Overexpression of Promyelocytic Leukemia Protein and Alteration of PML Nuclear Bodies in Early Stage of Hepatocarcinogenesis

Promyelocytic leukemia protein (PML) is a major component of PML nuclear bodies (PML NBs). Fusion of promyelocytic leukemia gene (PML) with retinoic acid receptor $\alpha$ (RAR $\alpha$) gene with the t(15;17) translocation causes disassembly of PML NBs, leading to development of acute promyelocytic leukemia. In contrast, PML overexpression as well as different morphological changes of PML NBs were described in a few solid tumors. In this study, the expression of PML through the multistep hepatocarcinogenesis was analyzed in 95 cases of human hepatocellular carcinomas (HCCs) for comparison along with dysplastic nodules (DNs) and background liver cirrhosis (LC) or chronic hepatitis by immunohistochemistry and immunoblot. In addition, cases of HCCs were further evaluated according to their histologic grade and etiology. The amount of PML as well as the number and size of PML NBs increased gradually through the progression from LC, DNs to HCCs. The overexpression of PML in HCCs was much more closely associated with HBV infection than HCV infection or alcoholic liver disease. The PML expression, however, was not correlated with histologic grade of HCCs. These results suggest that PML is involved in the early stage of multistep hepatocarcinogenesis, and HBV infection may be associated with the overexpression of PML and the morphological alteration of PML NBs.

Key Words: Leukemia, Promyelocytic; Nuclear Body; Carcinoma, Hepatocellular; Dysplastic Nodule; Liver Cirrhosis; Hepatitis B Virus

INTRODUCTION

Promyelocytic leukemia protein (PML) was first identified as the product of the promyelocytic leukemia gene (PML) that fuses with the retinoic acid receptor $\alpha$ (RAR $\alpha$) gene in the t(15;17) translocation of acute promyelocytic leukemia (1). PML-RAR $\alpha$ is known to be a dominant negative oncoprotein that exerts its putative leuke-mogenic effect by inhibiting assembly of the distinctive nuclear structure, PML nuclear body (NB) (2). Meanwhile, immunolocalization studies using sera from patients with autoimmune diseases showed that the PML was diffusely present within the nucleus and/or localized to distinctive "multiple nuclear dots", which were originally described as an autoantigenic target in a patient with primary biliary cirrhosis (3). PML NBs vary in number between 10 and 30 per nucleus, and typically have a diameter of between 0.2 and 1 mm. The number, size, and morphology of PML NBs alter throughout the cell cycle (4, 5) and are also dynamically changed by diverse extracellular environment including DNA and RNA viral infection (6-8).

Although it is well documented the proper organization of PML NBs is essential for normal cell proliferation and hematopoietic differentiation (9), the functions of PML or PML NBs still remain elusive in the pathogenesis of inflammatory and neoplastic diseases except APL. The PML expression was considerably upregulated in inflammatory tissues such as hepatitis, and PML NBs were not disturbed, unlike in cases of APL (13-15). Variable PML expression in human solid tumors suggests that the PML may play another role in addition to as a tumor suppressor, inducer of apoptosis and a depot of viral proteins (16, 17), and the mechanism of PML expression can be different from that of leukemogenesis.

Among solid neoplasms in which the PML is overexpressed, we selected hepatocellular carcinomas (HCCs) by the following indications: first, the expression of PML through the multistep hepatocarcinogenesis was analyzed in 95 cases of human hepatocellular carcinomas (HCCs) for comparison along with dysplastic nodules (DNs) and background liver cirrhosis (LC) or chronic hepatitis by immunohistochemistry and immunoblot. In addition, cases of HCCs were further evaluated according to their histologic grade and etiology. The amount of PML as well as the number and size of PML NBs increased gradually through the progression from LC, DNs to HCCs. The overexpression of PML in HCCs was much more closely associated with HBV infection than HCV infection or alcoholic liver disease. The PML expression, however, was not correlated with histologic grade of HCCs. These results suggest that PML is involved in the early stage of multistep hepatocarcinogenesis, and HBV infection may be associated with the overexpression of PML and the morphological alteration of PML NBs.

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cinogenesis is typical of a multistage process from chronic liver disease including liver cirrhosis (LC) through dysplastic nodule (DN) to HCCs (18), thus the PML expression can be properly correlated with the corresponding pathologic conditions.

**MATERIALS AND METHODS**

**Tissue samples**

Ninety-five cases of surgically resected HCCs were selected from the surgical pathology files of the Department of Pathology, Asan Medical Center, Seoul, Korea or Department of Pathology, Mt. Sinai Hospital, New York University, NY, U.S.A. Seventy-five cases were positive for HBV DNA or HBs antigen. Ten cases of HCCs were positive for HCV RNA and had no evidence of HBV infection and another 10 cases had past medical history of chronic alcoholism and were negative for HCV RNA, HBs or HBc antigens. Six cases of HCCs developed within a DN and three cases had a separate DN each. Serial sections of formalin-fixed and paraffin-embedded tissues from HCCs and non-neoplastic livers were cut for hematoxylin and eosin stain and immunohistochemistry.

**Histologic features of HCCs** were classified according to the grading system of Edmondson and Steiner (19) and those of non-neoplastic livers were reviewed.

Among 75 cases, fresh frozen tissues from HCCs and corresponding nontumor livers were available in 9 cases, one of which had both DN and HCC.

**Cell culture**

HepG2, HepG2.2.15 (20) and Hep3B cells were maintained in DMEM (Gibco, Gaithersburg, MD), supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere containing 5% CO₂.

**Immunohistochemical and immunofluorescent staining**

Immunohistochemistry was done following streptavidin biotin complex method after antigen retrieval by boiling twice in citrate buffer (pH 6.0) for 5 min as previously described (21). The primary antibody for PML used in this study was PG-M3 (Santa Cruz, California, U.S.A., 1:100 dilution). For immunofluorescent staining, cultured cells on coverslips were fixed in cold methanol for 10 min. Fluorescein-conjugated anti-mouse immunoglobulin was complexed with the primary antibodies for 1 hr. The tissue sections or cells were mounted with Fluromount G (Fisher Scientific Co., Pittsburgh, PA) and examined by using Olympus light microscope, Olympus Venox fluorescent microscope or Zeiss confocal laser scanning microscope.

**Expression of the PML** was described as follows: 1) average number of PML NB-positive cells per 1,000 hepatocytes or HCC cells; 2) average number of PML NBs in one nucleus of hepatocytes or HCC cells; 3) shape of PML NBs as fine dots, large dots greater than 1.0 μm in diameter, or ball-shaped NBs.

**Immunoprecipitation and immunoblotting**

Preparation of nuclear proteins was done as previously described (21). Protein amount was calibrated by using BCA protein assay. Twenty μg from the supernatant of each sample was incubated with the anti-PML antibody, PG-M3 at 1:200 dilution coupled to protein A-sepharose for 1 hr at 4°C. After extensive washing with the buffer, bound proteins were separated by SDS-PAGE and transferred to nitrocellulose papers which were allowed to react with the anti-PML antibodies for 1 hr. Immune complexes were detected by the chemiluminescence detection system (Amersham Pharmacia Biotech Ltd, UK).

**Statistical analysis**

The significance of differences in the expression of PML of HCCs, DNs, LC and chronic hepatitis (CH) was estimated by paired t-test, Two-sample test, one way analysis of variance (ANOVA) procedure. The level of statistical significance was set at 95% for all evaluations.

**RESULTS**

**PML expression in non-neoplastic liver**

Seventy-one cases of HCCs were associated with LC and 24 cases occurred in the background of chronic hepatitis (CH). In non-neoplastic liver, rare hepatocytes showed one or two PML NBs that were slightly variable in size and shape (Fig. 1A). The average number of PML NB-positive hepatocytes between CH and LC was not statistically significant (Table 1). Most of the reactive hepatocytes in CH and regenerating hepatocytes in LC that revealed PML NBs were unevenly distributed and closely associated with portal inflammatory cells. Average number of PML NBs in one hepatocyte nucleus was also higher in LC than that in CH, which was not statistically significant (Table 1).

**PML expression in dysplastic nodules**

Nine cases of DN were associated with HCCs in the background of LC. Average number of PML NB-positive hepatocytes was significantly increased in DN's compared with that in adjacent LC or CH (p<0.05) (Table 1). Six of nine cases of DN were classified low grade and three were high
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There was also a difference in number of PML NB-positive hepatocytes between low (31.27 ± 43.26/1,000 cells) and high grade DNs (72.72 ± 29.69/1,000 cells) (p<0.05 by paired t-test). The number of PML NBs in one hepatocyte nucleus was higher in DN than in LC, which was not statistically significant. Most of PML NBs were enlarged and some of them were ball-shaped with an empty center (Fig. 1B).

PML expression in hepatocellular carcinomas

The average number of PML NB-positive HCC cells per 1,000 HCC cells was markedly increased. The differences in average number of PML NB-positive cells between HCC, DN and LC were statistically significant (Table 1). The number of PML NBs per nucleus was also higher in HCC than in DN, LC and CH, which was not statistically significant (Table 1). Shape of PML NBs was quite different as well: greater than 50% of PML NBs in HCCs were large solid dots or ball-shaped with an empty core (Fig. 1C). The average number of PML NB-positive HCC cells was greater in HCCs associated with HBV than in HCCs associated with HCV or alcohol (Table 2). The number of PML NBs per one tumor cell nucleus was also higher in HBV-associated HCCs than in HCV-associated HCCs or alcohol-associated HCCs (Table 2). The shape of PML NBs, however.

Table 1. Expression of the PML in hepatocellular carcinomas, dysplastic nodules, liver cirrhosis and chronic hepatitis

| Diagnosis (No.of cases) | No. of PML NB + cells/1,000 cells | No. of PML NB/nucleus |
|-------------------------|-----------------------------------|-----------------------|
| HCC (95)                | 92.81 ± 78.31                     | 3.8 ± 1.86            |
| DN (9)                  | 45.09 ± 46.43                     | 2.8 ± 1.26            |
| LC (71)                 | 8.95 ± 7.66                       | 1.8 ± 1.54            |
| CH (24)                 | 4.70 ± 6.15                       | 0.5 ± 0.50            |

*HCC, hepatocellular carcinoma; DN, dysplastic nodule; LC, liver cirrhosis; CH, chronic hepatitis; PML, promyelocytic leukemia protein; PML NB, PML nuclear body; HBV vs HCV and HBV vs alcoholic, p<0.05 according to one way Analysis of Variance (ANOVA) procedure; Duncan’s multiple range test.

Table 2. Expression of the PML in 95 cases of hepatocellular carcinoma according to etiologies

| Etiology (No.of cases) | No. of PML NB + cells/1,000 cells | No. of PML NB/nucleus |
|------------------------|-----------------------------------|-----------------------|
| HBV (75)               | 100.31 ± 83.71                    | 4.2 ± 1.84            |
| HCV (10)               | 80.62 ± 39.43                     | 2.4 ± 0.56            |
| Alcohol (10)           | 48.43 ± 45.28                     | 2.7 ± 1.79            |

*HCC, hepatocellular carcinoma; DN, dysplastic nodule; LC, liver cirrhosis; CH, chronic hepatitis; PML, promyelocytic leukemia protein; PML NB, PML nuclear body.

Table 3. Expression of the PML in 95 cases of hepatocellular carcinomas according to histologic grade

| Histologic grade (No.of cases) | No. of PML NB + cells/1,000 cells | No. of PML NB/nucleus |
|-------------------------------|-----------------------------------|-----------------------|
| 1 (12)                        | 93.75 ± 106.5                     | 4.5 ± 2.40            |
| 2 (40)                        | 99.37 ± 80.93                     | 3.5 ± 1.72            |
| 3 (33)                        | 88.43 ± 68.25                     | 3.8 ± 1.80            |
| 4 (10)                        | 80.62 ± 65.31                     | 4.6 ± 1.64            |
| Total cases (95)              | 92.81 ± 78.31                     | 3.8 ± 1.86            |

*HCC, hepatocellular carcinoma; DN, dysplastic nodule; LC, liver cirrhosis; CH, chronic hepatitis; PML, promyelocytic leukemia protein; PML NB, PML nuclear body.

Fig. 1. PML expression in liver cirrhosis (A), dysplastic nodule (B), and hepatocellular carcinoma (C): A. Only one or two dots of PML NBs (arrows) are noted in regenerating hepatocytes. B. PML NBs are enlarged and some of them are ball-shaped (arrow). C. Most of PML NBs in HCC cells are markedly enlarged with empty cores (original magnification, × 1,000).
er, was similar in all HCCs. There was no significant correlation between the PML expression and histologic grade (Table 3). Pleomorphic nuclei in high grade HCCs, however, had greater number of much larger or ball-shaped PML NBs than in lower grade HCCs.

Differential overexpression of the PML in HCC compared with those in LC and DN was confirmed by immunoblotting in a case that had both DN and HCC in the background of LC (Fig. 2).

**DISCUSSION**

The development and progression of HCC is typical of a multistep process from chronic liver disease caused by viral infection or toxic substance through DN to early or advanced HCC with genetic and/or epigenetic alterations. Thus, we analyzed the PML expression in HCCs as well as DNs and adjacent non-neoplastic liver tissues in the present study. Although the PML is known to be present diffusely in the nucleoplasm or in PML NBs, the PML was localized mainly to PML NBs as revealed by immunohistochemical staining with the monoclonal antibody, PG-M3. Thus, we described the PML expression according to the number of PML NB-positive hepatocytes or HCC cells per 1,000 hepatocytes or HCC cells and the number of PML NBs in one nucleus of hepatocyte or HCC cell.

The differential overexpression of the PML in DN and HCC in this study indicates that the PML expression is differentially regulated during the multistep hepatocarcinogenesis, and the alteration of the PML expression may occur first in the early stage of hepatocarcinogenesis, from LC to DN formation. In contrast to our results, Chan et al. (15) described overexpression of the PML in the PML NBs in 50% of human HCCs and most of non-tumorous cirrhotic liver, thus indicated that the PML overexpression was associated with both LC and HCC formation. Gambacorta et al. (13) reported the PML expression in 10/10 liver tumors, but neither histologic grading of the HCCs nor comparative analysis between preneoplastic lesions and HCCs has been made. The immunoprecipitation with immunoblot analysis in a few representative cases of HCCs supported also a distinct overexpression of the PML in DN and HCC compared with non-neoplastic liver. To clarify a possibility of transcriptional upregulation, northern blot analysis using RT-PCR products of the PML in LC, DN and HCC has been undergoing. At this moment, however, it can not be determined whether the overexpression of the PML in DNs
and HCCs is a cause for hepatocellular carcinogenesis or an epiphenomenon during hepatocarcinogenesis.

Statistically significant difference of the PML expression between HBV-associated HCCs and HCV- or alcohol-associated HCCs and morphological characteristics of PML NBS in HepG2.2.15, a stably transfected cell line by HBV genome, suggest a strong relationship between HBV infection and PML NBS. PML NBS are known to be the site of DNA virus transcription and replication (8), thus HBV infection may affect the size or shape of PML NBS. Aoki et al. (22) reported that a subgenomic HBV DNA sequence (15 AB) is a hot spot for genomic recombination and a portion of 15AB-like sequence is homologous to break-point clusters of the human PML gene. Considering a close relationship between HBV infection and HCCs, it may be suggested that the PML might be a recombinogenic candidate triggering genomic instability in H BV-associated hepatocarcinogenesis. The structure of ball-shaped PML NBS is known to be associated with a recruitment of other PML NBS-associated proteins such as SUMO-1 (23, 24) and TRF1 or TRF2 (25), and assembly with PML within PML NBS. Thus a further analysis of coimmunoprecipitates with PML and PML isoforms in LC, DN and HCC using two-dimensional gel electrophoresis may elucidate an alternative mechanism of the PML overexpression and the morphological alteration of PML NBS.

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