Heterogeneity of Proliferative Activity in Nodule-in-Nodule Lesions of Small Hepatocellular Carcinoma

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Surgically resected small hepatocellular carcinomas showing "nodule-in-nodule" formation were analyzed in terms of cell proliferative activity. The analysis was achieved by successful immunohistochemical demonstration of proliferating cell nuclear antigen in formalin-fixed paraffin-embedded tissue sections. Eight nodules (up to 3 cm in diameter) examined were either atypical adenomatous hyperplasia or hepatocellular carcinoma of low histologic grade, containing a discrete inner nodular area composed of obvious hepatocellular carcinoma of higher histologic grade. In all cases, the proliferating cell nuclear antigen labeling index of the latter area was much higher than that of the former, which in turn was slightly higher than that of the non-cancerous liver of the patient in 6 cases. The data presented here provide supporting evidence that the successive emergence and expansion of a more rapidly proliferating subclone within a nodule result in the stepwise progression of malignancy of human hepatocellular carcinoma.

Key words: Proliferating cell nuclear antigen — Hepatocellular carcinoma — Tumor progression — Immunohistochemistry

Recently, morphological details of small hepatocellular carcinoma (HCC) have been well described.¹⁾ Arakawa *et al.*²⁾ first described nodule-in-nodule lesions, which were histologically composed of obvious invasive HCC surrounded by adenomatous hyperplasia. Tsuda *et al.*³⁾ analyzed hepatitis B viral genome integration in such lesions, and clearly demonstrated that both the outer adenomatous and inner carcinomatous lesions were derived from an identical hepatocyte clone. These two observations suggested that, at least in certain cases, obvious invasive HCC might emerge as a result of subclonal expansion within an adenomatous hyperplastic nodule or HCC of low histologic grade. One piece of supportive evidence for this suggestion would be to prove that the proliferative activity of the emerged subclone exceeded that of the original one. However, it has been almost impossible to evaluate cell proliferative activity in histopathologically well-observed, formalin-fixed paraffin-embedded tumor tissues.

Proliferating cell nuclear antigen (PCNA), also known as cyclin, is an auxiliary protein of DNA polymerase- δ ,⁴⁾ and shows prominent accumulation in S-phase nuclei of proliferating cells.⁵⁾ In the present study, the growth characteristics of nodule-in-nodule HCC were analyzed by immunohistochemical demonstration of PCNA in routinely processed tissue.

MATERIALS AND METHODS

Tumors Eight cases of HCC showing nodule-in-nodule formation, up to 3 cm in diameter, were examined. All of

them were surgically resected at the National Cancer Center Hospital, Tokyo, during the period between 1986 and 1989. Three areas were distinguishable within each nodule: A, non-cancerous liver surrounding the lesion; B, nodular hypercellular area excluding C; C, a distinct inner nodular area within the nodule. Histological diagnoses for each area are listed in Table I. The histologic grading of HCC was done according to the Edmondson Grading System.⁶⁾

Immunohistochemistry and PCNA labeling index (PCNA-LI) PCNA was detected immunohistochemi-

Table I. Histology and PCNA Labeling Indices of the Cases

Case	Area ^{a)}					
	A		B		C	
1	LC	(0.7%)	G1	(6.1%)	G2	(13.3%)
2	LC	(0.6)	G1	(6.3)	G2	(19.6)
3	LC	(1.5)	G1	(10.0)	G2	(14.5)
4	PC	(2.8)	AAH	(1.4)	G1	(12.4)
5	LC	(3.6)	G1	(5.9)	G2	(67.8)
6	LC	(5.1)	G1	(5.0)	G2-3	(22.3)
7	LC	(3.7)	G1-2	(5.3)	G2-3	(33.4)
8	CAH	(6.2)	G1	(20.8)	G2	(28.5)

LC, liver cirrhosis; PC, precirrhosis; CAH, chronic active hepatitis; G1, hepatocellular carcinoma (HCC) grade 1; G2, HCC grade 2; G3, HCC grade 3; AAH, atypical adenomatous hyperplasia; in parenthesis, PCNA-labeling index.

a) A, non-cancerous liver surrounding the lesion; B, nodular hypercellular area excluding C; C, a distinct inner nodular area within the nodule.

Fig. 1. Histology and immunostaining for PCNA on serial sections of a nodule-in-nodule lesion (case 5). a and c, boundary between A (upper left) and B areas (lower right). b and d, boundary between B (upper) and C areas (lower, indicated by arrows). (a and b, H-E, $\times 200$; c and d, immunostain for PCNA, $\times 200$)

cally on 10% formalin-fixed paraffin sections by the avidin-biotin-peroxidase complex method.⁷⁾ Mouse monoclonal antibody against human PCNA⁸⁾ was generously provided by Dr. Y. Takasaki, Department of Internal Medicine, Juntendo University, School of Medicine, Tokyo. It was applied at a 1:1000 dilution as a primary antibody. Visualization was performed using 3,3'-diaminobenzidine tetrahydrochloride and hydrogen peroxide. After light counterstaining with methyl green, PCNA-LI was determined by counting at least 500 hepatocyte nuclei (3–8 high-power fields) in each area.

RESULTS

Paraffin section immunohistochemistry showed that PCNA was localized diffusely in the nuclei of proliferating cells in normal tissues, such as germinal centers of reactive lymph follicles, the basal cell layer of the epidermis, and the neck zone of foveolar epithelium of the gastric mucosa (data not shown). In the HCC cases examined, various percentages of hepatocyte nuclei were labeled with various degrees of intensity (Fig. 1). PCNA-positive nuclei were found occasionally in lymphocytes, Kupffer cells, endothelial cells of sinusoids, and epithelial cells of bile ducts.

The PCNA-LI of atypical adenomatous hyperplasia (AAH) or HCC of low histologic grade (area B) was slightly to moderately higher than that of the surrounding non-cancerous liver tissue (area A) in most cases (Fig. 1a, c and Table I). In cases 4 and 6, the PCNA-LI of area B was slightly lower than that of area A. Most hepatocytes in area B of these two cases had vacuolated cytoplasm, demonstrating fatty change. In contrast, an obviously higher PCNA-LI was observed in area C compared with the surrounding area B in each of the eight cases (Fig. 1b, d and Table I).

DISCUSSION

PCNA, originally described as one of the nuclear antigens reactive with autoantibodies in sera of some patients with systemic lupus erythematosus,^{9,10)} had been identified as an auxiliary protein of DNA polymerase-delta.⁴⁾ Based on the facts that PCNA becomes markedly accumulated in S-phase nuclei of proliferating cells,⁵⁾ and that at least some antigenic epitopes can survive tissue fixation and paraffin embedding,^{11,12)} PCNA can be used as an immunohistochemical marker of proliferating cells on routine paraffin sections. Garcia *et al.*¹¹⁾ recently

reported a reliable correlation between PCNA labeling score and DNA flow cytometry data in various human tumors. They also referred to remarkable field-to-field heterogeneity of proliferative activity within a single solid tumor.¹¹⁾ In addition to their finding, the data presented here indicate that it is necessary to observe the morphological detail of a tumor sample when attempting to evaluate its proliferative activity.

From the data described here, it seems that some of AAH and low-grade HCC may have only a little, if any, growth advantage over non-cancerous liver. However, it is clear that hepatocytes in the inner nodular area proliferate much more rapidly than those in the surrounding lesion. Tsuda *et al.*³⁾ reported that AAH is already a clonal neoplastic lesion of probable borderline malignancy. They also demonstrated clonal identity between AAH and HCC within a nodule, by demonstrating identical clonal integration of the hepatitis B virus genomic DNA sequence in both tissues. The heterogeneity of PCNA-LI corresponding to the morphological alteration within nodule-in-nodule lesions implies that successive emergence and expansion of a highly proliferative subclone occur within preexisting clonal lesions, such as AAH or low-grade HCC. In the natural history of HCC, subclonal emergence and expansion as described above would be of considerable importance as one of the possible mechanisms for progression of malignancy.

In cases 4 and 6, the PCNA-LI of area B was lower or the same, in spite of its higher cellularity, than that of area A. Most hepatocytes in area B of both cases showed remarkable fatty change in their cytoplasm. Within such lesions, however, obvious HCCs with a high PCNA-LI emerged. Therefore, lesions of this type should also be evaluated as a background for emergence of higher malignancy. The discrepancy between the high cellularity and low PCNA-LI of these lesions remains to be explained.

ACKNOWLEDGMENTS

This research was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, and also by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare of Japan. The authors thank Dr. Y. Takasaki (Department of Internal Medicine, Juntendo University, School of Medicine) for providing a monoclonal anti-PCNA antibody, and also Mr. S. Osaka for photographic work.

(Received May 15, 1990/Accepted August 30, 1990)

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