To elucidate the role of proliferating cell nuclear antigen (PCNA) in vivo, PCNA immunohistochemistry combined with 3H-thymidine autoradiography was performed on 2 types of regenerating rat livers. One group of rats underwent 2/3 partial hepatectomy (PH), and the other group 1/3 PH. After 3H-thymidine labeling, the livers were fixed with paraformaldehyde (PFA) and methanol, and paraffin-embedded. In methanol-fixed tissues, the serial changes of the PCNA-positive index (PCNA-PI) corresponded well with those of the 3H-thymidine-labeling index (3H-thymidine-LI) after each PH. In PFA-fixed tissues, however, the PCNA-PI was 26% before PH, when the 3H-thymidine-LI was below 1%. Furthermore, the 1/3 PH group, despite a continued low 3H-thymidine-LI, showed a rise in PCNA-PI to a level comparable to that in the 2/3 PH group. From these results, we propose 3 phases of PCNA expression. In PFA-fixed tissues, PCNA that was detectable before PH was considered pooled in Go-phase nucleoplasm (Phase I), and some or many of the PCNA-positive cells failed to enter the S-phase in the 1/3 PH group, reflecting the increased PCNA not bound to DNA replicons (Phase II). In methanol-fixed tissues, PCNA was detected only in the S-phase, indicating its direct involvement in DNA synthesis (Phase III). Therefore, determining PCNA-PI in methanol-fixed tissues is useful to evaluate proliferative activity, whereas that in PFA-fixed tissues must be assessed with great caution.

Proliferating cell nuclear antigen (PCNA) has been reported to be a useful marker for identifying proliferating cells because it can be determined by immunohistochemical staining on routine formalin-fixed paraffin-embedded sections (7, 8, 11-13, 15, 16). However, this method poses some problems in the assessment of cell proliferation. For instance, there are some reports concerning histologically normal tissues that show markedly high percentages of PCNA-positive cells with poor correlation to other proliferative markers (12, 13). Moreover, two "populations" of PCNA are recognized by immunohistochemistry; one which is tightly bound to DNA replicons and the other which is free in the nucleoplasm (2, 6). Fixation in formaldehyde preserves both populations, whereas methanol fixation preserves only the former (2, 4, 6, 9, 14, 17).

To clarify biological role of PCNA expression, we compared the frequency of immunohistochemically detected PCNA-positive cells with that of autoradiographically determined S-phase cells in the regenerating rat liver, and proposed three functional phases of PCNA expression in relation to DNA synthesis. Using two fixatives (formaldehyde and methanol), we attempted to interpret PCNA expression at each functional phase and to determine its most appropriate clinical application.

MATERIALS AND METHODS

Animal treatment and tissue preparation

Of a total of 52 rats (Wistar, male, 7 weeks of age), 4 were used as controls (sacrificed without hepatectomy). In the remaining rats, 2/3 partial
hepatectomy (PH), consisting of resection of the median and left lobes, was performed in 24 rats, and 1/3 PH (resection of the median lobe alone) in 24 rats. In both PH groups, the rats were divided into 6 groups and sacrificed at 12, 24, 48, 96, 168 and 240 hr following surgery. Each rat was given 37 kBq (1 μCi)/g body weight of 3H-thymidine-6-T; Amersham) intraperitoneally 1 hr before sacrifice. The livers were resected immediately after sacrifice and cut into several pieces. Half were fixed with 4% buffered paraformaldehyde (PFA) and the other half with 100% methanol both at 4°C for 14-20 hr, then dehydrated and paraffin-embedded. The dehydration-embedding time was 17 hr.

**Immunohistochemical procedures**

Four-μm-thick sections were dried at 37°C, rehydrated and treated for 25 min with 0.3% H2O2 in absolute methanol to block endogenous peroxidase activity. After 3 washings with Tris-buffered saline (TBS), the sections were incubated with anti-PCNA antibody (PC10; Novocastra), diluted 1 : 100 in TBS containing 2% fetal bovine serum (FBS) and kept at 4°C overnight. They were then washed 3 times with TBS and incubated with biotinylated anti-mouse IgG (DAKO) diluted 1 : 100 in 2% FBS-TBS for 1 hr at room temperature. After 3 washings with TBS, they were incubated with peroxidase-conjugated streptavidin (DAKO) diluted 1 : 200 in 2% FBS-TBS. Following 3 more washings with TBS, peroxidase activity was revealed using 0.01% 3,3'-diaminobenzidine (DAB)-0.02% H2O2 in Tris-HCl buffer at pH 7.6.

**Autoradiographic procedures**

After immunohistochemical procedures, the histological preparations were washed in running tap water, and dipped in autoradiographic emulsion (NR-M2; Konica) diluted 2 times with distilled water. After exposure for 21 days, the autoradiographs were developed with FD-111 at 23.5°C for 7 min, fixed with sodium thiosulfate, washed in running tap water and counter-stained with Mayer’s hematoxylin.

**Evaluation of PCNA positivity**

The stained sections were examined under 200× magnification. A cell was considered to be PCNA-positive when yellowish-brown staining was recognized in the nucleus, regardless of its staining intensity. The PCNA-positive index (PCNA-PI) and 3H-thymidine labeling index (3H-thymidine-LI) were determined, in histological preparations for PCNA immunostaining combined with 3H-thymidine autoradiography, as the percentage (%, mean value with standard deviation) of PCNA-positive cells and that of 3H-thymidine-labeled cells, respectively, among 1,000 liver cells. Time-course changes in these indices for each fixation were examined.

**RESULTS**

3H-thymidine-LI after PH (Fig. 1-a, b)

3H-thymidine-LI was not affected by the kind of fixatives used and was 0.79±0.81% in control rats. After 2/3 PH, it increased to 26.5±9.1% at 24 hr, followed by a gradual decrease to the control level thereafter. After 1/3 PH, 3H-thymidine-LI reached a plateau of 7-9% at 24 hr, remained near at this level until 96 hr, then returned to the control level thereafter.

PCNA-PI after PH

(1) Tissue samples from methanol fixation

PCNA-PI showed a similar change to the 3H-thymidine-LI after each type of PH (Fig. 1-a). The percentage of double-positive cells (for both PCNA and 3H-thymidine) among total positive cells (for either PCNA or 3H-thymidine) was 80-90% (Fig. 2-a).

(2) Tissue samples from PFA fixation

The PCNA-PI of control rats was 25.8±2.0% (Fig. 1-b). After 2/3 PH, it was 67.5±7.5% at 24 hr. After 1/3 PH, there was a similar increase in PCNA-PI to a maximum of 59.7±9.5% at 24 hr and it remained high (48.5±2.1%) at 96 hr. The percentage of double-positive cells among the total positive cells was less than 40% (Fig. 2-b).

**DISCUSSION**

There have been many studies on PCNA expression in relation to DNA synthesis (2-4, 14, 17, 18). In quiescent cells, PCNA is homogeneously pooled throughout the nucleoplasm (2, 17). Under some proliferative stimulation, there is a 2- to 3-fold increase in PCNA content at the G1/S boundary (3, 17, 18). During the S-phase, 20-30% of PCNA is recruited from the nucleoplasmic pool and becomes tightly bound to DNA replicons, playing an important role as replicative complex (2, 17).

In the present experiment, the PCNA-PI in methanol-fixed tissues generally coincided with the 3H-thymidine-LI before and after both types of PH. These results indicate that PCNA immunostaining of methanol-fixed tissues specifically detects S-phase cells. This is supported by a previous study that
methanol can fix the population of PCNA which is tightly bound to DNA replicons during the S-phase (2). However, in a small number of cells, we found a discrepancy between PCNA positivity and $^{3}$H-thymidine-labeling. According to the speculation of Galand and Degraef (6), this may be attributed to the time interval between $^{3}$H-thymidine injection and tissue sampling, because some cells can enter or leave S-phase during that interval.

Most of the liver cells from control rats are con-
sidered to be in the G<sub>0</sub>-phase (10). In the present study, PCNA-PI in methanol-fixed rat livers was below 1% and almost equal to the $^3$H-thymidine-LI (Fig. 1-a), which suggests a cell population primarily in the G<sub>0</sub>-phase. In contrast, the PFA-fixed livers showed a PCNA-PI of 26%. These positive cells may

FIGS. 2a, b. PCNA immunostaining combined with $^3$H-thymidine autoradiography in regenerating rat livers 24 hr after 2/3 PH. $^3$H-thymidine-labeled cells are indicated by arrowheads.

a. Results in methanol-fixed tissues. The PCNA-positive cells with a granular intranuclear staining pattern corresponded well with $^3$H-thymidine-labeled cells. b. Results in PFA-fixed tissues. The PCNA-positive cells, mostly with a diffuse intranuclear staining pattern, increased in frequency to about 60%, only a small fraction of which showed $^3$H-thymidine uptake.

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reflect PCNA which is pooled in the nucleoplasm in the G0-phase (2, 17). Citterio et al. (5) reported that PCNA was weakly stained in differentiated leaf cells in the resting phase. Their finding may also reflect pooled PCNA detectable in the G0-phase.

In PFA-fixed tissues (Fig. 1-b), the increase from the control level of the PCNA-PI during the first 24 hr after 2/3 PH was 41.7%, and was much larger than that of the 3H-thymidine-LI (28%). In addition, after 1/3 PH, PFA-fixed tissues also showed a 33% increase in the PCNA-PI at 24 hr despite the low 3H-thymidine-LI during and after this period. These findings suggest that PCNA increased after proliferative stimulation but was not bound to DNA replicons, preventing most of the cells from entering the S-phase. Moreover, the PCNA-PI returned to the control level of the PCNA-PI at 24 hr despite the low 3H-thymidine-LI. Because we followed the PCNA-PI and 3H-thymidine-LI during almost the entire time-course of liver regeneration, these findings suggest that some PCNA-positive cells detectable in PFA-fixed tissues ultimately failed to enter the S-phase.

From these results, we propose three phases of PCNA expression: Phase I in which PCNA is pooled in the nucleoplasm during the G0-phase; Phase II in which PCNA is increased after proliferative stimulation but is not bound to DNA replicons; and Phase III in which PCNA is bound to DNA replicons and becomes involved in DNA synthesis.

Based on this proposal, we speculate that cells in Phase III PCNA expression, which corresponded to the rise of 3H-thymidine-LI, were more increased after 2/3 PH than after 1/3 PH. In the 1/3 PH group, Phase II PCNA expression seemed to be mainly observed. In addition, the PCNA-PI in methanol-fixed tissues indicated a frequency of S-phase cells that corresponded to Phase III expression. Thus, the PCNA-PI after methanol fixation permits assessment of the proliferative activity in situ in the same way as 3H-thymidine autoradiography or bromodeoxyuridine immunohistochemistry. Moreover, unlike those conventional methods for labeling S-phase cells, PCNA can be detected without injection of any agents before fixation. Therefore, PCNA immunostaining of methanol-fixed tissues is particularly useful in clinical practice, where labeling procedures are limited in vivo.

In contrast to methanol fixation, many questions remain concerning formaldehyde fixation, which has been widely used in PCNA immunohistochemistry for clinical purposes (7, 8, 11-13, 15, 16). Our findings suggest that the assessment of cell proliferative activity in formaldehyde-fixed materials should be made with caution, because 1) many or some of the PCNA-positive cells are in the G0-phase; and 2) the increased PCNA expression after proliferative stimulation does not necessarily allow the cells to enter the S-phase.

The amount and distribution of PCNA in each cell should be studied in relation to the cell cycle to clarify which PCNA-positive cells in PFA-fixed materials are able to progress Phase II to Phase III. Quantitative analysis by Bravo and Bravo (1) demonstrated that cells blocked at the G1/S boundary by hydroxyurea failed to enter the S-phase but showed almost the same increase in PCNA content as the untreated proliferating cells. Their study suggests that an increase in PCNA content by itself is not directly involved in the critical processes operating at the G1/S checkpoint. However, PCNA was reported to show dramatic changes in its distributional pattern during the cell cycle (1, 4). We are presently investigating these changes in its distribution in the stimulated G0 cells.

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