface, as detected by PCNA and autoradiographic labelling. Most of the labelled nuclei are double-labelled. (Arrows point to single PCNA labelling.)

*Table 1. PCNA index ($PCNA_F$-LI) and [^{3}H]thymidine index (TLI) in double-labelled human colorectal mucosa**

Patient Sample no.	Anapathology	a TLI %	b $PCNA_F$-LI %	b/a
A				
132	Adenocarcinoma	39.0	55.7	1.43
133	Normal	24.5	26.5	1.08
B				
134	Adenocarcinoma	25.6	27.2	1.06
135	Normal	19.9	26.8	1.35
C				
137	Crohn's disease	19.5	21.0	1.07
D				
138a	Adenocarcinoma	10.5	14.5	1.40
138b	Adenocarcinoma	26.5	45.0	1.70
139	Normal	22.0	24.3	1.10
E				
140a	Adenocarcinoma	22.0	27.0	1.22
140b	Adenocarcinoma	6.0	8.0	1.33
141	Normal	23.5	24.5	1.04
F				
143	Ulcerative colitis	29.5	42.0	1.42
G				
144	Adenocarcinoma	21.0	32.0	1.52
145	Normal	29.3	30.0	1.02

* Sections were successively submitted to immunostaining and autoradiography, scoring of the two labels thus being performed on a same, double-labelled cell population.

conclude that [^{3}H]thymidine labelling and PCNA positivity have the same ability to map topographical distribution of the proliferative cells in these tissues. This agrees with our previous observations on various animal tissues [11, 20].

Finally, as shown in Fig. 3, analysis of the set of available data revealed there was a good correlation ($r = 0.898$; $P < 0.0001$) between the TLI and the $PCNA_F$-LI.

DISCUSSION

It was suggested that in colorectal cancer a high LI and aneuploidy are associated with a poor disease-free or overall survival [3, 7]. In mucosa of patients at risk of malignant transformation (i.e. normal mucosa adjacent to a cancer or inflammatory mucosa) higher than normal TLI and extension of the proliferative compartment towards the surface of the crypt were suggested to be indicative of increased risk or even to represent a first step in the development of malignancy (see reviews in refs 2–6, 14–16). Whereas flow cytometry can quantify the DNA content of isolated cells or nuclei and thus provide useful information regarding ploidy and overall proliferative activity [3–5, 17–19], it gives no clue as to the proliferative heterogeneity. With [^{3}H]thymidine autoradiography or BrdU immunostaining, this type of information can be obtained together with indications about proliferation rate. However, the use of those labels is cumbersome, as they imply administration of a precursor or an *in vitro* labelling step. This explains the interest for intrinsic proliferative indicators. We show here that in archival tissues samples, fixed in formaldehyde and embedded in paraffin, there is a good correlation between the PCNA and the [^{3}H]thymidine indices. Our data show that there was a systematic excess of PCNA positivity over autoradiographic positivity, thus indicating that after formaldehyde fixation PCNA immunostaining labels more than the S phase compartment. This agrees with similar observations made on tissue sections from different organs of the mouse or of the rat [11, 20] and in cells in culture [9]. This has been interpreted as corresponding to the existence of two populations of PCNA molecules [11, 20]. The first one, detectable in S and G_1 cells, but also in quiescent cells, is nucleoplasmic and requires formaldehyde fixation to be preserved. The second one is tightly associated with DNA replication sites, which in part might explain how it resists detergent or high-salt extraction and is preserved after methanol fixation.

Our data show, in the absence of direct evaluation of tissue

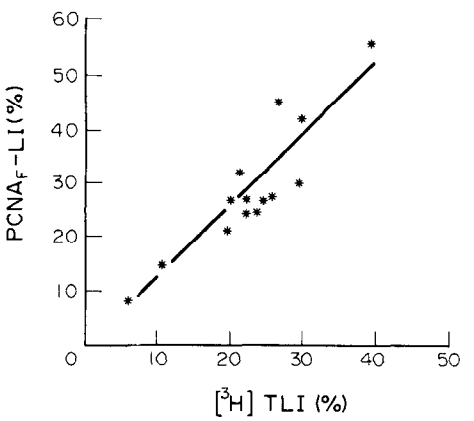

Fig. 3. Correlation ($r = 0.898$; $P < 0.0001$) between $PCNA_F$-LI and TLI measured in double-labelled tissue sections from formaldehyde-fixed archived samples from human colon mucosa.

turnover rate, PCNA immunostaining of formaldehyde fixed tissue affords an index (termed the $PCNA_F$-LI to insist on the importance of the fixation conditions) that likely correlates with it, as it is correlated with the S-phase index. Furthermore in this respect, a good correlation was shown between the $PCNA_F$-LI and the Ki-67 index (measured on cryostat sections), in malignant lymphomas [20].

Moreover, immunopositivity revealed the same topographical distribution of cycling cells (including abnormalities in this respect, incidentally observed in samples from the mucosa adjacent to cancers or from patients with ulcerative colitis or Crohn's disease) as detected by [^{3}H]thymidine labelling.

Our conclusions should be extrapolated with caution to other materials and/or methodological approaches and would certainly need to be verified if an alternative anti-PCNA antibody was used. Fixation conditions as well as pretreatments performed prior to the immunostaining, were shown to influence the intensity and distribution of the nuclear PCNA positivity [9, 11, 22–26]. The choice of the PCNA antibody is also a determinant [12, 24]. For example, using the PC10 antibody, in an otherwise identical assay, results in more intense nuclear staining than obtained with the 19A2 antibody, in human cell lines [24] as well as in animal or human tissues (our unpublished observations). There are also differences between tissues in the reactivity to certain anti-PCNA antibodies [9, 11, 22]. Nuclear staining intensity with a given antibody also seems to be less intense in non-S-phase than in S-phase cells under identical immunocytochemical conditions [25, 26]. All these considerations might explain the discrepancies between reports from different laboratories, such as for example concerning the presence or absence of a high incidence of PCNA positive nuclei in normal tissue adjacent to tumours [22], or the degree of superimposition of the [^{3}H]thymidine and PCNA labelling. This underlines the necessity of always referring to fixation and antibody in defining a PCNA labelling index.

In conclusion, PCNA immunostaining of formaldehyde fixed tissues appears useful in identifying and mapping the proliferative compartment, as well as detecting heterogeneities in the distribution of proliferating cells within tumours. This therefore offers the possibility of using archived unlabelled biopsy material for retrospective studies to rapidly estimate, from the $PCNA_F$-LI, the potential prognostic relevance of the S-phase index. The latter would then preferably be directly measured in prospective studies, by using PCNA immunostaining of methanol fixed tissues as a substitute to [^{3}H]thymidine labelling [11, 25].

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