TABLE II. Routes of Infection, Liver Histology, and Treatment Outcomes of the Seven Patients in Whom Infection With HBV Genotype A Persisted

| Case no. | Age/sex | Sexual contacts[a] | Liver histology[b] | Treatment[c] | HBeAg lost | HBsAg lost |
|---|---|---|---|---|---|---|
| 1 | 54/M | None | F1/A2 | Lam | Yes | Yes |
| 2 | 43/M | Hetero | F2/A1 | IFN | Yes | No |
| 3 | 46/M | None | F4/A2 | IFN | Yes | No |
| 4 | 21/M | Homo | F1/A1 | IFN/Lam | No | No |
| 5 | 24/M | Homo | F1/A2 | Lam | No | No |
| 6 | 28/M | Homo | F1/A1 | IFN/Lam | No | No |
| 7 | 21/M | Homo | F1/A1 | None | No | No |

[a]Extramarital sexual contacts in which infection with HBV genotype A was implicated.
[b]Pathology of the liver in the first biopsy; F, fibrosis stage; A, activity grade.
[c]Treatment received after admission to the Department of Gastroenterology in Toranomon Hospital; IFN, interferon; Lam, lamivudine.

lamivudine 3 months after the admission to our hospital. He lost both HBeAg and HBsAg, respectively, within 83 and 90 days on lamivudine; it was withdrawn at the disappearance of serum HBsAg. ALT levels normalized after the loss of HBeAg and HBsAg from serum.

Cases 2 and 3 did not receive treatment during 4 and 2 years, respectively, after they visited the hospital. They both responded to IFN 3 MU three times in week and lost HBeAg from serum, along with the disappearance of HBV DNA and normalization of ALT levels. They did not, however, clear HBsAg from serum. Cases 1–3 had received oral (Glycyron) or intravenous (SNMC) glycyrrhizin with or without ulsodeoxicholic acid (UDCA) while they were admitted to other institutions before referral to the Toranomon Hospital.

Clinical courses of the three patients who did not respond to antiviral therapies (Cases 4–6) are illustrated in Figure 2, along with that of a single patient who did not receive treatment (Case 7). Case 6 had received intravenous glycyrrhizin (SNMC) before his

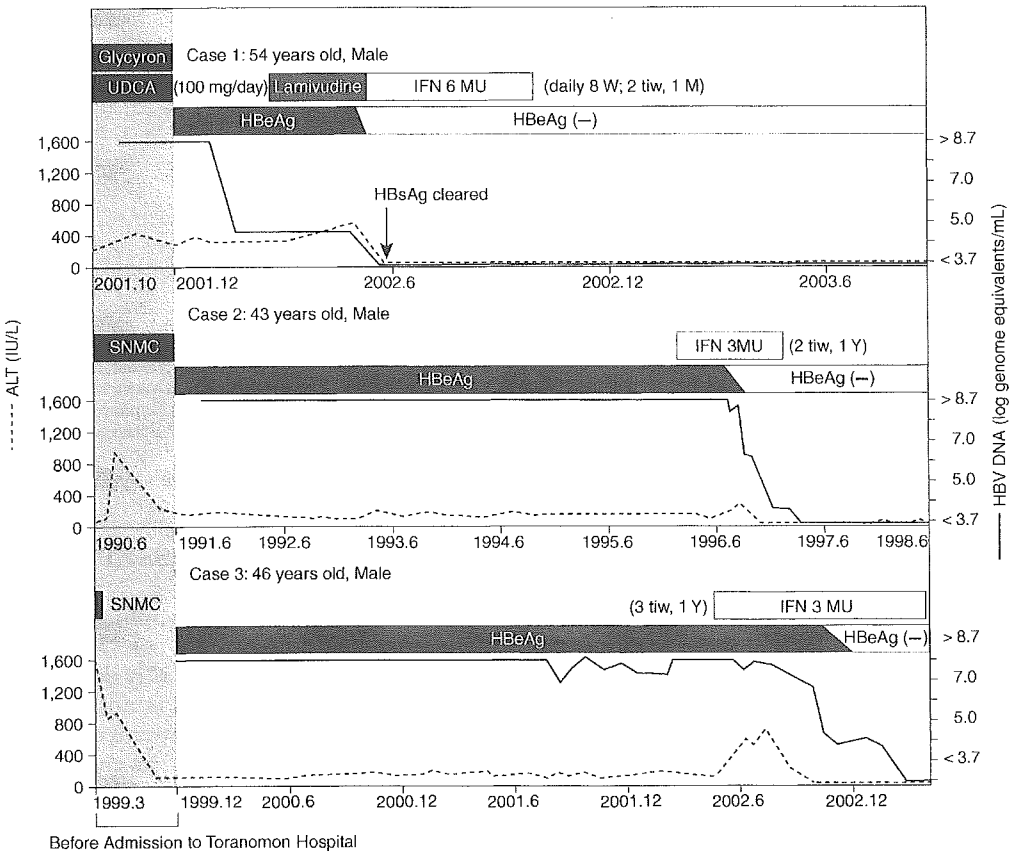


Fig. 1. Clinical courses of the three patients infected with HBV genotype A who responded to antiviral treatment with the loss of HBeAg. Courses and treatment they received before admission to Gastroenterology Department in Tornomon Hospital are shown in shaded areas on the **left**. The patient in Cases 1 lost HBsAg after treatment with lamivudine at time points indicated by arrows. IFN, interferon; SNMC, Stronger Neo-Minophagen C.

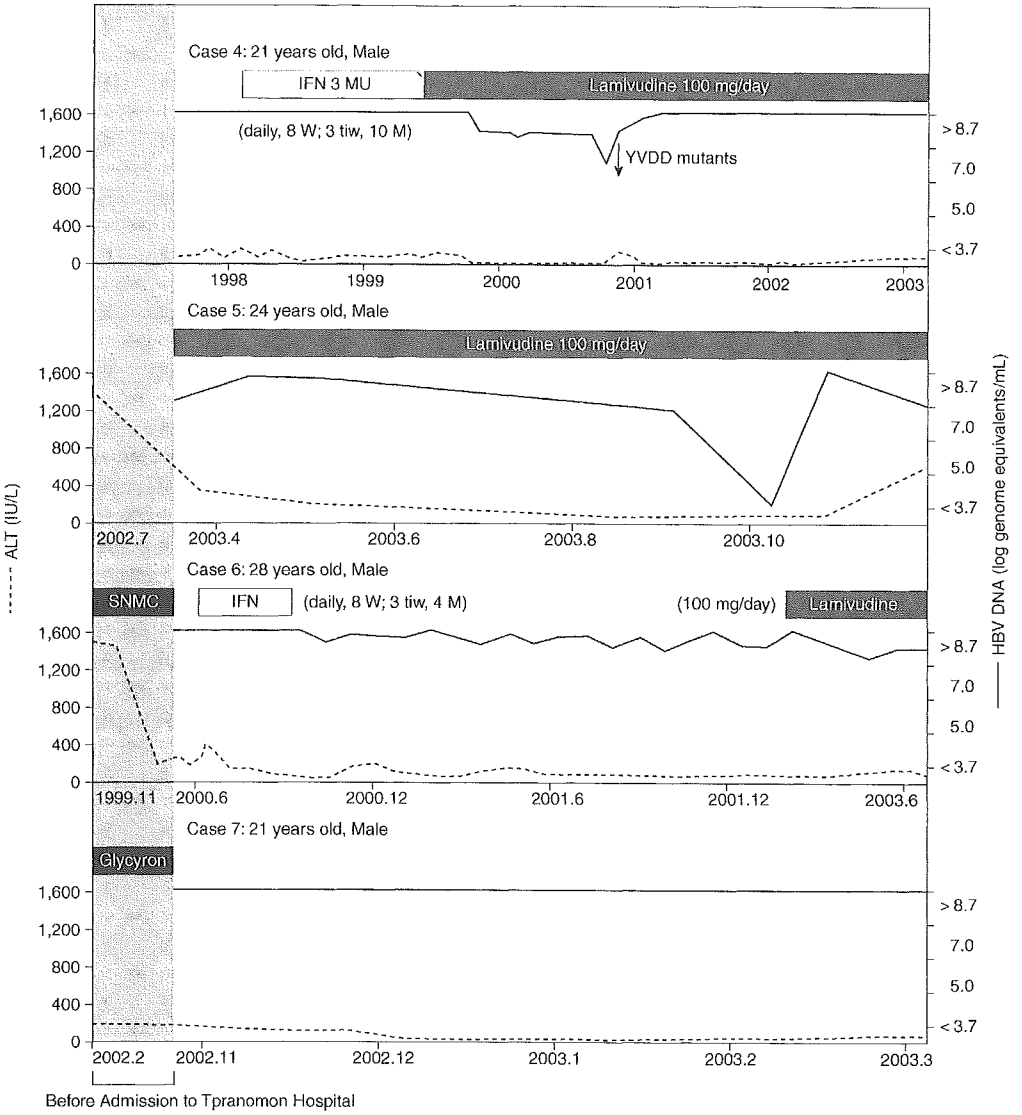


Fig. 2. Clinical courses of the four patients in whom HBeAg persisted after contracting infection with HBV genotype A despite antiviral treatment. The patient in Case 9 did not receive treatment after the admission. Courses and treatment they received before admission to Gastroenterology Department in Tornomon Hospital are shown in shaded areas on the **left**. IFN, interferon; SNMC, Stronger Neo-Minophagen C.

transfer to the Toranomon Hospital. Cases 4 and 6 did not respond to IFN or lamivudine that was commenced immediately after IFN or at an interval; lamivudine has been continued on them indefinitely. Case 5 was started on lamivudine soon after he was admitted to hospital and had been maintained on it for 1 year; he never responded to lamivudine. Variants with mutations in the YMDD motif of DNA polymerase/reverse-transcriptase developed in Case 4 while he was receiving lamivudine accompanied by a rise in ALT levels.

Although serum ALT returned to normal spontaneously (<50 IU/L) and then elevated only moderately in Case 7, high levels of HBV DNA (>8.7 LGE/ml) persisted through more than 1 year. Antiviral treatment was withheld because of the absence of active hepatitis.

## DISCUSSION

There are marked geographical differences in the distribution of HBV genotypes [Magnius and Norder, 1995; Lindh et al., 1997; Miyakawa and Mizokami, 2003]. Of them, genotype A is not indigenous in Japan where genotypes B and C prevail and account for by far the majority of acute as well as chronic HBV infections [Orito et al., 2001; Kobayashi et al., 2002]. Some characteristics of HBV genotype A infection are increasingly coming to the fore in Japan and have aroused concerns in hepatologists at hospitals in urban areas with cosmopolitan populations. Ogawa et al. [2002] found that 14 of the 25 (56%) patients with acute hepatitis B in a downtown Tokyo (Shinjuku) were

infected with HBV genotype A. Moreover, the frequency of acute hepatitis induced by HBV genotype A in our hospital is higher after than before 1991 (2/22 [9%] vs. 26/46 [57%], $P < 0.0001$) [Kobayashi et al., 2004]. The present study sums up our experiences on acute infection with HBV genotype A at the Department of Gastroenterology in Toranomon Hospital situated in the Metropolitan Tokyo during the past 28 years, to supplement our previous reports with additional findings and new insights [Kobayashi et al., 2002, 2003, 2004].

First, infection with HBV genotype A spreads principally by extramarital sexual contact in the adulthood in Japan [Kobayashi et al., 2002; Ogawa et al., 2002]. All the 31 patients of acute hepatitis B infected with HBV genotype A in the present series were men, and 16 (52%) of them confided having had extramarital heterosexual or homosexual contacts. Only one mother of 32 patients with acute or chronic infection with HBV genotype A possessed HBV DNA in serum; her genotype was B [Kobayashi et al., 2003], thereby excluding perinatal transmission of genotype A. In a molecular epidemiological survey of HBV in Amsterdam, a cluster of genotype A related in men having sex with men has been recognized [van Steenbergen et al., 2002].

Secondly, acute infection with HBV genotype A tends to persist. Of the 31 patients with acute genotype A infection, seven (23%) failed to clear it within 6 months, in comparison with one of the nine (11%) with acute genotype B or three of the 42 (7%) with acute genotype C infection. In our previous report [Kobayashi et al., 2002], infection persisted in all three patients infected with genotype A, in contrasted to the clearance of HBsAg in all four with genotype B (one) or C (three).

Low maximum ATL levels (<500 IU/L [83%] vs. ≥500 IU/L [4%], $P = 0.0001$) and the high baseline HBV DNA levels (median: >8.7 vs. 6.0 LGE/ml, $P = 0.004$) were predictive of the perpetuation of acute HBV genotype A infection. Hence, compromised immune responses toward lower inflammation activity in the liver and higher viral replication may have a role in evolving HBV genotype A infection. Four of the seven (57%) patients who progressed to chronic were homosexuals. It is tempting to speculate that derangement in cytotocxic T cell response contributed to the failure in clearing acute HBV infection toward persistence [Handzel et al., 1984]. Immunomodulatory treatments to cope with severe acute hepatitis, given to five of the seven (71%) patients before referral to hospital (Figs. 1 and 2), may have promoted the persistence of infection with HBV genotype A. We have reported that acute prolonged HBV infection occurs more often in patients with than without immunomodulatory treatments during acute illness, regardless of genotypes (86% [6/7] vs. 2.4% [1/42], $P = 0.01$).

Thirdly, HBV genotype A infection persisting in patients with acute hepatitis B is not cleared often by antiviral therapy. HBV genotype A infection was terminated in only one of the six (17%) patients who received antiviral treatment. He was one of the three patients who seroconverted with the loss of HBeAg; interferon (IFN) and lamivudine was given to him early in the course of infection (Cases 1 in Fig. 1). Since most (~95%) patients with acute adulthood hepatitis B resolve infection in Japan, antiviral treatment is rarely used for them. As far as acute infection with HBV genotype A is concerned, however, therapeutic intervention needs to be considered in view of the frequent chronic outcomes. Since many (76% [24/31]) patients even with HBV genotype A can clear infection spontaneously, the timing of starting antiviral therapy would have to be contemplated. The single patient who cleared HBsAg was started on IFN and then lamivudine within 3 months after he was referred to our hospital. It is not certain whether he could have cleared HBV infection, should he never be placed on lamivudine early. HBsAg was not cleared, however, in the remaining two patients with HBeAg seroconversion in whom IFN was started 4 and 2 years, respectively, after they came to our care (Fig. 1).

Early antiviral treatment deserves consideration in patients who are infected with HBV genotype A, especially because of its propensity to become chronic. It is not certain how long patients should receive lamivudine after HBV DNA has disappeared from the circulation. Inasmuch as cccDNA continues to be present in the liver [Brechot et al., 1980; Yotsuyanagi et al., 1998], even after HBsAg is cleared from serum, a therapeutic option would be to continue lamivudine until anti-HBs is detected in serum as in Case 1. In view of the poor immune responses with low ALT levels, which might be inherent to HBV genotype A infection among homosexual, such a special care would have to be taken for its treatment.

There are two genetic subgroups of genotype A designated Ae which is common in Europe (the original genotype A) and Aa which is frequent in Africa as well as Asia [Sugauchi et al., 2003]; Aa is equivalent to subgroup A′ described by Bowyer et al. [1997]. It strikes as a surprise that of the 68 patients who were infected acutely or chronically with HBV genotype A and admitted to the Toranomon Hospital, 54 (79%) possessed HBV of subgroup Ae (European type); HBV of subgroup Aa (African/Asian type) was found in only four (6%) [Kobayashi et al., 2004]; they all were infected persistently. Since subgroup Ae was not found in any patients with acute hepatitis B in our series, it remains unclear whether or not the outcome of primary infection with HBV genotype A would be influenced by subgroup Aa and Ae.

Although acute HBV infection of genotype A tends to persist in comparison with those of the other genotypes, only a minority (7/31 [23%]) develops chronic infection. An efficient therapeutic strategy has to be found, however, since the infection with HBV genotype A was terminated in only one of the six (17%) patients who were treated. Recently, adefovir dipivoxil was found to be effective for the treatment of chronic hepatitis B [Marcellin et al., 2003], and it may offer a reasonable option for resolving persistent HBV genotype A infection.

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# Virological Outcomes in Patients Infected Chronically With Hepatitis B Virus Genotype A in Comparison With Genotypes B and C

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In a single hospital in Tokyo, the 87 patients infected persistently with hepatitis B virus (HBV) genotype A, the 413 with B, and the 3,389 with C were compared for virological outcome. Hepatitis B surface antigen (HBsAg) was cleared from the serum in 12% (3/26), 2% (2/112), and 3% (23/826) of patients with genotypes A, B, and C, respectively, at 5 years of follow-up ($P=0.0395$). Hepatitis B e antigen (HBeAg) was cleared from serum more frequently in patients with genotype B than those with A or C (78% [32/41] vs. 58% [11/19] or 45% [251/562], $P=0.00001$) at 5 years. Of the 45 individuals infected with genotype A and followed for 3 years or longer, HBeAg was more frequent (16% [3/19] vs. 73% [19/26], $P=0.0002$) and levels of HBV DNA higher (median <2.6 [range: <2.6–5.6] vs. >7.6 [<2.6–>7.6] log copies/ml, $P=0.001$) in the 26 patients with biopsy-proven chronic hepatitis than the 19 asymptomatic carriers. Among the 26 hepatitis patients infected with HBV genotype A, decreases in HBV DNA were less frequent (20% [1/5] vs. 93% [13/14] or 86% [6/7], $P=0.0095$) and increases in serum levels of hyaluronic acid ≥10 ng/ml commoner (80% [4/5] vs. 14% [2/14] or 14% [1/7], $P=0.017$) in the patients who kept HBeAg than in those who seroconverted or who remained HBeAg-negative. In conclusion, patients persistently infected with HBV genotype A fare better than those with genotype B or C. However, high levels of HBV DNA continue in those in whom HBeAg persists along with fibrosis in the liver. ***J. Med. Virol. 78:60–67, 2006.*** © 2005 Wiley-Liss, Inc.

**KEY WORDS:** chronic hepatitis; cirrhosis; hepatitis B e antigen; hepatitis B surface antigen; hepatocellular carcinoma; sexual transmission

## INTRODUCTION

There are an estimated 350 million people in the world who are persistently infected with hepatitis B virus (HBV), some of whom develop a spectrum of chronic liver disease ranging from chronic hepatitis through cirrhosis to hepatocellular carcinoma [Lee, 1997]. New HBV infections have been prevented by mass vaccination of neonates [Tsen et al., 1991; Chen et al., 1996] and immunopropylaxis of babies born to mothers carrying HBV [Noto et al., 2003]. However, there are individuals who have been infected, and they need to be identified for receiving treatment as required. Clinical outcomes and the response to antiviral treatment are influenced by many host factors, such as ethnicity, gender, and the age at infection, as well as viral factors represented by HBV genotypes.

HBV has a partially double-stranded DNA genome of approximately 3,200 nucleotides (nt) [Tiollais et al., 1981]. Eight HBV genotypes have been classified by a sequence divergence in the entire genome exceeding 8% [Okamoto et al., 1988], and they are named by capital Alphabet letters from A to H [Okamoto et al., 1988; Norder et al., 1992; Stuyver et al., 2000; Arauz-Ruiz et al., 2002]. Recently, HBV genotypes have attracted an increasing attention because they influence the clinical outcome and treatment response in patients with chronic liver disease [Tsubota et al., 2001; Kao, 2002; Miyakawa and Mizokami, 2003; Schaefer, 2005; Yu et al., 2005]. Due to their uneven geographical

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distribution, however, only two HBV genotypes prevail in most countries; the United States is the only exception with seven (A–G) genotypes [Chu et al., 2003; Westland et al., 2003]. Thus, genotypes A and D are common in Europe and India, while genotypes B and C are frequent in Asia [Magnius and Norder, 1995; Miyakawa and Mizokami, 2003]. Therefore, comparison has been restricted between patients infected with genotypes A and D, as well as those with B and C [Zhang et al., 1996; Mayerat et al., 1999; Kao et al., 2000; Orito et al., 2001; Chu et al., 2002; Thakur et al., 2002].

During 31 years from 1973 to 2003, 4,121 patients visited Toranomon Hospital in the Metropolitan Tokyo, and HBV genotypes were determined in them. There were 128 patients with genotype A, of whom 87 were chronically infected with HBV at the presentation. They were followed along with the 413 patients chronically infected with genotype B and the 3,389 with genotype C for seroclearance of hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg). Furthermore, patients with genotype A were grouped by the presence or absence of HBeAg at the presentation, as well as seroconversion during the follow-up, and they were compared for virological and clinical outcomes.

## MATERIALS AND METHODS

### Patients Chronically Infected With HBV

During 31 years from April 1973 to December 2003, genotypes of HBV DNA were determined in 4,121 patients with HBsAg in the Department of Gastroenterology at Toranomon Hospital in the Metropolitan Tokyo. Genotypes were A in 128 (3.11%) patients, B in 431 (10.46%), C in 3,434 (83.32%), D in 4 (0.97%), E in 1 (0.02%), and F in 3 (0.07%); they were not classifiable in the remaining 120 (2.91%) patients.

Of the 128 patients infected with HBV genotype A, 41 (32%) presented with acute hepatitis B as diagnosed by high-titered IgM antibody to hepatitis B core antigen. The remaining 87 (68%) patients were chronically infected with HBV genotype A when they visited our hospital. Their diagnoses were asymptomatic carriers with persistently normal ALT levels in 38 (44%) and chronic hepatitis in 39 (45%). In addition, nine (10%) patients presented with cirrhosis and one (1%) with hepatocellular carcinoma. Chronic hepatitis was diagnosed by liver biopsies performed under laparoscopy, and liver cirrhosis by liver biopsy and/or ultrasonographic images plus laparocopic findings. Hepatocellular carcinoma was diagnosed by imaging modalities, such as ultrasonography, computed tomography, and magnetic resonance imaging, and by liver biopsy if necessary.

The 87 patients infected chronically with HBV genotype A had the median age of 34 years (range: 11–67 years), included 72 (83%) men and were followed for the median of 5.0 years (0.1–22 years). Only two (2%) had a history of blood transfusion, and three (3%) were co-infected with hepatitis C virus. They had the median serum HBV DNA level at 4.2 log copies/ml, and HBeAg was detected in sera from 32 (37%). Subgenotypes of A [Bowyer et al., 1997; Sugauchi et al., 2004] were Aa (Asian or African type) in 5 (6%) and Ae (European type) in 65 (75%); they were not classifiable in the remaining 17 (19%).

### Serological Markers of HBV Infection

HBsAg was determined by hemagglutination (MyCell; Institute of Immunology Co., Ltd., Tokyo, Japan) or enzyme-linked immunosorbent assay (ELISA) (ELISA, F-HBsAg; Sysmex, Kobe, Japan), and HBeAg by ELISA (ELISA, F-HBe; Sysmex). HBV DNA was determined by quantitative polymerase chain reaction (PCR) (Amplicor HBV Monitor Test; Roche Molecular Systems, Inc., New Jersey) and the results were expressed in log copies/ml within a detection range from 2.6 to 7.6.

### Genotypes of HBV

The six major genotypes (A–F) were determined serologically by ELISA (HBV GENOTYPE EIA; Institute of Immunology). The method utilizes the combination of epitopes on preS2-region products that is specific for each genotype [Usuda et al., 1999, 2000]. Genotype G was determined by preS2 serotype for genotype D and HBsAg subtype adw, and H was recognized by serotype for genotype C and subtype adw, respectively; these combinations were specific for genotypes G and H, respectively [Kato et al., 2001, 2004].

Subgenotypes of A designated Ae prevalent in Europe and Aa frequent in Africa as well as Asia [Sugauchi et al., 2004] (corresponding to A′ originally reported by Bowyer et al. [1997]), were determined by the nucleotide sequence in the *S* gene [Sugauchi et al., 2004]. Briefly, nucleic acids were extracted from serum and a sequence of the large *S* gene was amplified by PCR with nested primers. The first-round PCR was performed with BGF1 (sense, 5′-CTG TGG AAG GCT GGC ATT CT-3′ [nt 2757–2776]) and BGR2 (antisense, 5′-GGC AGG ATA GCC GCA TTG TG-3′ [nt 1050–1079]) primers, and the second-round PCR with PLF5Bm (sense, 5′-TGT GGA TCC TGC ACC GAA CAT GGA GAA-3′ [nt 136–162]) and BR112 (antisense, 5′-TTC CGT CGA CAT ATC CCA TGA AGT TAA GGG A-3′ [nt 865–895]) as well as BGF5 (sense, 5′-TGC GGG TCA CCA TAT TCT TG-3′[nt 2811–2830]) and BGR6 (antisense, 5′-AGA AGT CCA CCA CGA GTC TA-3′ [nt 249–268]) for 35 cycles each (94°C, 1 min [5 min in the first cycle]; 53°C, 2 min; and 72°C, 3 min [7 min in the last cycle]). Amplification products were run on gel electrophoresis and stained with BIG Dye (Applied Biosystems, California), purified by Qiquick PC purification kit (Qiagen, Hilden, Germany) and then sequenced in AGI Prism 310 Genetic Analyzer (Applied Biosystems). The large S-gene sequences were analyzed phylogenetically along with reference Aa and Ae sequences by six-parameter and neighbor-joining methods [Gojobori et al., 1982; Saitou and Nei, 1987].

### Determination of Hyaluronic Acid in Serum

Hyaluronic acid was determined by the agglutination of microparticles coated with proteins that specifically bind with it (Elpia-Ace HA, Fujirepio, Tokyo, Japan).

### Statistical Analysis

Frequencies were compared between groups by the Mann–Whitney *U*-test and Fisher's exact test, and means by the Wilcoxon signed rank test. Loss of HBeAg or HBsAg was compared in the Kaplan–Meier life table, and differences were evaluated by log-rank test after the production limit method. A *P*-value less than 0.05 was considered significant.

## RESULTS

### Patients Infected Chronically With HBV Genotype A

There were 45 patients who were infected chronically with HBV genotype A and had been followed for 3 years or longer. Of them, 19 had persistently normal ALT levels (asymptomatic carriers), while the remaining 26 with elevated ALT levels possessed biopsy-proven chronic hepatitis. Table I compares demographic and virological characteristics at the baseline between the 19 asymptomatic carriers and 26 patients with chronic hepatitis. HBeAg was more frequent and the median HBV DNA level higher in patients with chronic hepatitis than asymptomatic carriers. The majority of asymptomatic carriers (79% [15/19]) and patients with chronic hepatitis (73% [19/26]) were infected with subgenotype Ae. There were three (12%) patients infected with subgenotype Aa and two of them had chronic hepatitis. Subgenotypes were not classifiable in the remaining four (21%) asymptomatic carriers and four (15%) patients with chronic hepatitis. Liver disease worsened in a single patient with chronic hepatitis. He was 47 years old at the presentation and infected with subgenotype Ae. Cirrhosis developed followed by hepatocellular carcinoma in him.

### HBsAg and HBeAg in Patients With Chronic Hepatitis Infected With HBV Genotype A

Of the 26 patients infected with HBV genotype A, 4 (15%) lost HBsAg during follow-up, in comparison with 16 of the 116 (14%) patients with genotype B and 68 of the 862 (8%) with genotype C. Figure 1 compares seroclearanace of HBsAg among patients with genotype A, B, or C. The loss of HBsAg at 5 years was significantly more frequent in patients with genotype A than B or C (12% vs. 2% or 3%, $P = 0.0395$).

Of the 26 hepatitis patients with genotype A, 19 (75%) possessed HBeAg at the presentation. HBeAg was cleared from serum in 14 (74%) of them during follow-up, in comparison with the seroclearance in 36 of the 41 (88%) patients with genotype B and in 347 of the 562 (62%) with genotype C. Figure 2 compares seroclearance of HBeAg among patients with genotype A, B, or C. At 5 years of follow-up, HBeAg was cleared more frequently in patients with genotype B than in those with genotype A or C (78% vs. 58% or 45%, $P = 0.00001$).

### Development of Cirrhosis and Hepatocellular Carcinoma in Patients Infected With HBV of Various Genotypes

Figure 3 compares the development of cirrhosis in patients infected with genotype A, B, or C. Of the patients with genotype A, cirrhosis developed in only one at 5 years, but not any more during follow-up for 20 years. In contrast, cirrhosis increased steadily in patients with genotype B or C; it developed twice more often in patients with genotype C than B (30% vs. 14%).

Hepatocellular carcinoma developed in the single cirrhotic patient with genotype A, but did not in any others with genotype A during follow up for 20 years (Fig. 4). It increased with time, however, in patients with genotype B or C. Hepatocellular carcinoma tended to develop more frequently in patients with genotype C than B at 20 years (15% vs. 11%).

### Changes in HBV DNA Levels and Hyaluronic Acid in the Patients Infected With HBV Genotype A

Of the 26 patients with genotype A, 14 (54%) seroconverted for the loss of HBeAg, while 5 (19%) kept it throughout follow-up longer than 3 years; the remaining 7 (27%) patients were without HBeAg at the presentation and thereafter. Table II compares demographic and virological characteristics of the three

TABLE I. Baseline Characteristics of the 45 Patients Infected With HBV Genotype A Who Were Followed for Longer Than 3 Years

| Feature | Asymptomatic carriers (n = 19) | Chronic Hepatitis (n = 26) | Differences |
|---|---|---|---|
| Age (years)[a] | 29 (11–48) | 32 (13–59) | NS[c] |
| Male | 15 (79%) | 24 (92%) | NS |
| Follow-up (years)[a] | 6.5 (3.4–17.7) | 6.8 (3.5–18.6) | NS |
| History of transfusion | 0 (0%) | 1 (4%) | NS |
| Anti-HCV | 0 (0%) | 1 (4%) | NS |
| HBeAg positive | 3 (16%) | 19 (75%) | $P = 0.0002$ |
| HBV DNA (log copies/ml) | <2.6 (<2.6–5.9) | >7.6 (<2.6–>7.6) | $P = 0.001$ |
| Subgroups (Aa/Ae/ND[b]) | 0%/79%/21% | 12%/73%/15% | NS |

[a]Median values are shown with the range in parentheses.
[b]Not determined.
[c]Not significant.

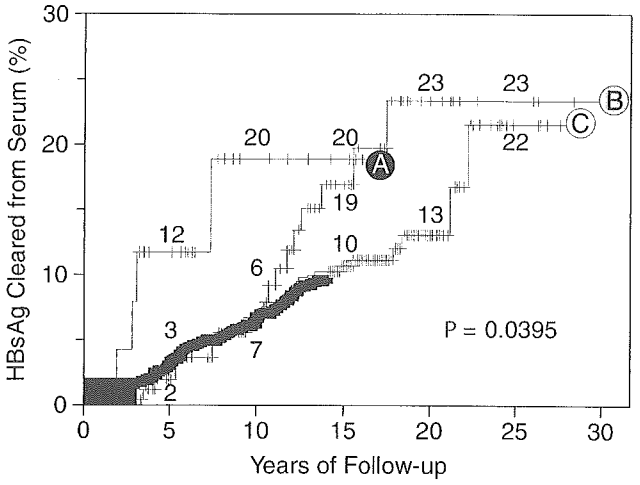


Fig. 1. Seroclearance of HBsAg during follow-up. Clearance rates of HBsAg are compared among patients with chronic hepatitis B who were infected with genotypes A, B, or C by the Kaplan–Meier life table. Differences are significant between genotype A and genotypes B and C at 5 and 10 years, as well as between genotypes B and C at 20 years by the log-rank test. Seroclearance of HBsAg did not spontaneously occurr in all of them.

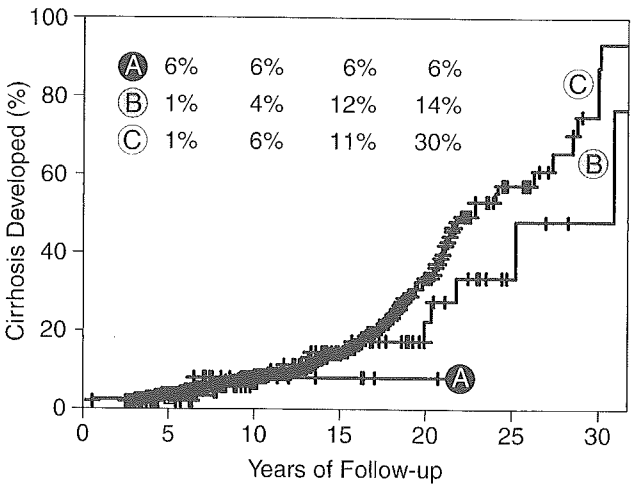


Fig. 3. Development of cirrhosis in patients infected with HBV genotype A, B, or C.

groups of patients at the baseline. Levels of HBV DNA were significantly lower in the patients without HBeAg than in those whom HBeAg persisted or who seroconverted within 3 years ($P = 0.03$).

Figure 5 compares changes in HBV DNA levels among patients infected with genotype A in whom HBeAg persisted, who seroconverted and who had remained negative for HBeAg. HBV DNA levels >7.6 log copies/ml continued for longer than 3 years in four of the five (80%) patients with persistent HBeAg. HBV DNA levels decreased in 13 of the 14 (93%) patients with seroconversion; they slightly changed from 6.7 to 7 log copies/ml in the remaining one patient. HBV DNA decreased to levels below the detection limit in 4 of the 14 (29%) patients with seroconversion and 1 of the 7 (14%) without HBeAg at the baseline. Of the 7 patients without HBeAg, 4 (57%) kept HBV DNA in detectable levels, comparable to 9 of the 14 (64%) patients with seroconversion. Decreases in HBV DNA during follow-up for 3 years or longer were significantly more frequent in the patients with seroconversion and those without HBeAg than in those with persistent HBeAg (93% [13/14] and 86% [6/7] vs. 20% [1/5], $P = 0.0095$ by the Fisher's exact test).

Figure 6 compares serum levels of hyaluronic acid among patients infected with genotype A in whom HBeAg persisted, who seroconverted and who had remained HBeAg-negative. Hyaluronic acid increased in four of the five (80%) patients in whom HBeAg persisted in contrast to only one of the seven (14%) patients without HBeAg. Increases in serum levels of hyaluronic acid ≥10 ng/ml was more frequent in the

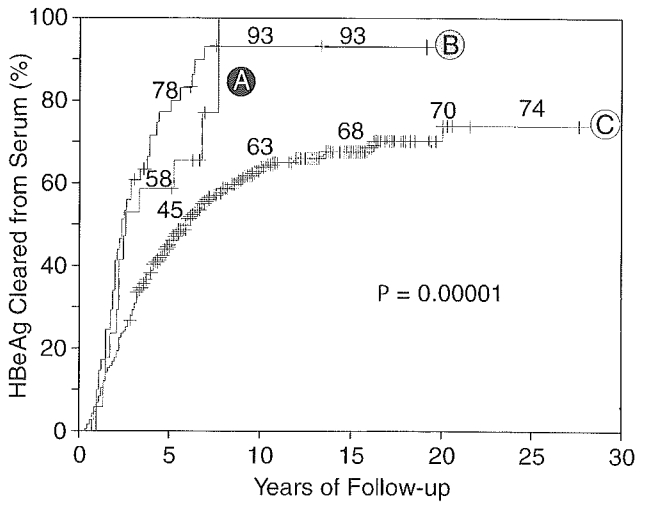


Fig. 2. Seroclearance of HBeAg during follow-up. Clearance rates of HBeAg are compared among patients with chronic hepatitis B who were infected with genotypes A, B, or C by the Kaplan–Meier life table. Differences are significant among genotypes A–C at 5 years as well as between genotypes B and C since 10 years or later by the log-rank test. Seroclearance of HBeAg did not spontaneously occurr in all of them.

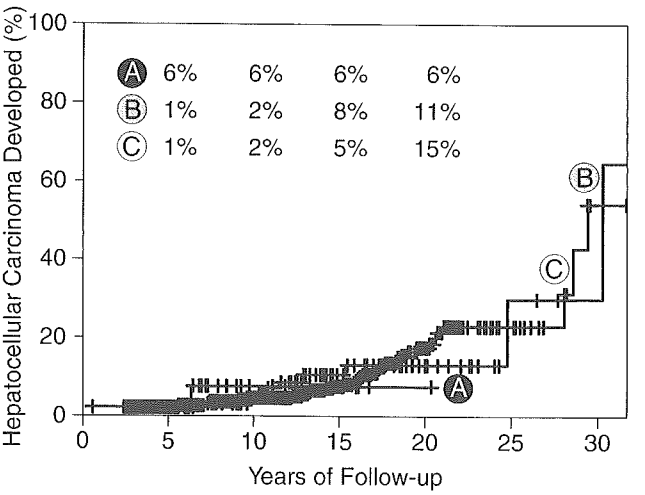


Fig. 4. Development of hepatocellular carcinoma in patients infected with HBV genotype A, B, or C.

TABLE II. Baseline Characteristics of the 26 Patients Infected With HBV Genotype A in Whom HBeAg Persisted, Who Seroconverted and Who Were Without HBeAg at the Presentation

| Feature | HBeAg persisted (n = 5) | Seroconverted (n = 14) | Without HBeAg (n = 7) | Differences |
|---|---|---|---|---|
| Age (years)[a] | 49 (24–59) | 30 (13–60) | 33 (14–41) | NS[c] |
| Male | 5 (100%) | 14 (100%) | 5 (71%) | NS |
| Follow-up (years)[a] | 6.2 (3.7–7.4) | 9.2 (3.0–21) | 8.1 (3.9–17) | NS |
| History of transfusion | 0 | 1 (7%) | 0 | NS |
| Anti-HCV | 0 | 0 | 1 (14%) | NS |
| HBV DNA (log copies/ml) | >7.6 (all patients) | >7.6 (6.7–>7.6) | 4.1 (<2.6–7.1) | $P = 0.03$ |
| Subgroups (Aa/Ae/ND[b]) | (0%/80%/20%) | (7%/79%/14%) | (29%/57%/14%) | NS |

[a]Median values are shown with the range in parentheses.
[b]Not determined.
[c]Not significant.

patients with persistent HBeAg than in those with seroconversion and those without HBeAg (80% [4/5] vs. 14% [2/14] and 14% [1/7], $P = 0.017$ by the Fisher's exact test).

Of the 19 hepatitis patients presenting with serum HBeAg, 16 received antiviral and/or steroid withdrawal therapies, and 11 (69%) responded by the loss of HBeAg, while the remaining 4 failed to do so (Table III). There were three patients in whom HBeAg disappeared without receiving treatments. In total, therefore, seroconversion was accomplished in 14 of the 19 (74%) patients with genotype A.

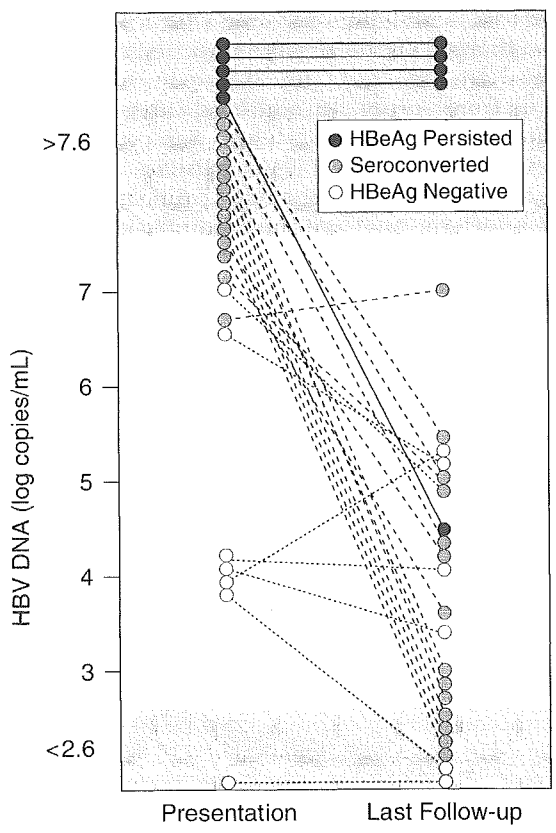


Fig. 5. Changes in serum levels of HBV DNA from the baseline to the last follow-up. Patients in whom HBeAg persisted, who seroconverted and who were without HBeAg at the baseline are compared.

## DISCUSSION

Of the eight genotypes of HBV, E, and F are local, and confined to Central Africa and Central/South America, respectively [Magnius and Norder, 1995; Miyakawa and Mizokami, 2003]. Genotype H is genetically close to F and distributes in Central America [Arauz-Ruiz et al., 2002]. Genotype G occurs very rarely [Stuyver et al., 2000; Chu et al., 2003; Kato et al., 2004], and is always

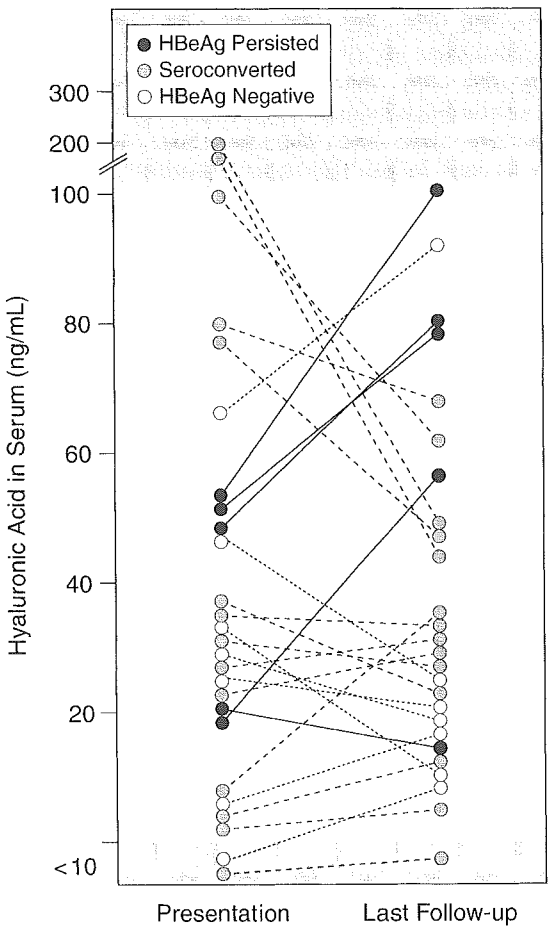


Fig. 6. Changes in serum levels of hyaluronic acid from the baseline to the last follow-up. Patients in whom HBeAg persisted, who seroconverted and who were without HBeAg at the baseline are compared.

TABLE III. Loss of HBeAg in the 19 Hepatitis Patients Infected With HBV Genotype A Who Had Been Followed for Longer Than 3 Years

| Case No. | Sex/age | Pathology | Sub-group | Treatment | HBeAg Lost |
|---|---|---|---|---|---|
| 1 | M23 | F1/A1 | Ae | Interferon | Yes |
| 2 | M33 | F2/A1 | Ae | Interferon | Yes |
| 3 | M44 | F3/A1 | Ae | Interferon | Yes |
| 4 | M57 | F2/A1 | Ae | Interferon | Yes |
| 5 | M13 | F1/A1 | Ae | Steroid withdrawal | Yes |
| 6 | M16 | F1/A1 | Ae | Steroid withdrawal | Yes |
| 7 | M28 | F1/A1 | ND | Steroid withdrawal | Yes |
| 8 | M47 | F2/A1 | Aa | Steroid withdrawal | Yes |
| 9 | M17 | F1/A1 | Ae | Steroid/Interferon | Yes |
| 10 | M29 | F1/A1 | Ae | Lamivudine | Yes |
| 11 | M38 | F1/A1 | Ae | Lamivudine | Yes |
| 12 | M30 | F1/A0 | Ae | None | Yes |
| 13 | M39 | F1A1 | Ae | None | Yes |
| 14 | M47 | F3/A2 | Ae | None | Yes |
| 15 | M24 | F2/A2 | Ae | Interferon and others[b] | No |
| 16 | M43 | F2/A1 | Ae | Steroid/Interferon | No |
| 17 | M48 | F1/A2 | Ae | Interferon/Lamivudine | No |
| 18 | M49 | F1/A1 | ND[a] | Steroid withdrawal | No |
| 19 | M59 | F1A1 | Ae | Interferon | No |

[a]Not determined.
[b]The patient received interferon, lamivudine interferon/lamivudine, and then lamivudine plus entecavir.

co-infected with HBV of the other genotypes [Kato et al., 2002, 2003]. Thus, only four genotypes (A–D) are left for comparison in epidemiological and clinical studies in most countries of the world. Since even these four genotypes have distinct geographical distributions, comparison with respect to severity of liver disease or response to antiviral treatment is hardly feasible among them, except in multi-national studies on patients of diverse ethnicities [Westland et al., 2003; Janssen et al., 2005].

In the Toranomon Hospital in Tokyo, by far the most patients presenting with HBsAg were infected with HBV of genotype B (10.5%) or C (83.3%), and genotype A infected only a minority (3.10%) of them. During 31 years, 128 patients with genotype A visited there. Unlike most infections with genotype B and C transmitted perinatally from carrier mothers with HBeAg [Okada et al., 1976], genotype A infection in Japan is often acquired in the adulthood by men having extramarital sexual contacts either with men or women; there has been no evidence for maternal transmission of HBV genotype A in Japan [Kobayashi et al., 2002, 2003; Ogawa et al., 2002; Suzuki et al., 2005]. HBV infection prevails among homosexuals in Western countries where genotype A is frequent, who poorly respond to vaccines [Goilav and Piot, 1989]. Genotype A infection in Japan has a propensity to become chronic and tends to respond to antiviral therapies better than genotype B or C infection [Kobayashi et al., 2002, 2003 Suzuki et al., 2005].

In the present study, we have compared the virological outcome among infections with HBV genotypes A, B, and C, and found substantial differences. Patients with genotype A fared better than those with genotype B or C in that they cleared HBsAg and HBeAg faster during follow-up (Figs. 1 and 2). It is not certain, however, whether or not the observed differences are influenced by the duration of HBV infection. HBV genotype A is contracted predominantly by men in the adulthood and genotypes B or C had been transmitted perinatally until 1986 when the national immunoprophylaxis started. It needs to be pointed out that this study is retrospective in nature, and most patients with HBeAg had received interferon, lamivudine or steroid withdrawal, or combination thereof. Of the 16 patients with genotype A who received treatment, 11 (69%) responded and cleared HBeAg from serum. In addition, three patients lost HBeAg spontaneously. Hence seroconversion was achieved in 14 of the 19 (74%) patients with genotype A. In view of lamivudine, adefovir dipivoxil, and pegylated interferon that are reported efficacious in treatment of chronic hepatitis B [Perrillo et al., 2000; Hadziyannis et al., 2003; Kumada, 2003; Janssen et al., 2005], it would be unethical to evaluate genotype-dependent differences in the natural course of persistent HBV infection.

Of the 45 individuals chronically infected with HBV genotype A and had been followed for 3 years or longer, HBeAg was more frequent and HBV DNA levels higher in the 26 patients with biopsy-proven chronic hepatitis than in the 19 asymptomatic carriers. Among the 26 patients with genotype A, HBeAg persisted throughout the observation in 5 (19%) and disappeared in 14 (54%); HBeAg remained negative in the other 7 (27%) patients. HBV DNA stayed in high levels more frequently ($P = 0.0095$) in the patients with persistent HBeAg (80% [4/5]) than in those who seroconverted (7% [1/14]) or remained HBeAg-negative (29% [2/7]). Furthermore, increases in serum hyaluronic acid $\geq$10 ng/ml were more frequent ($P = 0.017$) in the patients with persistent HBeAg (80% [4/5]) than in those with seroconversion (14% [2/17]) or HBeAg-negative (14% [1/7]). Although the patients with genotype A fare better than those with genotype B or C, persistent HBeAg refractory to

treatment would predict ongoing liver disease with fibrosis in progress.

Recently, subgenotypes have been recognized and they may influence the biology of HBV and liver disease. For instance, a subgenotype of B having the recombination with genotype C (Ba) induces more severe liver disease with poorer response to lamivudine than that without the recombination (Bj) [Sugauchi et al., 2002, 2003; Akuta et al., 2003]. As for genotype A, there are two subgenotypes with different geographical distributions. Subgenotype Ae is common in Europe and the United States, while Aa is prevalent in Asia and Africa [Bowyer et al., 1997; Sugauchi et al., 2004]. In a case-control study, HBeAg was more frequent and HBV DNA levels higher in carriers of Ae than Aa [Tanaka et al., 2004]. The majority of genotype A strains from our patients (86%) were found to be Ae; they were probably introduced to Japan by immigrants and visitors from foreign countries [Kobayashi et al., 2004]. Cirrhosis and hepatocellular carcinoma developed in only one of the 19 (5%) patients infected with subgenotype Ae, in remarkable contrast to frequent hepatocellular carcinoma in Africa where infection with subgenotype Aa is common during the infancy [Kew et al., 2005].

Although there have been accumulating lines of evidence for virological and clinical influence of HBV genotypes, there are conflicting views on them. Differences between genotypes B and C in Asia [Kao et al., 2000; Orito et al., 2001; Tsubota et al., 2001; Chan et al., 2004; Yu et al., 2005] have not been reproduced, probably due to selection bias for the patients with severe disease [Sumi et al., 2003] or subgenotypes of B different between Japan (Bj) and Hong Kong (Ba) [Yuen et al., 2004]. Liver disease, once advanced beyond a certain severity, will progress spontaneously irrespective of HBV genotypes. Subgenotype Ba having the recombination with genotype C may be endowed with a higher disease-inducing capacity than subgenotype Bj without the recombination [Sugauchi et al., 2002].

Of patients infected with three different genotypes in Japan, the virological outcome of persistent HBV infection was more favorable for those with genotype A than B and C in that order. It is not known where genotype D stands, although it fares worse than genotype A in chronic HBV infection [Thakur et al., 2002; Janssen et al., 2005]. In ranking the four major genotypes (A–D) in disease-inducing capacity and response to antiviral therapies, perinatals, or adulthood transmission, as well as subgenotypes inherent to countries, would have to be taken into considerations [Sugauchi et al., 2002, 2004; Norder et al., 2004].

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# NAD(P)H Oxidase Plays a Crucial Role in PDGF-Induced Proliferation of Hepatic Stellate Cells

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**The proliferation of hepatic stellate cells (HSCs) is a critical step in hepatic fibrogenesis. Platelet-derived growth factor (PDGF) is the most potent mitogen for HSCs. We investigated the role of nonphagocytic NAD(P)H oxidase–derived reactive oxygen species (ROS) in PDGF-induced HSC proliferation. The human HSC line, LI-90 cells, murine primary-cultured HSCs, and PDGF-BB were used in this study. We examined the mechanism of PDGF-BB-induced HSC proliferation in relation to the role of a ROS scavenger and diphenylene iodonium, an inhibitor of NAD(P)H oxidase. We also measured ROS production with the aid of chemiluminescence. We showed that PDGF-BB induced proliferation of HSCs through the intracellular production of ROS. We also demonstrated that HSCs expressed key components of nonphagocytic NAD(P)H oxidase ($p22^{phox}$, $gp91^{phox}$, $p47^{phox}$, and $p67^{phox}$) at both the messenger RNA and protein levels. Diphenylene iodonium suppressed PDGF-BB–induced ROS production and HSC proliferation. Coincubation of $H_2O_2$ and PDGF-BB restored the proliferation of HSCs that was inhibited by diphenylene iodonium pretreatment. Phosphorylation of the mitogen-activated protein kinase (MAPK) family constitutes a signal transduction pathway of cell proliferation. Our data demonstrate that NAD(P)H oxidase–derived ROS induce HSC proliferation mainly through the phosphorylation of p38 MAPK. Moreover, an *in vivo* hepatic fibrosis model also supported the critical role of NAD(P)H oxidase in the activation and proliferation of HSCs. In conclusion, NAD(P)H oxidase is expressed in HSCs and produces ROS via activation of NAD(P)H oxidase in response to PDGF-BB. ROS further induce HSC proliferation through the phosphorylation of p38 MAPK.** (HEPATOLOGY 2005;41:1272-1281.)

In chronic liver diseases, regardless of their nature (viral infection, alcohol abuse, metal overload), the progression of hepatic fibrosis is associated with the development of portal hypertension and the occurrence of hepatocellular carcinoma. Hepatic stellate cells (HSCs) are increasingly being recognized as the key mediators of the progression of hepatic fibrosis.[1] In chronic liver diseases, HSCs undergo a process of activation, developing a myofibroblast-like phenotype that is associated with increased proliferation, chemotaxis, and collagen synthesis.[2] Of these, the degree of fibrogenesis that occurs in liver diseases is most likely to be affected by an increased number of HSCs, which results from their proliferation.[3] The results of *in vitro* and *in vivo* studies suggest that platelet-derived growth factor (PDGF) is the most potent mitogen of HSCs and is therefore likely to be an important mediator of the increased proliferation of HSCs during hepatic fibrogenesis in chronic liver diseases.[4,5] Immunohistochemistry and *in situ* hybridization studies have revealed that PDGF and PDGF receptors are overexpressed at both the messenger RNA (mRNA) and protein levels in liver tissue from patients with chronic hepatitis or cirrhosis, and are positively correlated with the severity of histological lesions and collagen deposition.[6,7] These reports suggest strongly that PDGF facilitates the progression of hepatic fibrosis in human chronic liver diseases. There-

*Abbreviations: HSC, hepatic stellate cell; PDGF, platelet-derived growth factor; NAD(P)H, nicotinamide-adenine dinucleotide phosphate; ROS, reactive oxygen species; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; Mn-TBAP, manganese (III) tetrakis (benzoic acid) porphyrin chloride; DPI, diphenylene iodonium; PMAC, 10-methyl-9-(phenoxycarbamoyl)acridinium fluorosulfonetae; ERK, extracellular-signal-related kinase; PCNA, proliferating cell nuclear antigen; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; IP, intraperitoneal; DMN, dimethylnitrosamine.*

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fore, clarification of the cellular and molecular mechanisms underlying the PDGF-induced proliferation of HSCs will aid in the development of a new antifibrotic therapy for chronic liver diseases.

Reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide ($H_2O_2$), and hydroxyl radical can stimulate HSC proliferation and collagen synthesis.[8,9] Although both PDGF and ROS have been implicated in the initiation and progression of hepatic fibrogenesis, the relationship between PDGF and ROS in HSC proliferation has yet to be clarified. Recently, it has been shown that nonphagocytic nicotinamide-adenine dinucleotide phosphate [NAD(P)H] oxidase exists in vascular smooth muscle cells and endothelial cells and is involved in the proliferation of these types of cells through the continuous, low-level intracellular production of ROS.[10-12] A previous *in vivo* study has shown that $p22^{phox}$ and $gp91^{phox}$, which are anchored in the cell membrane, are expressed in HSCs at both the mRNA and protein levels.[13] However, it is presently uncertain whether PDGF causes HSC proliferation through NAD(P)H oxidase–derived ROS. In the present study, we examined whether key components of NAD(P)H oxidase—$p22^{phox}$, $gp91^{phox}$, $p47^{phox}$, and $p67^{phox}$—are expressed in HSCs and investigated whether PDGF enhances the production of NAD(P)H-derived ROS. Moreover, we clarified the role of NAD(P)H oxidase–derived ROS in the process of PDGF-induced HSC proliferation. Here we have shown that NAD(P)H oxidase–derived ROS play a critical role in PDGF-mediated HSC proliferation and the *in vivo* hepatic fibrosis model.

## Materials and Methods

***Reagents.*** Human recombinant PDGF-BB was purchased from Austral Biologicals (San Ramon, CA). Manganese (III) tetrakis (benzoic acid) porphyrin chloride (Mn-TBAP) was purchased from Cayman Chemical Company (Ann Arbor, MI). Diphenylene iodonium (DPI) and 10-methyl-9-(phenoxycarbamoyl)acridinium fluorosulfonate (PMAC) were purchased from Dojindo Laboratories (Kumamoto, Japan). Allopurinol, indomethacin, apocynin, PD98059, and SB203580 were obtained from Sigma Chemical Company (St. Louis, MO). All other chemicals and reagents were of analytical grade and, if not stated, were purchased from Sigma or from Wako Pure Chemical Industries (Osaka, Japan).

***Antibodies.*** Rabbit anti-human $p22^{phox}$, $gp91^{phox}$, $p47^{phox}$, and $p67^{phox}$ antibodies were kindly provided by Dr. S. Imajoh-Ohmi.[14] Rabbit anti-phospho–extracellular signal-related kinase (ERK) and anti-phospho–p38 mitogen-activated protein kinase (p38 MAPK) antibodies were obtained from Cell Signaling Technology, (Beverly, MA). Mouse anti–α-smooth muscle actin and anti-proliferating cell nuclear antigen (PCNA) were obtained from DAKO (Kyoto, Japan).

***Cell Culture.*** The HSC line LI-90, which exhibits characteristics compatible with those of human HSCs, was used in this study.[15] LI-90 was kindly provided by Human Science Cell Bank (Saitama, Japan). The experiments were performed on cells between the third and tenth serial passages (1:3 split ratio) using originally supplied LI-90.

Primary-cultured HSCs were isolated from female BALB/c mice (6 weeks old) via *in situ* collagenase perfusion and differential centrifugation on Nycodenz (Pharma AS, Oslo, Norway) density gradients, as previously described.[16] Cultured HSCs at second passage were used in this study. Both LI-90 cells and primary-cultured HSCs were cultured in a 5% $CO_2$ humidified incubator at 37°C. Dulbecco's Modified Eagle Medium (DMEM) (GIBCO BRL, Rockville, MD) containing 10% fetal bovine serum (FBS) (Filton, Brooklyn, Australia) was used as the growth medium. After cells became subconfluent (at 70%-80% confluence), the cells were cultured with DMEM without containing phenol red and FBS for 24 hours (serum starvation) before the start of all experiments.

***Cell Proliferation Assay.*** LI-90 cells or primary-cultured HSCs were plated in 96-well microplates (Sumitomo Bakelite Co., Tokyo, Japan) at a density of $7 \times 10^3$ cells/well in complete culture medium. The cells proliferation assay was performed using the Prex WST-1 cell proliferation assay system (Takara, Osaka, Japan).[17] The number of viable cells was estimated at a wavelength of 450 nm on an enzyme-linked immunosobent assay plate reader 1 hour after the addition of WST-1. To confirm that the cell proliferation assay reflected the increase in cell number, the number of cells was counted by the trypan blue exclusion method after trypsinization.

DNA synthesis was also measured using a Biotrack cell proliferation ELISA system (Amersham, Little Chalfont, UK).[18] During the last 4 hours, the cells were labeled with bromodeoxyuridine. After removing the culture medium, the cells were fixed and the incorporated bromodeoxyuridine was detected according to the manufacturer's recommended protocol.

***Reverse-Transcription Polymerase Chain Reaction.*** Total cellular RNA was extracted from the cells, using Isogen (Nippon Gene, Tokyo, Japan) as described in the product protocol. cDNA was generated from 1 μg of total RNA, using random hexanucleotide primers, and reverse-transcription polymerase chain reaction was performed. The specific primers were the same as previously reported.[11]

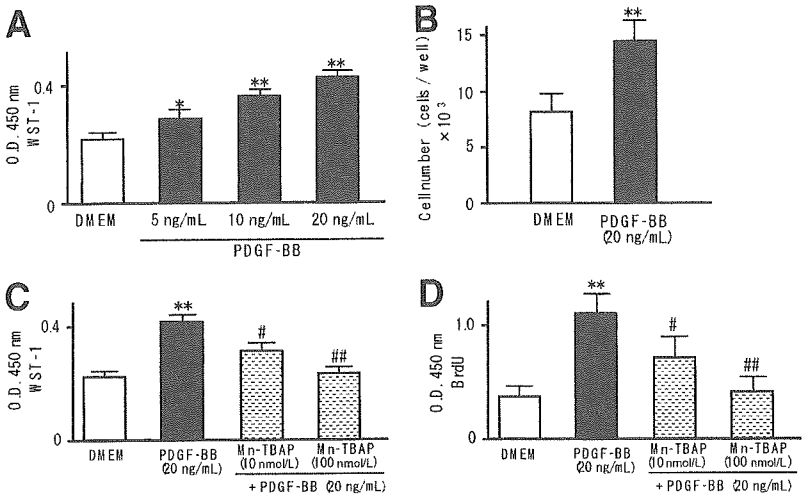


Fig. 1. ROS-mediated proliferation of HSCs induced by PDGF-BB. (A) PDGF-BB dose-dependently increased LI-90 proliferation as demonstrated via WST-1 assay. (B) PDGF-BB also increased the number of cells as demonstrated by the trypan blue exclusion method. (C) Mn-TBAP significantly reduced the PDGF-BB–mediated proliferation of cells. (D) Mn-TBAP dose-dependently inhibited the DNA synthesis induced by PDGF-BB. Data are expressed as the mean ± SD of 5 independent experiments. $*P <$ .01 and $**P <$ .001 compared with the unstimulated control group. $^{\#}P <$ .01 and $^{\#\#}P <$ .001 compared with the PDGF-BB group. DMEM, Dulbecco's Modified Eagle Medium; PDGF, platelet-derived growth factor; Mn-TBAP, manganese (III) tetrakis (benzoic acid) porphyrin chloride; BrdU, bromodeoxyuridine; O.D., optical density.

***Immunoblots and Immunocytochemistry.*** Western blotting (10% SDS-PAGE) was performed as described elsewhere.[19] The bands were visualized using a Lumiglo Substrate Kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

Immunocytochemistry was performed using antibodies recognizing $p22^{phox}$ (1:100 dilution), $gp91^{phox}$ (1:100 dilution), $p47^{phox}$ (1:100 dilution), or $p67^{phox}$ (1:100 dilution). Immunostaining of the cells was accomplished using a commercial kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol.

***Measurement of ROS Production in HSCs.*** To measure intracellular ROS production in LI-90 cells and primary-cultured HSCs, we used a modification of a previously described method using PMAC-enhanced chemiluminescence.[20] This method is based on the reaction between reduced PMAC and ROS, which results in the emission of light. The chemiluminescence images were acquired with a high-performance intensified CCD camera (PI-MAX 512RB Princeton Instruments, Nippon Roper, Chiba, Japan) that was placed 30 cm above a black polystyrene 96-well plate containing HCSs set in a dedicated black box.[21] Chemiluminescence was measured every 2 minutes for 0 to 16 minutes after the addition of phosphate-buffered saline (PBS) containing PDGF-BB and PMAC.

***Animals and Treatment.*** The *in vivo* experiments were performed in accordance with the Yamagata University School of Medicine Guidelines for the Care and Use of Laboratory Animals. Specific pathogen-free BALB/c female mice (6 weeks old) were used. Hepatic fibrosis was induced by intraperitoneal (IP) injection of 10 mg/kg dimethylnitrosamine (DMN) in saline three times a week for the indicated periods for 6 weeks. Daily IP administration of Mn-TBAP (5 mg/kg) or DPI (1 mg/kg) was begun simultaneously with DMN administration. Animals were divided into the following groups: (1) control (daily IP administration of 0.1 mL PBS [n = 6]); (2) DMN (daily IP administration of 0.1 mL PBS [n = 6]); (3) DMN + Mn-TBAP (daily IP administration of 0.1 mL Mn-TBAP solution in PBS [1 mg/mL] [n = 6]); and (4) DMN + DPI (daily IP administration of 0.1 mL DPI solution in PBS [0.2 mg/mL] [n = 6]).

***Histological and Immunohistochemical Examination.*** Liver specimens were routinely fixed in 4% formaldehyde in PBS and embedded in paraffin. Tissue sections (4 $\mu$m thick) were either stained with Sirius red or subjected to immuno-histostaining with antibodies against PCNA. Specific staining was visualized using a commercial kit (Vector Laboratories).

***Statistical Analysis.*** Data are expressed as the mean ± SD. Statistical comparisons were made using the Student *t* test for unpaired samples. For studies involving more than two groups, a two-way ANOVA was determined using Scheffe's test. The level of statistical significance was set at $P <$ .05 for all cases.

## Results

***ROS Scavenger Suppressed the PDGF-BB–Induced Proliferation of HSCs.*** PDGF-BB induced a dose-dependent increase in LI-90 proliferation, as measured via WST-1 assay (Fig. 1A). PDGF-BB at a concentration of 5 ng/mL, 10 ng/mL, and 20 ng/mL significantly increased cell proliferation after 24 hours of incubation compared with the unstimulated control cells. To assess whether the PDGF-BB–induced increase in formazan formation measured via WST-1 assay was really associated with cell growth, cell count experiments were also performed with the aid of trypsinization. As shown in Fig. 1B, the addition of PDGF-BB (20 ng/mL) in FBS-free DMEM caused a significant increase in cell number.

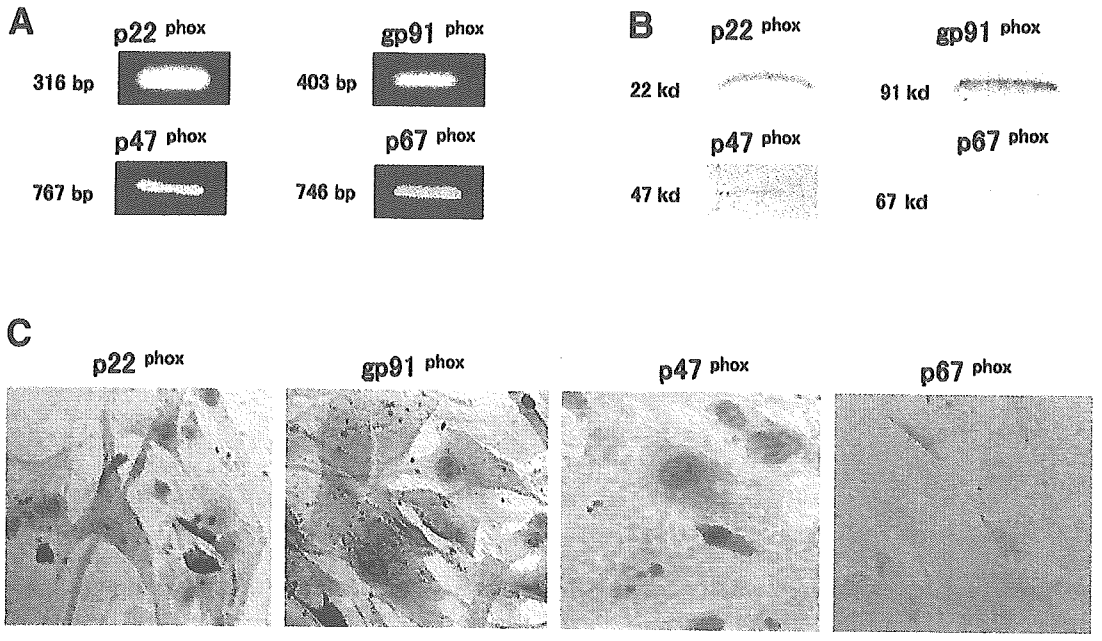


Fig. 2. Expression of NAD(P)H oxidase components in HSCs. LI-90 cells were seeded onto chamber slides (Nunc Lab-TekTM II-Chamber Slide; Nalge Nunc, Rochester, NY) and were cultured with DMEM containing 10% FBS. The cells were then fixed with 4% formaldehyde in PBS for 15 minutes at room temperature. (A) Cultured cells expressed 4 enzymatic subunits of NAD(P)H oxidase at the mRNA level. (B) Western blotting analysis showed that cells express $p22^{phox}$, $gp91^{phox}$, $p47^{phox}$, and $p67^{phox}$ at the protein level. Whole cell extracts were obtained in lysis buffer containing protease and phosphatase inhibitors. Proteins (50 $\mu$g) were run on 10% SDS polyacrylamide gels. (C) Immunocytochemistry of cells showed positive staining for the key enzymatic components of NAD(P)H oxidase: $p22^{phox}$, $gp91^{phox}$, $p47^{phox}$, and $p67^{phox}$ (original magnification, ×400).

We pretreated LI-90 cells with Mn-TBAP, a cell-permeable SOD/catalase mimetic, as intracellular ROS scavenger for 30 minutes. As shown in Fig. 1C, Mn-TBAP dose-dependently suppressed the PDGF-BB–induced proliferation of cells. Particularly, Mn-TBAP at a concentration of 100 nmol/L significantly suppressed the PDGF-BB–induced proliferation of cells, and this same dose did not exert any toxic effects on cells after incubation times as long as 36 hours (data not shown). Bromodeoxyuridine analysis showed that PDGF-BB at a concentration of 20 ng/mL significantly stimulated DNA synthesis. Mn-TBAP dose-dependently inhibited the DNA synthesis induced by PDGF-BB (Fig. 1D).

***HSCs Express Four Enzymatic Subunits of NAD(P)H Oxidase.*** Reverse-transcription polymerase chain reaction analysis showed that four enzymatic subunits of NAD(P)H oxidase were expressed at the mRNA level (Fig. 2A). Western blot analysis revealed that four enzymatic subunits of NAD(P)H oxidase were expressed at the protein level in LI-90 cells (Fig. 2B). Immunocytochemical analysis of cultured cells showed positive staining for $p22^{phox}$, $gp91^{phox}$, $p47^{phox}$, and $p67^{phox}$ (Fig. 2C). No cultured cells showed positive immunostaining when the antibodies were replaced with a control serum (data not shown).

***NAD(P)H Oxidase Is the Main Source of PDGF-BB–Induced ROS Production by HSCs.*** To investigate whether LI-90 cells produced ROS in response to PDGF-BB, we incubated cells with PDGF-BB (20 ng/mL) and PMAC (500 $\mu$mol/L) and measured the PMAC-enhanced chemiluminescence with a high-performance intensified CCD camera at an interval of 2 minutes. PDGF-BB caused a marked increase in the chemiluminescence from PMAC immediately after the incubation. The chemiluminescence observed in response to PDGF-BB peaked at 6 minutes after the incubation and declined gradually thereafter (Fig. 3A). To confirm that the chemiluminescence accurately reflected the increased production of ROS, we examined whether Mn-TBAP, a cell-permeable ROS scavenger, was able to decrease the enhanced chemiluminescence by PDGF-BB. The chemiluminescence was completely abolished when cells were preincubated with Mn-TBAP for 30 minutes (Fig. 3B). However, superoxide dismutase, a cell-nonpermeable enzyme, failed to inhibit the enhanced chemiluminescence induced by PDGF-BB (data not shown). Pretreatment with DPI for 30 minutes, an inhibitor of NAD(P)H oxidase, remarkably reduced PDGF-BB–induced chemiluminescence (Fig. 3B). Pretreatment with apocynin (100 $\mu$mol/L), another type of NAD(P)H oxidase inhibitor, for 1 hour also reduced the enhanced chemiluminescence induced by PDGF-BB (data not shown).

A high concentration of DPI inhibits flavoproteins including NAD(P)H oxidase, mitochondrial oxidases, xanthine oxidase, and cyclooxygenase. To ascertain that the

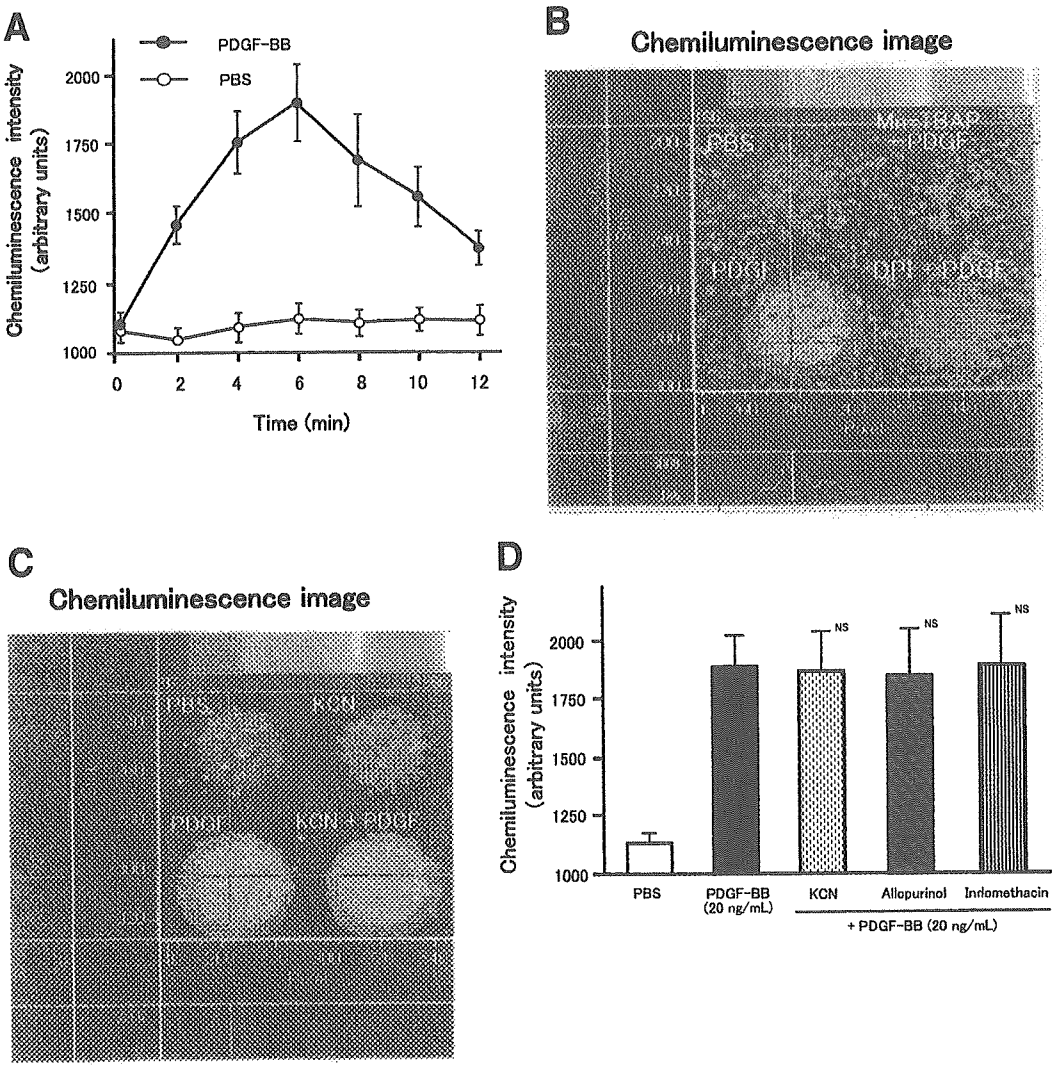


Fig. 3. ROS formation in HSCs as a result of stimulation by PDGF-BB. LI-90 cells were grown on black polystyrene 96-well microplates (Corning, NY) in DMEM containing 10% FBS. After cells became subconfluent, they were cultured with DMEM not containing phenol red or FBS for 24 hours. The cells were rinsed twice with PBS. The cells were then incubated with the fresh PBS containing PDGF-BB and PMAC, and the resulting chemiluminescence was measured. (A) Time course of the changes in chemiluminescence. These changes were monitored in the presence of PDGF-BB and PMAC using a high-performance intensified camera. The chemiluminescence in cells in response to PDGF-BB gradually increased and peaked at 6 minutes after the incubation. Data are given as the mean ± SD of 4 independent experiments. (B) Pretreatment with Mn-TBAP (100 nmol/L) or DPI (25 μmol/L) suppressed the PDGF-BB-induced chemiluminescence. (C) Pretreatment of cells with KCN (500 μmol/L) did not reduce the formation of PDGF-BB-derived ROS in cells. (D) Pretreatment of cells with either allopurinol (100 μmol/L) or indomethacin (100 μmol/L) did not significantly reduce the PDGF-BB-derived ROS formation. Data are expressed as the mean ± SD of 5 independent experiments. The figures are representative of at least 4 independent experiments. PDGF, platelet-derived growth factor; PBS, phosphate-buffered saline; Mn-TBAP, manganese (III) tetrakis (benzoic acid) porphyrin chloride; DPI, diphenylene iodonium; KCN, potassium cyanide; NS, not significant compared with PDGF-BB group.

enhanced chemiluminescence did not originate from a mitochondrial electron transport system, we examined whether the mitochondrial poison potassium cyanide (KCN) inhibited the PDGF-BB–enhanced chemiluminescence. As shown in Fig. 3C, pretreatment with KCN (500 μmol/L) for 30 minutes had no significant effect on the enhanced chemiluminescence induced by PDGF-BB. Preincubation for 1 hour with a maximally effective concentration of allopurinol (an inhibitor of xanthine oxidase: 100 μmol/L) or indomethacin (an inhibitor of cyclooxygenase: 100 μmol/L) failed to inhibit the PDGF-BB–mediated enhanced chemiluminescence (Fig. 3D).

***NAD(P)H Oxidase–Derived ROS Are Essential to the PDGF-BB–Induced Proliferation of HSCs.*** To elucidate whether PDGF-BB–induced proliferation of LI-90 cells was mediated by the activation of NAD(P)H oxidase, we examined the effect of DPI on the PDGF-BB–induced proliferation of cells. Pretreatment of the cells with DPI (25 μmol/L) for 30 minutes significantly inhibited the PDGF-BB–induced proliferation of cells

(Fig. 4). DPI at a concentration of 25 μmol/L did not exert any toxic effects on cells after incubation times as long as 36 hours (data not shown). Pretreatment with apocynin (100 μmol/L), another type of NAD(P)H oxidase inhibitor, for 1 hour also inhibited the PDGF-BB–induced proliferation of cells (Fig. 4). Preincubation with neither indomethacin (an inhibitor of cyclooxygenase) nor allopurinol (an inhibitor of xanthine oxidase) for 1 hour attenuated the PDGF-BB–induced proliferation of cells (Fig. 4). Coincubation of $H_2O_2$ and PDGF-BB restored the LI-90 proliferation that was inhibited by DPI pretreatment (Fig. 4).

***PDGF-BB Mediates the Phosphorylation of p38 MAPK Through the Activation of NAD(P)H Oxidase.*** PDGF-BB–induced LI-90 proliferation was significantly suppressed by preincubation for 1 hour with PD98059 (an inhibitor of MEK upstream activator of ERK: 25 μmol/L) or SB203580 (an inhibitor of p38 MAPK: 25 μmol/L). The degree of suppression was more significant with SB203580 than with PD98059 (Fig. 5A). To investigate the relationship between NAD(P)H oxidase–derived ROS and the phosphorylation of MAPKs, we first examined the effect of DPI or Mn-TBAP on the phosphorylation of ERKs and p38 MAPK in cells. PDGF-BB caused the phosphorylation of ERKs and p38 MAPK in cells, with a peak at 10 to 15 minutes (Fig. 5B). Preincubation of cells with PD98059 or SB203580 for 1 hour led to the inhibition of PDGF-induced ERKs or p38 MAPK phosphorylation, respectively (data not shown). As shown in Fig. 5B, preincubation with DPI inhibited the phosphorylation of p38 MAPK but had no effect on the

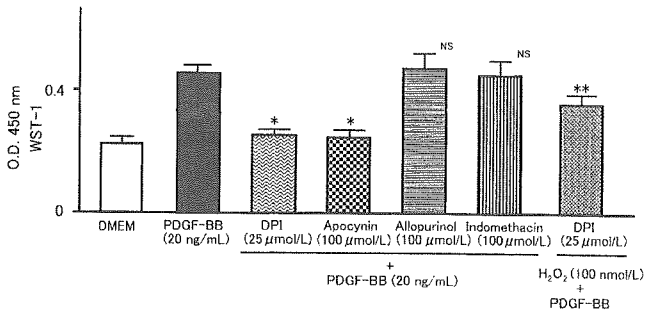


Fig. 4. The role of NAD(P)H oxidase-derived ROS on the PDGF-BB-induced proliferation of HSCs. Two different NAD(P)H oxidase inhibitors, DPI and apocynin, significantly reduced the PDGF-BB-mediated proliferation of LI-90 cells. Preincubation with neither allopurinol nor indomethacin attenuated the PDGF-BB-induced proliferation of cells. Coincubation with $H_2O_2$ (100 nmol/L) and PDGF-BB (20 μmol/L) restored the PDGF-BB-induced proliferation of cells that was inhibited by DPI pretreatment. Data are expressed as the mean ± SD of 5 independent experiments. $*P < .001$ compared with the PDGF-BB group. $**P < .001$ compared with the DMEM and DPI + PDGF-BB groups. NS, not significant compared with PDGF-BB group; DMEM, Dulbecco's Modified Eagle Medium; PDGF, platelet-derived growth factor; DPI, diphenylene iodonium; O.D., optical density.

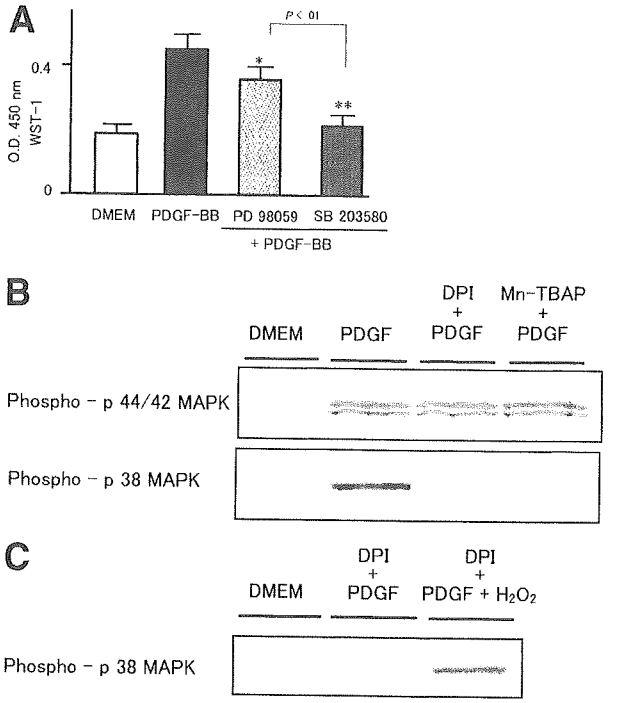


Fig. 5. Involvement of p38 MAPK in PDGF-BB-mediated HSC proliferation. (A) PDGF-BB-induced LI-90 proliferation was significantly suppressed by pretreatment with PD98059 or SB203580. The degree of suppression was more significant with SB203580 than with PD98059. (B) PDGF-BB (20 ng/mL) induced the phosphorylation of ERK and p38 MAPK. Pretreatment with DPI (25 μmol/L) or Mn-TBAP (100 nmol/L) inhibited the phosphorylation of p38 MAPK. However, DPI or Mn-TBAP had no significant effect on the PDGF-BB-mediated phosphorylation of ERK. (C) Coincubation of $H_2O_2$ and PDGF-BB restored the phosphorylation of p38 MAPK that was inhibited by DPI pretreatment. Data are expressed as the mean ± SD of 5 independent experiments and are representative of at least 4 independent experiments. $*P < .01$ and $**P < .001$ compared with the PDGF-BB group. DMEM, Dulbecco's Modified Eagle Medium; PDGF, platelet-derived growth factor; MAPK, mitogen-activated protein kinase; DPI, diphenylene iodonium; Mn-TBAP, manganese (III) tetrakis (benzoic acid) porphyrin chloride; O.D., optical density.

phosphorylation of ERK induced by PDGF-BB. Preincubation with Mn-TBAP also inhibited the phosphorylation of p38 MAPK induced by PDGF-BB but did not affect the phosphorylation of ERKs (Fig. 5B). Coincubation of $H_2O_2$ (100 nmol/L) and PDGF-BB (20 ng/mL) restored the phosphorylation of p38 MAPK that was inhibited by DPI pretreatment (Fig. 5C).

***NAD(P)H Oxidase–Derived ROS Are Essential for the PDGF-BB–Induced Proliferation of Primary Cultured HSCs.*** To ascertain whether the phenomenon observed was not an artifact of the cell line used, we examined the role of NAD(P)H oxidase in PDGF-BB–induced proliferation of murine primary-cultured HSCs. Reverse-transcription polymerase chain reaction analysis showed that four enzymatic subunits of NAD(P)H oxidase were expressed at the mRNA level (Fig. 6A). Western blot analysis revealed that 4 enzymatic subunits of NAD(P)H oxidase were expressed at the protein level in

the primary-cultured HSCs (data not shown). PDGF-BB caused a marked increase in the chemiluminescence from PMAC immediately after the incubation with primary-cultured HSCs, which peaked at 6 to 8 minutes (Fig. 6B). The chemiluminescence was abolished when cells were preincubated with Mn-TBAP or DPI for 30 minutes (Fig. 6C).

As shown in Fig. 6D, PDGF-BB stimulated the DNA synthesis of primary-cultured HSCs. This PDGF-BB–induced DNA synthesis was inhibited by pretreatment with Mn-TBAP, DPI for 30 minutes, or apocynin for 1 hour. Coincubation of $H_2O_2$ and PDGF-BB restored the DNA synthesis that was inhibited by DPI pretreatment for 30 minutes (Fig. 6D). Moreover, PDGF-BB–induced DNA synthesis of primary-cultured HSCs was significantly suppressed by pretreatment with SB203580 or PD98059 for 1 hour. Similar to the result for LI-90, the degree of suppression was more significant with SB203580 than with PD98059 (Fig. 6D).

***Mn-TBAP and DPI Attenuate the Activation and Proliferation of HSCs in a Hepatic Fibrosis Model.*** Murine chronic liver injury was induced via IP injection of DMN to assess the role of NAD(P)H oxidase in hepatic fibrosis. Mice in DMN + Mn-TBAP and DMN + DPI groups showed no evidence of connective tissue septa (Fig. 7A). Western blotting analysis indicated that α-smooth muscle actin expression was increased in DMN-induced fibrotic liver but that Mn-TBAP or DPI reduced the expression of α-smooth muscle actin in DMN-induced hepatic fibrosis (Fig. 7B). Immunostaining for PCNA, an index of HSC proliferation, also showed an increased number of S-phase HSCs in the DMN group and a reduced number of S-phase HSCs in the DMN + Mn-TBAP and DMN + DPI groups (Fig. 7C).

## Discussion

Because PDGF is the most potent mitogen for HSCs, clarification of the mechanisms underlying the PDGF-induced proliferation of HSCs is clinically important for the establishment of new antifibrotic therapies of chronic liver diseases. In the present study, we investigated the mechanisms underlying PDGF-BB–induced HSC proliferation, using not only the human HSC line, LI-90, but also primary-cultured HSCs. PDGF is a dimer of two polypeptide chains, A and B, forming three isoforms known as PDGF-AA, PDGF-AB, and PDGF-BB. Of these, PDGF-BB has the most potent activity.[22] In this study, PDGF-BB induced proliferation of HSCs through the intracellular production of ROS. Moreover, NAD(P)H oxidase–derived ROS stimulated the phosphorylation of p38 MAPK, a stress-activated protein ki-

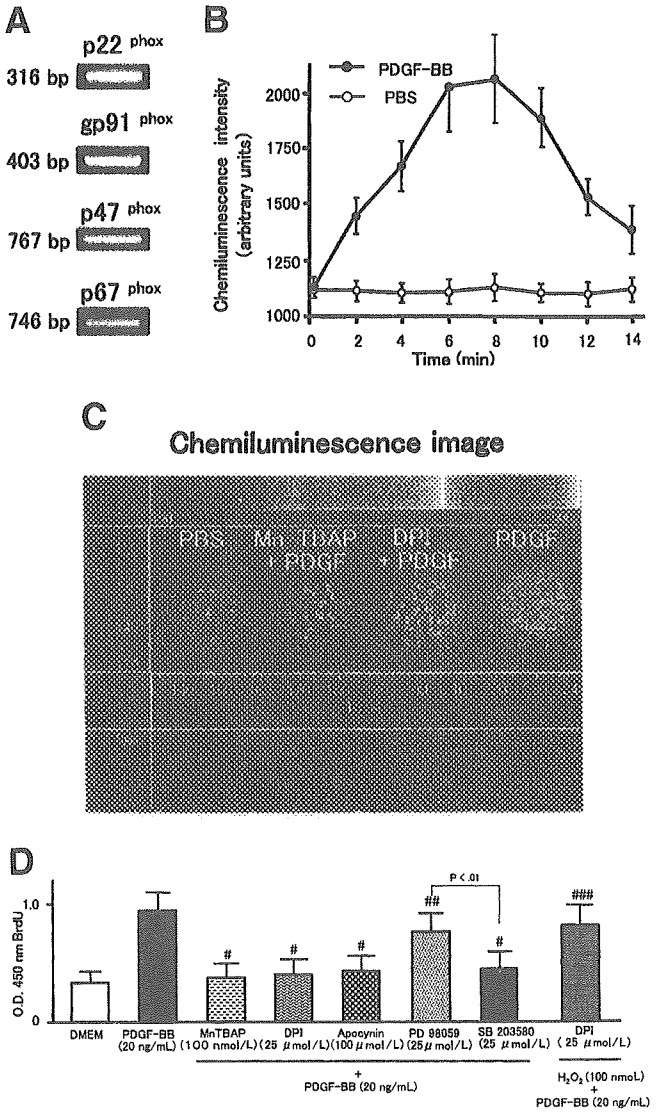


Fig. 6. Critical role of NAD(P)H oxidase–derived ROS in the PDGF-BB–induced proliferation of primary-cultured HSCs. (A) Primary-cultured HSCs expressed 4 enzymatic subunits of NAD(P)H oxidase at the mRNA level. (B) The chemiluminescence in HSCs in response to PDGF-BB (20 ng/mL) gradually increased and peaked at 6 to 8 minutes after the incubation. (C) Pretreatment with Mn-TBAP (100 nmol/L) or DPI (25 μmol/L) suppressed the PDGF-BB–induced chemiluminescence. (D) PDGF-BB (20 ng/mL) significantly increased DNA synthesis of HSCs. Pretreatment with Mn-TBAP (100 nmol/L), DPI (25 μmol/L), or apocynin (100 μmol/L) significantly reduced the PDGF-BB–mediated DNA synthesis of HSCs. PDGF-BB–induced DNA synthesis was significantly suppressed by pretreatment with SB203580 (25 μmol/L) or PD98059 (25 μmol/L) for 1 hour. The degree of suppression was more significant with SB203580 than with PD98059. Coincubation of $H_2O_2$ (100 nmol/L) and PDGF-BB (20 ng/mL) restored the phosphorylation of p38 MAPK that was inhibited by DPI pretreatment. The figure is representative of at least 4 independent experiments. Data are expressed as the mean ± SD of 5 independent experiments. $^{\#}P < .001$ and $^{\#\#}P < .05$ compared with the PDGF-BB group. $^{\#\#\#}P < .001$ compared with the DMEM and DPI + PDGF-BB groups. PDGF, platelet-derived growth factor; PBS, phosphate-buffered saline; Mn-TBAP, manganese (III) tetrakis (benzoic acid) porphyrin chloride; DPI, diphenylene iodonium; DMEM, Dulbecco's Modified Eagle Medium; BrdU, bromodeoxyuridine; O.D., optical density.

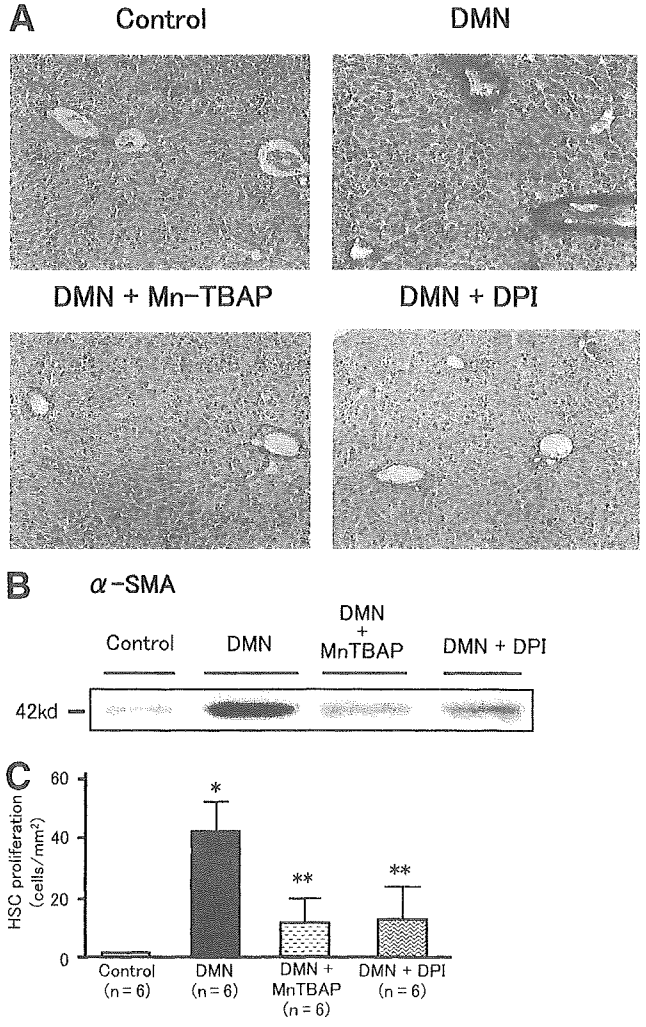


Fig. 7. *In vivo* effect of Mn-TBAP or DPI on activation and proliferation of HSCs induced by DMN. (A) Representative photomicrographs of Sirius red-staining of liver tissues from control, DMN alone, DMN + Mn-TBAP, and DMN + DPI (original magnification, ×100). (B) α-Smooth muscle actin protein expression in liver homogenates via Western blotting. Proteins (50 μg) were run on 10% SDS polyacrylamide gels. (C) HSC proliferation evaluated by immunostaining for PCNA. A total area of 50 $mm^2$ was examined in each liver, and PCNA-positive cell number per square millimeter was calculated. Mn-TBAP and DPI reduced HSC proliferation. The figure is representative of 1 experiment. Data are expressed as the mean ± SD of 6 mice used in the experiment. $*P < .001$ compared with the control group. $**P < .01$ compared with the DMN group. DMN, dimethylnitrosamine; Mn-TBAP, manganese (III) tetrakis (benzoic acid) porphyrin chloride; DPI, diphenylene iodonium; α-SMA, α-smooth muscle actin; HSC, hepatic stellate cell.

nase, and formed the signaling pathway that mediated the PDGF-induced proliferation of HSCs. The selective suppression of NAD(P)H oxidase in HSCs may be relevant to the development of new antifibrotic therapy for chronic liver diseases.

ROS—which include the superoxide anion, $H_2O_2$, and hydroxyl radical—are highly reactive molecules. It is now well established that lower, more "physiological" levels of ROS can exert regulatory roles within the cells. Recently, a series of reports has documented that the intracellular redox system can also modulate protein phosphorylation and dephosphorylation as a result of the presence of redox-sensitive functional groups in the structures of both protein kinases and phosphatases.[23,24] Redox homeostasis also regulates the activation of transcription factors. Our present results show that Mn-TBAP, a cell-permeable ROS scavenger, suppresses the PDGF-BB–induced proliferation of HSCs. The effective concentration of Mn-TBAP was in the nanomole order and is clinically achievable in humans. Moreover, $H_2O_2$ significantly increased HSC proliferation. These results suggest that a low level of ROS induces the proliferation of HSCs and is essential for PDGF-BB–mediated HSC proliferation.

To clarify the mechanism of ROS production in HSCs by PDGF-BB, we detected ROS production by PMAC-enhanced chemiluminescence with a high-performance intensified CCD camera. The ROS detection system we employed in the present study can minimize sampling error and has the advantage of being able to quantify intracellular ROS production by various types of cultured cells.[21] The chemiluminescence was completely abolished when cells were incubated with Mn-TBAP, a cell-permeable ROS scavenger. However, superoxide dismutase, a cell-nonpermeable enzyme, failed to inhibit the enhanced chemiluminescence induced by PDGF-BB. Taken together with the finding that Mn-TBAP significantly inhibited PDGF-induced HSC proliferation, ROS produced after incubation with PDGF-BB appear to be implicated in PDGF-BB–induced HSC proliferation.

NAD(P)H oxidase is a four-subunit enzyme consisting of two membrane components, $p22^{phox}$ and $gp91^{phox}$, and two cytosolic components, $p47^{phox}$ and $p67^{phox}$. A previous *in vivo* study showed that $p22^{phox}$ and $gp91^{phox}$, anchored in the cell membrane, are expressed in HSCs at both the mRNA and protein levels.[13] However, the role of NAD(P)H oxidase in HSCs has not been understood until recently. Bataller et al. reported that NAD(P)H oxidase–derived ROS were involved in the signal transduction pathway of angiotensin II.[25] They showed $p47^{phox}$, $gp91^{phox}$, and Nox1, a homologue of $gp91^{phox}$, were expressed at the mRNA level. In the present study, we demonstrated that $p22^{phox}$, $gp91^{phox}$, $p47^{phox}$, and $p67^{phox}$ were expressed in HSCs at both the mRNA and protein levels. PDGF-BB induced ROS production through the activation of NAD(P)H oxidase, and this was confirmed by the inhibition of ROS production by NAD(P)H oxidase inhibitors, DPI, and apocynin. Because ROS production by NAD(P)H oxidase in HSCs is observed not only in angiotensin II but also in PDGF, as we showed in the present study, NAD(P)H oxidase–