**ABSTRACT**

A proliferating cell nuclear antigen (PCNA) was identified with autoantibodies from a patient with systemic lupus erythematosus. Specific antibodies were purified by affinity chromatography in which Novikoff hepatoma nucleolar proteins were conjugated to Sepharose-4B. The purified anti-PCNA antibodies produced bright nucleolar fluorescence in tumor cells as shown by indirect immunofluorescence. PCNA was found in nucleoli of human cell lines, HeLa, Hep-2, and Namalwa, and a solid human renal and a prostate carcinoma. Both strong and weak nucleolar fluorescence areas were found in the renal and prostate carcinoma indicating that there are varying degrees of proliferation among tumor cells. Two human colon carcinoma cell lines, Ω (an aggressive, fast-growing clone of human colon carcinoma cell line HCT 116) and CBS (a slow-growing human colon carcinoma cell line [group 3]), with different growth rates were compared. The fast-growing colon carcinoma cells, Ω, exhibited a higher percentage of nucleolar fluorescence (28.5%) than that of the slow growing colon cells (13.6%). By enzyme-linked immunosorbent assays, the Ω cell extract had a higher PCNA antigen content (2.8-fold) than that of the CBS cell extract which, in turn, was higher than that of human liver extract. PCNA was also found in a human fetal lung fibroblast cell line (IMR-90). Very weak or negative nucleolar fluorescence was observed in several normal human tissues including liver, kidney, prostate, and cheek cells. Nucleolar fluorescence was also observed in rat Novikoff hepatoma cells. Although normal rat livers do not have PCNA nucleolar fluorescence, nuclear and nucleolar fluorescence were observed at 18 hr after partial heptectomy.

**INTRODUCTION**

Sera from patients of systemic rheumatic disease containing autoantibodies with high titer and specificity to cellular molecules may have significant diagnostic value to the clinician (22). Using sera from patients with SLE, Miyachi et al. (17) and Takasaki et al. (21) found a PCNA enriched in nucleoli during G1-S phase of the cell cycle. This paper reports a study of PCNA in human tumor and normal tissues. Results of this study point to the possibility that immunofluorescence assay of proliferative associated proteins in tumor cells may provide another means to study tumor growth and tumor heterogeneity.

**MATERIALS AND METHODS**

**Sera and Tissues.** PCNA serum was obtained from a patient [E. B.] with SLE by Dr. Eng Tan. The antiserum reacted with PCNA and Sm antigen (17, 21). The IgG fraction was purified by a DEAE-Affi-Gel blue column (Bio-Rad Laboratories Bulletin 1062). Antiserum to human tumor nucleolar antigens was raised in rabbits immunized with HeLa nucleoli (5, 12). Tissues were obtained either from postmortem or surgical samples. The steps for obtaining and analyzing samples were approved by the Human Research Committee of Baylor College of Medicine and affiliated hospitals. Holzman rats (male, 180 to 200 g) were partially hepatectomized (75%). After 18 hr, regenerating liver was removed, and thin sections (3 to 4 µm) were cut.

**Protein Extraction.** Novikoff hepatoma nucleoli were isolated as described previously (6, 19) and extracted with 3 M NaCl:1 mM phenylmethylsulfonyl fluoride:0.1 mM leupeptin (19). The extract was recovered after centrifugation (100,000 × g for 16 hr).

**Purification of Antibodies.** Nucleolar proteins were conjugated to CNBr-Sepharose 4B which was then washed with 8.5 mM NaH2PO4:1.6 mM Na2HPO4:0.145 M NaCl (pH 7.2):0.05% Tween 20 (PBS:Tween buffer) before use. The crude IgG was applied onto the affinity column and tumbled end-over-end for 18 hr at 4°. Approximately 90% of the IgG was washed from the column with PBS:Tween buffer, and specific IgG was eluted with 4 M MgCl2.

**Immunofluorescence.** Tissue sections and cell smears were fixed with acetone for 11 min at 4°. Immunofluorescence was analyzed as described earlier (7). For double labeling immunofluorescence, 10 µg each of human anti-PCNA antibodies and rabbit anti-HeLa nucleolar antibodies were incubated with the tissue slide at 37° for 60 min followed by 10° for 16 hr. After the unbound antibodies were washed off, a mixture of FITC-conjugated goat anti-human IgG and Rhodamine-conjugated goat anti-rabbit IgG was added and incubated for 60 min at 37°. After the unbound antibodies were washed off, the slide was mounted with glycerin PBS (1:1 v/v), pH 9.5, and observed with a fluorescence microscope.

**Synchronization of HeLa Cells.** HeLa cells were synchronized with double thymidine method (1). HeLa cells (100 ml; 0.4 x 10⁶/ml) in minimum essential medium (Eagle's) were blocked with 3 mM thymidine for 16 hr. The cells were then incubated in fresh medium without thymidine and grown for 8 hr. They were blocked again for 16 hr. After the block was released, aliquots of cells were taken out for assays at different time points (every 2 hr).

For [3H]thymidine uptake determination, 1 ml of HeLa cells (duplicate) was added with 1 µCi of [3H]thymidine and incubated at 37° for 2 hr. Cells were collected on a Millipore membrane (0.45 µm), washed with cold PBS, and precipitated with 1 ml of cold 5% trichloracetic acid (10 min). They were then washed with cold 5% trichloracetic acid and ethanol. After they were solubilized in ethyleneglycol monoethyl ether, the radioactivity in the membrane was counted with a scintillation counter.

The mitotic index calculation was done by the method of Puck and Steffen (18) with slight modifications. One ml of culture cells was incubated with colcemide (0.25 µg/ml) at 37° for 2 hr before the fixation and
staining. The mitotic index was determined by counting until 1000 cells had been scored.

For immunofluorescence assay, 1 ml of cells (5 × 10⁵/ml) was washed 3 times with PBS. The cells were suspended in 100 μl of PBS. About 5 μl of the cell suspension were spread on a glass slide (1 sq cm) and air dried (30 min). The slides were then fixed and immunostained as described earlier (7). The percentage of cells with positive nucleolar fluorescence was determined by scoring 500 cells.

Li. Two human colon carcinoma cells, Ω (3) and CBS (2), were grown on slides with McCoy’s Medium 5A supplemented with 10% fetal calf serum. Cells were labeled in medium with 1 μCi [³H]thymidine per ml for 60 min. Cells were washed with cold PBS twice, air dried, and fixed with cold methanol/acetic acid (3:1) for 10 min. The slide was dipped in ilford L4 emulsion (1:1, diluted with water) and exposed for 1 week. It was developed in Kodak D-19, fixed with Kodak fixer, and stained with Giemsa stain.

ELISA. Human colon carcinoma cells, Ω, CBS, and human liver, were suspended in PBS andsonicated until nucleoli were not observed. The cell extract was recovered in the supernatant after centrifugation at 27,000 × g for 30 min. The quantity of PCNA in these cell extracts was determined by ELISA assay described by Kelsey et al. (14).

RESULTS

The IgG fraction of the serum was used to identify PCNA in various cell types. This serum reacted with both PCNA and Sm antigen which is in nucleoplasm and outside the nucleoli. The nucleolus-specific PCNA antibodies were purified by affinity chromatography. Approximately 5% of the input IgG was bound to the Novikoff nucleolar protein column and eluted with 4 M MgCl₂. The crude IgG gave both nucleoplasmic and nucleolar fluorescence in HeLa cells (Fig. 1A). With the purified antibodies, bright nucleolar fluorescence was retained, but the extranucleolar fluorescence was mostly removed (Fig. 1B).

With the purified anti-PCNA antibodies, nucleolar fluorescence was also found in other tumor cell lines including HeP-2 (Fig. 1C), Namalwa (Fig. 1D), and Novikoff hepatoma (Fig. 1E). It was also found in nucleoli of IMR-90 cells (Fig. 1F), a fetal lung fibroblast line.

Two solid tumor tissues also examined were a renal carcinoma and a prostate carcinoma. Fig. 1G shows the PCNA in renal carcinoma. Bright nucleolar fluorescence was observed in most of the cells. At higher magnification (Fig. 1H), localized nucleolar fluorescence with relatively less nuclear fluorescence was detected. Cytoplasmic fluorescence was observed in many of the tissue sections studied. This fluorescence was also observed with normal human IgG. In most areas of the prostate carcinoma section, nucleolar fluorescence was found (Fig. 1I).

Nucleolar fluorescence was not observed with anti-PCNA antibodies in normal human liver (2 samples), kidney (2 samples), prostate (one sample), or cheek cells (one sample) (Fig. 2, A to D, respectively). In one liver sample, weak nucleolar fluorescence was observed.

When rats were partially hepatectomized to study the induction of PCNA in regenerating liver, bright nuclear and some nucleolar fluorescence (27 ± 17%; S.D.) was observed at 18 hr after partial hepatectomy (Fig. 2, E and F). This fluorescence was not observed in normal rat liver (Fig. 2G).

To demonstrate the varying amount of PCNA in tumor cells, a double labeling experiment was performed. Sections from the same renal or prostate carcinoma were stained with PCNA and human tumor nucleolar antibodies. As shown in Fig. 3 (A and B for renal carcinoma; E and F for prostate carcinoma), most of the tumor section had both FITC and Rhodamine fluorescence in nucleoli, suggesting dual localization of PCNA and human tumor nucleolar antigen in the nucleoli. However, in certain areas of the same section, nucleolar Rhodamine fluorescence was bright, but the FITC fluorescence in nucleoli was weak or absent, which suggests that the amounts of PCNA were small in those nucleoli (Fig. 3: C and D, renal carcinoma; G and H, prostate carcinoma). With the PCNA antibodies, some cytoplasmic and intercellular fluorescence was observed in the human tissue sections studied. Such fluorescence was also observed with normal human IgG or the goat anti-human IgG alone, which indicates the presence of residual human IgG in these tissue sections.

Two human colon carcinoma cell lines, Ω and CBS (2, 3), with different growth rates were investigated (Table 1). The fast-growing line, Ω, had a shorter doubling time (13.9 hr) and Li (46.5%) than did the slow-growing line CBS (doubling time, 21.5 hr; Li = 17.5%). When immunostained with both HeLa nucleolar antibodies and PCNA antibodies, both antibodies stained the nucleoli of Ω cells equally well (Fig. 4, A and B). In CBS cells, most nucleoli were stained with HeLa nucleolar antibodies (Fig. 4, ▲) but fewer were stained by the anti-PCNA antibodies (▲).
fluorescence was observed. Antigen-HeLa nucleolar antibodies produced a bright nucleolar fluorescence in most tumors studied (Fig. 1). However, residual network and speckled nuclear fluorescence was still observed, especially in the regenerating rat liver. In a previous study of Wil-2 cells (17, 21), nucleoplasmic PCNA (speckled fluorescence) migrated to nucleoli during G1-S phase. It is possible that the residual network nuclear fluorescence represents a nuclear PCNA available for transportation into the nucleoli. It is not clear whether the nuclear fluorescence results from the same molecule as those in the nucleoli or different molecules which share common epitopes. Although only one precipitin line was observed in Ouchterlony immunodiffusion assays (17, 21), it is important to determine the molecular species which account for the immunofluorescence in different tissues.

Although the mechanisms that lead to antibody production against the cell nucleus and its relation to rheumatic diseases are not understood (22), sera from patients with rheumatic diseases may become useful in analysis of tumor heterogeneity and tumor growth. Meyer and Hixon (16) and Tubiana et al. (23) found that LI provides a significant prognostic information on breast cancer. Hoshino and Wilson (13) also reported a poor prognosis for patient of glioma with labeling index of more than 5%. As pointed out by Meyer (15), pulse-labeling studies with [3H]-thymidine of human tumor cells have been tedious, and this hampers the studies on tumor growth and cell kinetics. Studies on PCNA localization in nucleoli may be an alternative to evaluate the presence and number of proliferating tumor cells.

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REFERENCES


DISCUSSION

PCNA was originally found by Tan et al. (22) with serum from patients with SLE. It was related to cell proliferation as demonstrated in mitogen-stimulated normal lymphocytes and was localized in nucleoli of Wil-2 cell line during the G1-S phase of the cell cycle (17, 21).

The present study was designed to evaluate: (a) the presence of PCNA in human tumor and normal tissues; and (b) the relationship between PCNA and tumor growth. Using PCNA antibodies and fluorescein-conjugated goat anti-human IgG, nucleolar fluorescence was observed in most tumors studied (Fig. 1). PCNA was localized in nucleoli throughout the cell cycle except during G2 and M phase (Chart 2). Over 90% of the HeLa cells contain PCNA in nucleoli during late G2 and early S phase. This is in good agreement with the previous study on Wil-2 and stimulated normal lymphocytes (17, 21).

Anti-HeLa nucleolar antibodies produce bright nucleolar fluorescence in a broad range of tumor tissues (4–12). Two antigens, 68/6.3 and 54/6.3 (molecular weight × 10−3) the pH value of the isoelectric point), from Namalwa cells, have been purified and characterized (9, 10). These antigens do not distinguish the proliferating tumor cells. A potential value of analysis for PCNA was shown by double labeling studies on tumor sections. The anti-HeLa nucleolar antiserum produced nucleolar fluorescence in almost all tumor cells. Positive and negative areas were identified by the PCNA antibodies, suggesting that they differed in proliferation. Accordingly, detection of PCNA on tumor nucleoli may be useful in identifying actively growing as compared to nonproliferating areas in solid tumors.

In a separate study, lymphoma and leukemia cells were examined (20). Over 80% of the tumor cells contained the tumor nucleolar antigen, but only 20% of tumor cells contained nucleolar PCNA. This result also suggests that only a portion of these tumor cells was proliferating.

The antibodies used in this study were affinity-purified and retained selective binding to nucleoli as shown by immunofluorescence on HeLa cells (Fig. 1B). However, residual network and speckled nuclear fluorescence was still observed, especially in the regenerating rat liver. In a previous study of Wil-2 cells (17, 21), nucleoplasmic PCNA (speckled fluorescence) migrated to nucleoli during G1-S phase. It is possible that the residual network nuclear fluorescence represents a nuclear PCNA available for transportation into the nucleoli. It is not clear whether the nuclear fluorescence results from the same molecule as those in the nucleoli or different molecules which share common epitopes. Although only one precipitin line was observed in Ouchterlony immunodiffusion assays (17, 21), it is important to determine the molecular species which account for the immunofluorescence in different tissues.

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Fig. 2. Immunofluorescence in normal tissues. A, liver. × 400. B, kidney. × 400. C, prostate gland. × 400. D, cheek cell. × 400. E, rat regenerating liver. × 400. F, rat regenerating liver. × 630. G, normal rat liver. × 400.
Fig. 3. Double immunofluorescence with anti-PCNA and anti-HeLa nucleolar antibodies. Renal carcinoma (A to D) and prostate carcinoma (E to H) sections were incubated with human anti-PCNA and rabbit anti-HeLa nucleolar antibodies. Second antibodies are a mixture of FITC-conjugated goat anti-human IgG and rhodamine-conjugated goat anti-rabbit IgG. A and B, C and D, E and F, and G and H are the same area of the section. A, C, E, and G show rhodamine fluorescence. B, D, F, and H. show FITC fluorescence. Most of the tumor section (A and B and E and F) had both FITC and rhodamine fluorescence in nucleoli. In certain areas of the tumor section (C and D and G and H), nucleolar rhodamine fluorescence is intense (C and G), but the FITC fluorescence in nucleoli is weak (D and H) or not noticeable. A to D, ×400; E to H, ×100.
Fig. 4. Comparison of nucleolar fluorescence in cells of different growth rate. Two human colon carcinoma cells with different growth rate, O and CBS, were immunostained with both HeLa nucleolar and PCNA antibodies as described in "Materials and Methods." A, O cells with HeLa nucleolar antibodies; B, O cells with PCNA antibodies. Nucleoli were stained equally well with both antibodies; C, CBS cells with HeLa nucleolar antibodies; D, CBS cells with PCNA antibodies; E, CBS cells phase-contrast picture; D, nucleoli stained with both HeLa nucleolar antibodies and PCNA antibodies. A, nucleoli stained with HeLa nucleolar antibodies but not with PCNA antibodies; F, autoradiogram of O cells for analysis of LI.
Indirect Immunofluorescence Studies of Proliferating Cell Nuclear Antigen in Nucleoli of Human Tumor and Normal Tissues

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