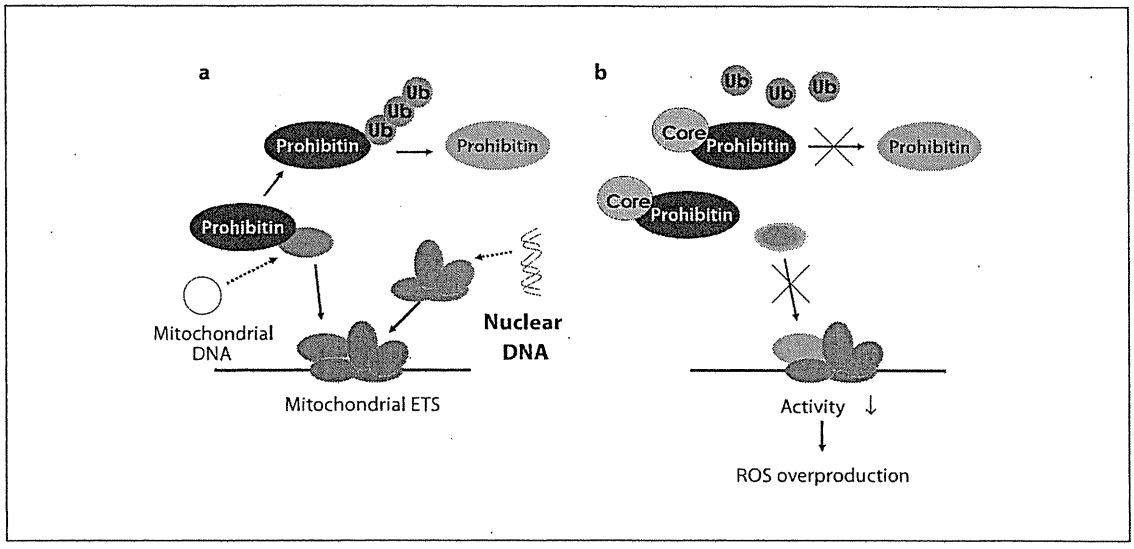

Fig. 1. The HCV core protein binds prohibitin and impairs its chaperone function leading to ROS overproduction. **a** Mitochondrial proteins consist of nuclear DNA-encoded proteins as well as mitochondrial DNA-encoded ones. Prohibitin acts as a protein chaperone for the mitochondrial proteins that are encoded by mitochondrial DNA by stabilizing newly synthesized mitochondrial translation products through direct interaction. **b** The HCV core interacts with prohibitin, disturbing its molecular chaperone function, and leads to a decrease in the activity of ETS complex IV, COX. Subunit II of COX is encoded by the mitochondrial DNA, while other subunits are encoded by the nuclear DNA. This is a new mechanism for oxidative stress overproduction in viral infection in that HCV induces mitochondrial ETS dysfunction by inhibiting chaperone function. Ub = Ubiquitin.

identified several proteins of different expressions when compared with control HepG2 cells. Among upregulated proteins in the core-expressing cells, we focused on prohibitin, which functions as a mitochondrial protein chaperone, and found that the core protein interacts with prohibitin and represses the interaction between prohibitin and subunit proteins of cytochrome c oxidase (COX), which may lead to decreases in the expression level of the proteins and in COX activity.

Prohibitin, a mitochondrial protein chaperone, was identified as an upregulated protein in core-expressing cells. Prohibitin is a ubiquitously expressed and highly conserved protein that was originally determined to play a predominant role in inhibiting cell cycle progression and cellular proliferation by attenuating DNA synthesis [14]. It is present in the nucleus and interacts with transcription factors that are important in cell cycle progression. In core-expressing cells, prohibitin was also detected in the nucleus and its expression level was also higher than that in control Hepswx cells or HepG2 cells. Mitochondrial prohibitin acts as a protein chaperone by stabilizing newly synthesized mitochondrial translation products through direct interaction [15]. We examined the interaction between prohibitin and the mitochondrially encoded subunit II of COX and found a suppressed interaction between these proteins in core-expressing cells. In addition, there are several studies that showed the association of prohibitin with the assembly of mitochondrial respiratory complex I as well as complex IV (COX) [15] (fig. 1). Complex I also consists of both nuclear- and mitochondrial-DNA-encoded subunits; therefore, it is probable that the assembly and function of complex I are impaired by the core protein. In respect to the complex I function, we previously found a decreased complex I activity in core-expressing cells. Other groups have also shown that complex I activity is decreased in cultured cells [16]. Based on these findings, the interaction between prohibitin and the core protein may impair the function of complex I as well as complex IV, leading to an increase in ROS production. In fact, the suppression of prohibitin function has been shown to result in an increased production of ROS [17], a phenomenon observed in the core-expressing cells used in this study as well as in the liver of core-gene transgenic mice [2]. Interestingly, Shelly Lu et al. [18] recently reported that the liver-specific deletion of prohibitin resulted in morphological abnormality and HCC.

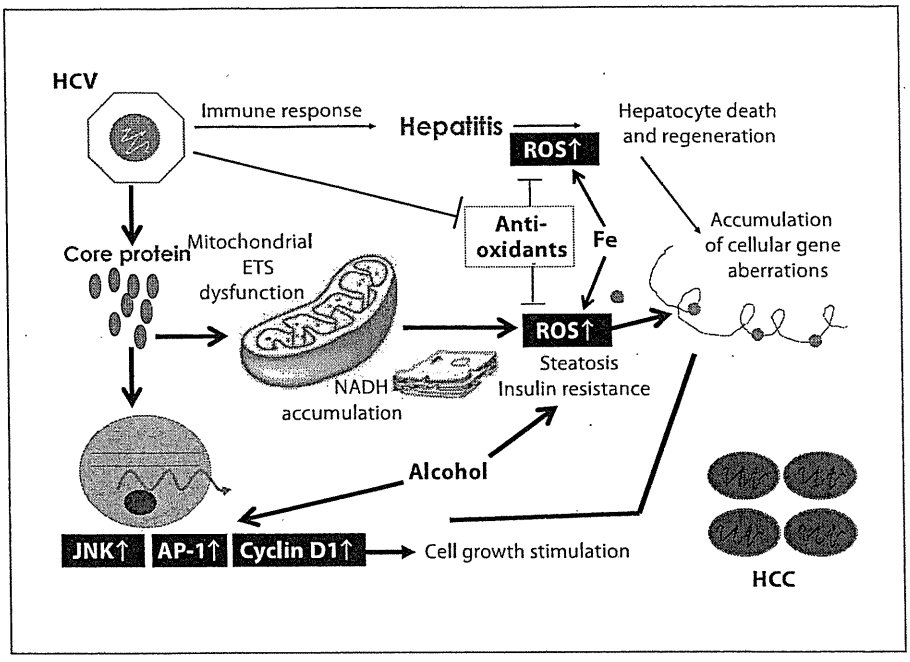

Fig. 2. Molecular pathogenesis of HCC development in HCV infection. Inflammation should contribute to hepatocarcinogenesis by producing genetic aberrations via continual cell death and regeneration. In the case of HCV infection, the virus itself contributes to hepatocarcinogenesis via two pathways. In one pathway, the core protein acts on the function of the mitochondrial ETS, leading to the overproduction of oxidative stress. The core protein also compromises some antioxidants and exacerbates ROS generation. Fe accumulation is an aggravating factor. The presence of steatosis and insulin resistance augments oxidative stress production. The other pathway is the modulation of cellular gene expression and signal transduction including the JNK pathway, which would give a growth advantage to hepatocytes. The combination of these alterations would escalate the development of HCC in HCV infection.

This is a new mechanism for ROS overproduction in viral infection in that HCV induces mitochondrial dysfunction through the inhibition of chaperone function in the mitochondria [19].

HCV Compromises the Antioxidant System

As discussed above, chronic hepatitis C is characterized by its prominent augmentation of oxidative stress. Related to this, iron accumulation in the liver has been shown to aggravate the oxidative stress as shown by the increase in the amount of DNA adducts in the liver [2, 9]. Iron is accumulated in the liver of HCV core gene transgenic mice [20]. The accumulation of iron observed in the liver of the core gene transgenic mice fed with normal chow corroborates the observation in chronic hepatitis C patients [9, 10]. Then, the impact of iron overloading on the oxidant/antioxidant system was examined using this mouse model and cultured cells. Iron overloading caused the induction of ROS as well as antioxidants. However, some of the key antioxidant enzymes, including HO-1 and NADH dehydrogenase quinone 1 (NDQ-1), were not augmented sufficiently by iron overloading, while other antioxidant enzymes such as catalase and GST were augmented more strongly in the iron-overloaded core gene transgenic mice than in the iron-overloaded control or non-iron-overloaded core gene transgenic mice. The attenuation of iron-induced augmentation of HO-1 was also confirmed in HepG2 cells expressing the core protein. HO-1 catalyzes the initial and rate-limiting reaction in heme catabolism and cleaves prooxidant heme to form biliverdin, which is converted into bilirubin in mammals; both of these have been known to have very strong antioxidant activities [21]. In addition, HO-1 has been also suggested to be a central antioxidant in conditions of GSH depletion [22]. Thus, HO-1 is an essential protective endogenous mechanism against oxidative stress, particularly in the case of iron overload. Therefore, it is probable that the attenuation of HO-1 and NQO-1 would hamper the antioxidant system and lead to a robust production of oxidative stress in HCV infection.

Thus, HCV infection not only induces ROS but also hampers antioxidant activation in the liver, thereby exacerbating oxidative stress that would facilitate hepatocarcinogenesis.

Conclusion

Pathways other than oxidative stress provocation in HCV-related hepatocarcinogenesis are alteration of the expression of cellular genes and modulation of intracellular signaling pathways. For example, tumor necrosis factor (TNF)-α and interleukin-1β have been found transcriptionally activated [23]. The mitogen-activated pro-

tein kinase (MAPK) cascade, which is involved in numerous cellular events including cell proliferation, is also activated in the liver of the core gene transgenic mouse model. In the liver prior to HCC development, only the c-Jun N-terminal kinase (JNK) route is activated. Downstream of the JNK activation, transcription factor activating protein (AP)-1 activation is markedly enhanced [23, 24]. Far downstream, both the mRNA and protein levels of cyclin D1 and cyclin-dependent kinase (CDK)4 are increased. Thus, the HCV core protein modulates the intracellular signaling pathways and gives advantage for cell proliferation to hepatocytes. The combination of these pathways that are activated in HCV infection, i.e. ROS overproduction, attenuation of antioxidants, cell growth stimulation via MAPK activation, metabolic disturbances such as hepatic steatosis, and insulin resistance [25], which are all induced by HCV itself, would contribute to hepatocarcinogenesis, together with moderate but long-lasting inflammation in chronic hepatitis C (fig. 2).

The results of our studies on transgenic mice have indicated a carcinogenic potential of the HCV core protein in vivo; thus, HCV would be directly involved in hepatocarcinogenesis. In research studies of carcinogenesis, the development of colorectal cancer is induced by the accumulation of a complete set of cellular gene mutations [26]. Their theory has been extended to the carcinogenesis of other cancers as well, called 'Vogelstein-type' carcinogenesis. On the basis of the results we obtained for the induction of HCC by the HCV core protein, we would like to introduce a different mechanism for hepatocarcinogenesis in HCV infection. We do allow multistages in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that many mutations accumulate in hepatocytes. Some of these steps, however, may be skipped in the development of HCC in HCV infection to which the core protein would contribute. The overall effect achieved by expression of the viral protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations, required for carcinogenesis.

By considering such a 'non-Vogelstein-type' process for the induction of HCC, a plausible explanation may be given for many unusual events which occur in HCV carriers. It no longer seem so difficult to determine why HCC develops in persistent HCV infection with an outstandingly high incidence. Our theory may also give an account of the multicentric de novo occurrence characteristics of HCC, which would be the result of persistent HCV infection.

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Disclosure Statement

The authors have nothing to disclose.

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ORIGINAL ARTICLE

Pandemic 2009 Influenza A (H1N1) Virus among Japanese Healthcare Workers: Seroprevalence and Risk Factors

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OBJECTIVE. To evaluate the seroprevalence and risk factors for 2009 influenza A (H1N1) virus infection among healthcare personnel.

DESIGN. Observational cross-sectional study.

PATIENTS AND SETTING. Healthcare workers (HCWs) in an acute care hospital.

METHODS. Between September 14 and October 4, 2009, before 2009 H1N1 vaccination, we collected serological samples from 461 healthy HCWs. Hemagglutination-inhibition antibody assays were conducted. To evaluate the risk factors of seropositivity for 2009 H1N1 virus, gender, age, profession, work department, usage of personal protective equipment, and seasonal influenza vaccination status data were gathered via questionnaires.

RESULTS. Our survey showed that doctors and nurses were at highest risk of seropositivity for the 2009 H1N1 virus (odds ratio [OR], 5.25 [95% confidence interval {CI}, 1.21–22.7]). An increased risk of seropositivity was observed among pediatric, emergency room, and internal medicine staff (adjusted OR, 1.98 [95% CI, 1.07–3.65]). Risk was also higher among HCWs who had high titers of antibodies against the seasonal H1N1 virus (adjusted OR, 1.59 [95% CI, 1.02–2.48]).

CONCLUSIONS. Seropositivity for the 2009 H1N1 virus was associated with occupational risk factors among HCWs.

Infect Control Hosp Epidemiol 2012;33(1):58-62

Pandemic 2009 influenza A (H1N1) virus infections were first reported during April in Mexico and the United States.[1] A few weeks later, the virus had spread worldwide. On June 11, 2009, the World Health Organization announced a phase-6 pandemic alert as the 2009 H1N1 outbreak achieved pandemic status.[2] In Japan, the incidence of 2009 H1N1 infection began to increase in August 2009, that is, 3–4 months prior to the usual influenza season. The indigenous transmission of 2009 H1N1 infection in Japan, as well as in many other countries, largely involved children.[3]

Because of their constant close interaction with infected patients, healthcare workers (HCWs) are most likely to be at greater risk for contracting influenza than the general public.[4] Patient-to-HCW transmission and infection with 2009 H1N1 virus have been reported in the United States[5] and in Germany.[6] It is not clear, however, which HCWs were most affected by the 2009 H1N1 virus. Moreover, in hospitals, the prevention of 2009 H1N1 outbreaks was an important issue. In particular, concerns were raised by the possibility that even mild disease could result in staff absenteeism, with subsequent reduction of staff strength at a time of increased demand for health services. Nevertheless, cases of patient-to-HCW transmission may have occurred due to inadequate use of personal protective equipment (PPE). The objective of this study was, therefore, to identify which HCWs were at highest risk for 2009 H1N1 virus infection in an acute-care hospital setting.

METHODS

Subjects and Study Procedures

A cross-sectional study comprising different divisions of HCWs was performed at the University of Tokyo Hospital, an acute-care hospital with 1,250 beds and a medical team of approximately 2,000 workers. HCWs who were scheduled to receive the 2009 H1N1 vaccine and agreed to participate in the study were enrolled. From August 2009 onward, HCWs who received a diagnosis of or were highly suspected of having influenza A were excluded from vaccination because of the vaccine shortage faced in that year.

We especially focused on doctors and nurses for precise analysis. On the basis of the frequency of exposure to patients with influenza-like illness, recruited doctors (n = 262) and nurses (n = 176) were subdivided into 4 groups: pediatric staff (group 1, n = 147), emergency room staff (group 2,

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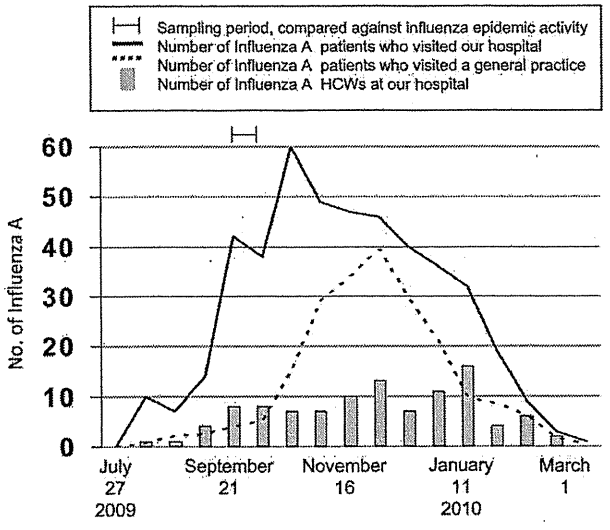

FIGURE 1. Epidemic curves constructed from the number of patients with influenza A and the number of healthcare workers (HCWs) at the University of Tokyo Hospital.

$n = 66$), internal medicine staff (group 3, $n = 142$), and other medical division staff (group 4, $n = 83$). In addition, for comparative purposes, a group of comedical staff ($n = 23$) with no direct contact with patients was also included. Personal data, including gender, age, profession, work department, usage of PPE, and seasonal influenza vaccination status, were gathered with appropriate questionnaires. Compliance with PPE rules (ie, use of surgical masks and gloves) was assessed as the frequency of PPE use during a 4-month period beginning in May 2009, through a 4-point scale: 1, always; 2, most of the time; 3, sometimes; 4, never. We defined appropriate PPE use as categories 1 and 2.

The numbers of patients and HCWs who received a diagnosis of influenza A were reported daily to our infection control team. In addition, we obtained data on Japanese influenza epidemic activity from the national sentinel surveillance.

Sample Collection and Laboratory Procedures

Serum samples were collected before vaccination, between September 14 and October 4, 2009. After collection, serum samples were pretreated with receptor-destroying enzyme (RDE; RDE II, Denka Seiken, Tokyo, Japan) for 18 hours at 37°C to inactivate nonspecific inhibitors. After incubation for 30 minutes at 56°C to inactivate the RDE, the samples were further treated by absorption onto chicken red blood cells for 1 hour at room temperature to remove nonspecific agglutinins. After centrifugation, supernatants were tested with 1 : 2 serial dilutions at an initial dilution of 1 : 10 for the hemagglutination-inhibition (HI) antibody assays. The serum samples were tested against the 2009 H1N1 virus (A/California/7/2009) and seasonal H1N1 virus (2008–2009 seasonal vaccine strain A/Brisbane/59/2007). The tests were performed at Mitsubishi Chemical Medience, Japan.

Data Analysis

Geometric mean titers (GMTs) were estimated by assigning the value of 1 : 5 for titers lower than 1 : 10. Seropositivity against 2009 H1N1 was defined as titers above 1 : 10 on the basis of previous seroepidemiologic studies.[7] Regarding seasonal H1N1, seropositivitity was defined as titers above 1 : 40.[8]

Univariate and multivariate logistic-regression analyses were performed, using the following variables: age, gender, profession, work department, contact with patients, and seasonal H1N1 influenza vaccination status. Multivariate analysis was based on a stepwise approach in which variables that did not improve the model fit at $P < .2$ were discarded. Odds ratios (ORs) with asymptotic Wald 95% confidence intervals (CIs) and 2-sided P values were reported. Statistical significance was set at $P \leq .05$. All statistical analyses were performed using SPSS Statistics 18 (IBM).

Ethics Review

Written informed consent forms were obtained from all participants. The ethics review board of the University of Tokyo approved the study.

RESULTS

Figure 1 shows that at our hospital, the influenza A epidemic curves peaked earlier than did those in other general-practice hospitals. This is because our hospital was designated as specialized in the treatment of 2009 H1N1 influenza. Between

TABLE 1. Cohort Characteristics

	Doctor	Nurse	Comedical staff
No. (%) of participants	262 (57)	176 (38)	23 (5)
Median (range) age, years	32 (24–56)	30 (22–58)	36 (25–59)
Age in years, no. (%)			
20–29	119 (45)	94 (54)	6 (26)
30–39	98 (37)	66 (37)	12 (52)
40–59	44 (17)	16 (9)	5 (22)
No. (%) of men	179 (68)	13 (7)	7 (30)
No. (%) of seasonal influenza vaccine recipients	159 (60)	145 (83)	16 (70)

TABLE 2. Participants' Characteristics and Distribution of Antibody Titer against 2009 Influenza A (H1N1) Virus before Vaccination

	No. (%) of participants	Distribution of antibody titer, no. (%) of patients			GMT (95% CI)	P
		<10	10–20	≥40		
Department						
Group 1 (pediatric staff)	147 (34)	90 (61)	44 (30)	13 (9)	13.2 (8.5–17.9)	.005[a]
Group 2 (emergency workers)	66 (15)	39 (59)	21 (32)	6 (9)	10.8 (7.8–13.9)	.006[a]
Group 3 (internal medicine staff)	142 (32)	96 (68)	38 (27)	8 (5)	10.1 (8.0–12.2)	.001[a]
Group 4 (other medical division staff)	83 (19)	67 (81)	16 (19)	0 (0)	6.3 (5.6–7.0)	
Contact with patients with influenza-like illness						
Yes, without PPE	62 (14)	39 (63)	15 (24)	8 (13)	17.2 (6.6–27.7)	.17[b]
Yes, with PPE	179 (41)	119 (66)	51 (28)	9 (5)	9.9 (8.2–11.7)	.87[b]
No	197 (45)	134 (68)	53 (27)	10 (5)	9.8 (8.0–11.5)	
Age groups						
20–29	213 (49)	150 (70)	51 (24)	12 (6)	10.4 (7.2–13.6)	
30–39	164 (37)	102 (62)	51 (31)	11 (7)	11.1 (9.1–13.1)	.72[c]
40–59	61 (14)	40 (66)	17 (28)	4 (6)	12.0 (7.6–16.3)	.57[c]
Seasonal influenza vaccination in the past 3 years						
≥2 times	375 (88)	245 (65)	107 (27)	23 (8)	10.0 (8.4–11.6)	.04
<2 times	63 (12)	47 (75)	12 (19)	4 (6)	7.3 (5.2–9.4)	
Antibody titer of seasonal H1N1 virus						
≥40	288 (66)	181 (63)	85 (29)	22 (8)	12.2 (9.6–14.8)	.01
<40	150 (34)	111 (74)	34 (23)	5 (3)	8.4 (6.8–10.1)	

NOTE. CI, confidence interval; GMT, geometric mean antibody titers; PPE, personal protective equipment.

[a] χ^2 test comparing groups 1–3 and group 4.

[b] χ^2 test comparing contact and noncontact groups.

[c] χ^2 test comparing the respective age group and the 20–29 years age group.

the beginning of the pandemic and the sampling period, fewer than 10 HCWs in our hospital were affected by influenza A.

A total of 461 HCWs were included in this study. The cohort characteristics are provided in Table 1. The median age of participants varied between 30 (nurses) and 36 (comedical staff) years. Doctors were predominantly men (68%), and the nurses and other comedical staff were predominantly women (93% and 70%, respectively). Only 60% of the doctors and 83% of the nurses had received the seasonal H1N1 influenza vaccination in 2009.

GMTs for 2009 H1N1 virus antibodies of doctors (GMT, 11.7 [95% CI, 8.9–14.6]) and nurses (GMT, 8.8 [95% CI, 7.4–10.2]) were higher than those of the comedical staff (GMT, 5.4 [95% CI, 4.8–6.1]). Higher seroprevalence was observed in doctors and nurses compared with comedical staff (OR, 5.25 [95% CI, 1.21–22.7]). Doctors and nurses ($n = 438$) were thus subjected to further analysis. GMTs for HI antibodies against the 2009 H1N1 virus were significantly higher ($P < .01$) in groups 1–3 (by approximately 1.6–2.1-fold) than in group 4 (Table 2). Second, doctors and nurses who had received seasonal influenza vaccines more than twice within the previous 3 years showed significantly higher ($P = .04$) GMT values than did the others. In addition, GMT values were also significantly higher ($P = .05$) in doctors and nurses who had high seasonal H1N1 virus antibody titers than in those who had low seasonal H1N1 antibody titers (Table 2).

Regarding the use of personal protective equipment, GMT values of doctors and nurses who had not used proper PPE (eg, surgical masks or gloves) while in contact with patients who had influenza-like illness tended to be slightly higher than those of workers who had no contact with patients who had influenza-like illness (Table 2). However, these differences were not statistically significant. Additionally, the GMT values of doctors and nurses who had used PPE while in contact with patients who had influenza-like illness did not differ from the values observed in the no-contact group.

The results of the univariate and multivariate analyses of doctors and nurses who were seropositive for 2009 H1N1 virus are presented in Table 3. An increased risk for seropositivity was found for doctors and nurses belonging to groups 1–3 compared with medical staff belonging to group 4 (adjusted OR, 1.98 [95% CI, 1.07–3.65]).

DISCUSSION

Our study shows that a higher seroprevalence was observed in doctors and nurses compared with comedical staff. The seroprevalences for doctors and nurses were 7% and 5%, respectively. One possible explanation for these low values is

TABLE 3. Univariate and Multivariate Analysis of Factors Associated with Seropositivity for the 2009 Influenza A (H1N1) Virus

	Proportion of seropositivity, %	Crude OR (95% CI)	*P*	Adjusted OR (95% CI)	*P*
Department (pediatric, emergency, internal medicine staff)	36.6	2.42 (1.35–4.35)	.003	1.98 (1.07–3.65)	.03
Contact with patients with influenza-like illness without PPE	37.1	1.18 (0.68–2.06)	.56	NA	NA
Seasonal influenza vaccination ≥2 times in the past 3 years	34.7	1.56 (0.85–2.86)	.15	0.97 (0.63–1.51)	.9
Antibody titer of seasonal H1N1 virus ≥40	37.2	1.68 (1.09–2.60)	.02	1.59 (1.02–2.48)	.04

NOTE. CI, confidence interval; NA, not applicable; OR, odds ratio; PPE, personal protective equipment.

that our study was conducted during the initial period of the epidemic. In this study, HCWs who worked as pediatric staff, emergency workers, or internal medicine staff also showed significantly higher seroprevalences than did other medical division staff. No significant differences between these 2 groups were found either for the history of seasonal H1N1 infection or for vaccination. This may suggest that occupational exposure is an important factor associated with preexisting antibodies. Our results are in agreement with the findings of other studies, which reported that HCWs in the emergency room had the highest risk of infection.[9,10]

Additionally, our cross-sectional study demonstrates that HCWs with high baseline seasonal H1N1 titers showed high seropositivity for the 2009 H1N1 virus. This is in agreement with the finding that elevated preexposure antibody titers to the 2009 H1N1 virus was associated with previous infection with the seasonal H1N1 virus occurring in 2007.[11] These findings are further supported by the evidence that memory cytotoxic T lymphocytes established by seasonal influenza A viruses may show cross-reactivity against the 2009 H1N1 virus.[12] On the other hand, others reported that there is no significant relationship between the 2009 H1N1 virus antibodies and the presence or absence of antibodies against the seasonal H1N1 virus.[13,14] It remains unclear whether the seasonal influenza vaccination provides cross-reactive antibody response to the pandemic H1N1 influenza. Thus, further studies are needed to determine whether these different results are due to exposure to the seasonal influenza virus, seasonal influenza vaccination, or community-specific factors.

For infection control, surgical masks and gloves may have helped to prevent the transmission of 2009 H1N1 influenza. Nevertheless, in our study, the specific effects of these measures are difficult to ascertain because statistically significant differences in GMT were not observed in workers using PPE as compared with their colleagues, and multivariate analysis did not identify this as a risk factor. In contrast, Cheng et al[15] reported that the failure to comply with the standard precautions such as wearing a surgical mask during contact with suspected influenza patients was associated with an increased risk of 2009 H1N1 infection.

Several limitations of our study may, however, have contributed to the results obtained. First, seropositivity in our study did not include data regarding HCWs who received a diagnosis of or were highly suspected of having influenza A from May 2009 to just before vaccination (ie, at the time of blood sample collection); these HCWs were excluded from the 2009 H1N1 vaccination because of vaccine shortage. This may have affected seroprevalence and risk analysis in our study. However, it is worthy of note that between the beginning of the pandemic and the sampling period, the number of HCWs affected by influenza A in our hospital was less than 10. Furthermore, these were evenly distributed by the job categories considered herein. Therefore, we believe that the effect of excluding the symptomatic HCWs from this study was limited. Second, the low seroconversion rates in our cohort reduced the power of the study to investigate exposures more weakly associated with the effect of PPE. Moreover, we did not collect detailed data on whether there was strict adherence to the use of PPE.

In the process of establishing vaccination policies and identifying vaccination priority groups, it is imperative to determine the relative risk of contracting the disease that the various groups face. HCWs were considered a priority group for vaccination against the 2009 H1N1 virus because they were at high risk of contracting the disease. Additionally, HCWs may serve as a target group for prevaccination surveillance in whom early seroepidemiological changes indicating novel, emergent infectious diseases may be detected and vaccine efficacy evaluated.

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LIPIDS IN HEALTH AND DISEASE

RESEARCH Open Access

LXR agonist increases apoE secretion from HepG2 spheroid, together with an increased production of VLDL and apoE-rich large HDL

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Abstract

Background: The physiological regulation of hepatic apoE gene has not been clarified, although the expression of apoE in adipocytes and macrophages has been known to be regulated by LXR.

Methods and Results: We investigated the effect of TO901317, a LXR agonist, on hepatic apoE production utilizing HepG2 cells cultured in spheroid form, known to be more differentiated than HepG2 cells in monolayer culture. Spheroid HepG2 cells were prepared in alginate-beads. The secretions of albumin, apoE and apoA-I from spheroid HepG2 cells were significantly increased compared to those from monolayer HepG2 cells, and these increases were accompanied by increased mRNA levels of apoE and apoA-I. Several nuclear receptors including LXRα also became abundant in nuclear fractions in spheroid HepG2 cells. Treatment with TO901317 significantly increased apoE protein secretion from spheroid HepG2 cells, which was also associated with the increased expression of apoE mRNA. Separation of the media with FPLC revealed that the production of apoE-rich large HDL particles were enhanced even at low concentration of TO901317, and at higher concentration of TO901317, production of VLDL particles increased as well.

Conclusions: LXR activation enhanced the expression of hepatic apoE, together with the alteration of lipoprotein particles produced from the differentiated hepatocyte-derived cells. HepG2 spheroids might serve as a good model of well-differentiated human hepatocytes for future investigations of hepatic lipid metabolism.

Keywords: Spheroid HepG2 cells, LXR agonist, Apolipoprotein E, ApoE rich HDL, VLDL

Background

Apolipoprotein E (apoE), a 34-kD glycoprotein produced mainly by hepatocytes and also secreted from several cells including macrophages and adipocytes, plays a crucial role in lipoprotein metabolism and atherosclerosis. It mediates the cellular uptake of several classes of lipoproteins by acting as a ligand for the chylomicron remnant receptor, the VLDL receptor, LDL receptor and the LDL receptor-related protein (LRP). ApoE produced by macrophages and those accessing macrophages from the bloodstream facilitate the reverse cholesterol transport by promoting the formation and maturation of HDL particles [1,2]. In addition to these functions, apoE produced in hepatocytes enhances the production of VLDL particles [3]. The increased production of hepatic VLDL particles, a phenomenon observed in insulin-resistant patients or some primary hyperlipidemia subjects, leads to the accumulation of atherogenic lipoproteins in the circulation resulting in the aggravation of atherosclerosis.

The genetic regulation of the apoE gene has been pursued extensively. Taylor et al has identified two hepatic enhancer elements located far-downstream of the apoE gene, and clarified the regions critical to the baseline expression of the apoE gene [4,5]. They also identified the duplicated downstream enhancer elements termed multienhancers (ME.1 and ME.2), and demonstrated that these elements are crucial for apoE expression in macrophages and adipocytes [6]. In addition, Laffitte

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et al elegantly clarified that the nuclear receptor liver × receptor (LXR) regulates apoE expression in adipocytes and macrophages through direct interaction of the LXR response element found in both ME.1 and ME.2 [7].

In spite of these extensive analyses on apoE gene regulation, physiological factors which affect gene regulation of apoE in the liver have not been elucidated so far. Previous in vivo studies utilizing guinea pig [8] and cebus monkey [9] have shown that cholesterol feeding to these animals resulted in the up-regulation of apoE gene in the liver, raising the possibility that the accumulation of cholesterol in hepatocytes would affect hepatic up-regulation of the apoE gene. In addition, investigation in mice also indicated the up-regulation of hepatic apoE gene by cholesterol feeding [10]. However, the contribution of LXR in the regulation of murine hepatic apoE was not demonstrated [7,10]. Furthermore, no study has clarified the role of LXR in the regulation of hepatic apoE gene in human-derived hepatocytes or hepatic cell lines, with only one exception which utilized artificial reporter gene construct, in which ME.1 or ME.2 was placed just before the -890 to +93 apoE promoter [7].

As for the model of human hepatocytes, HepG2 cells have been widely used for in vitro experiments; however HepG2 cells grown in monolayer form on a culture plate are different from the in vivo hepatocytes which exist in three dimensional form in the liver, and would not completely reflect the physiological functions of hepatocytes. HepG2 cells in spheroid culture, which grow in three dimensional form after being encapsulated in alginate beads [11], have been shown to be more differentiated than HepG2 cells cultured in monolayer form; the cells proliferating in alginate beads form cell-cell contact with each other, and normal hepato-cellular junctional complexes including canaliculi with microvilli are constructed [11]. In consequence, the production of several proteins and the detoxificatory functions [11] as well as the production of cholesterol and triglycerides [12] increased significantly in HepG2 cells in spheroid culture compared to those in monolayer culture.

In this report, we first compared the production of several apolipoproteins from HepG2 cells in spheroid culture with those in monolayer culture. Next, we examined the effect of TO901317, a synthetic LXR ligand, on the secretion of apoE as well as lipoproteins with HepG2 cells cultured both in three-dimensional form and in monolayer form.

Results

HepG2 cells cultured in spheroid form (S-Hep) secreted more albumin and apolipoproteins than HepG2 cells cultured in monolayer (M-Hep)

HepG2 cells cultured in spheroid form grew in three dimensional form (Figure 1A). To validate the differentiation of HepG2 cells prepared as spheroids in our procedure, we first examined the time-course changes in the secretion of albumin. As shown in Figure 1B, the secretion of albumin from S-Hep was enhanced; the highest secretion level was observed on Day 11, reaching as high as 4.5-fold compared to M-Hep, which was concordant with the previous report [11]. The secretions of apolipoprotein A-I (apoA-I), apoE, and apolipoprotein B (apoB) did also increase in S-Hep, and the time-course changes in their levels were almost the same as those found with albumin (Figure 1C-E). The mRNA levels of apoA-I and apoE on Day 11 of S-Hep revealed a 3-fold and 3.5-fold increase compared to M-Hep (Figure 2A). Because the levels of albumin and apoE secretions were highest on Day 11, for the subsequent experiments, we utilized S-Hep on Day 11.

PPAR-α, PPAR-γ, LXR-α, RXR-α were more abundant in the nuclear fractions of S-Hep than in those of M-Hep

In order to elucidate whether the up-regulation of the genes of apolipoproteins in S-Hep were associated with changes in the nuclear receptors, we next examined the nuclear protein levels in S-Hep in comparison with M-Hep. As shown in Figure 2B and 2C, the Western blot analyses of the proteins prepared from nuclear fractions revealed that peroxisome proliferator-activated receptor (PPAR)-α, LXR-α and retinoid × receptor (RXR)-α were more abundant in S-Hep than M-Hep. We did not observe differences in nuclear protein levels of hepatocyte nuclear factor (HNF)1-α and HNF4-α between S-Hep and M-Hep, however, PPAR-γ was also increased in S-Hep. This result suggested that the state of differentiation of hepatic derived cells would affect the expressions of several proteins associated with lipid metabolism at the level of DNA transcription.

TO901317 increased apoE secretion and suppressed apoA-I secretion from HepG2 spheroids more evidently than monolayer HepG2 cells

The increased nuclear level of LXRα in S-Hep together with the increased secretion of apoE from S-Hep prompted us to evaluate the effect of LXRα agonist on the secretion of apoE, because LXRα has been identified as a critical factor for the regulation of apoE in macrophages and adipocytes. Thus we next examined the effect of TO901317 (TO), a synthetic LXRα agonist, on the apolipoproteins' secretion from S-Hep as well as M-Hep. As was shown in Figure 3A, the incubation of cells with TO did not alter the levels of apoB secretion in both S-Hep and M-Hep. The secretion of apoA-I was decreased in both forms of HepG2 cells when the cells were incubated with TO, which was concordant with the previous finding by Huuskonen et al (Figure 3B) [13]. On the other hand, apoE secretion was enhanced

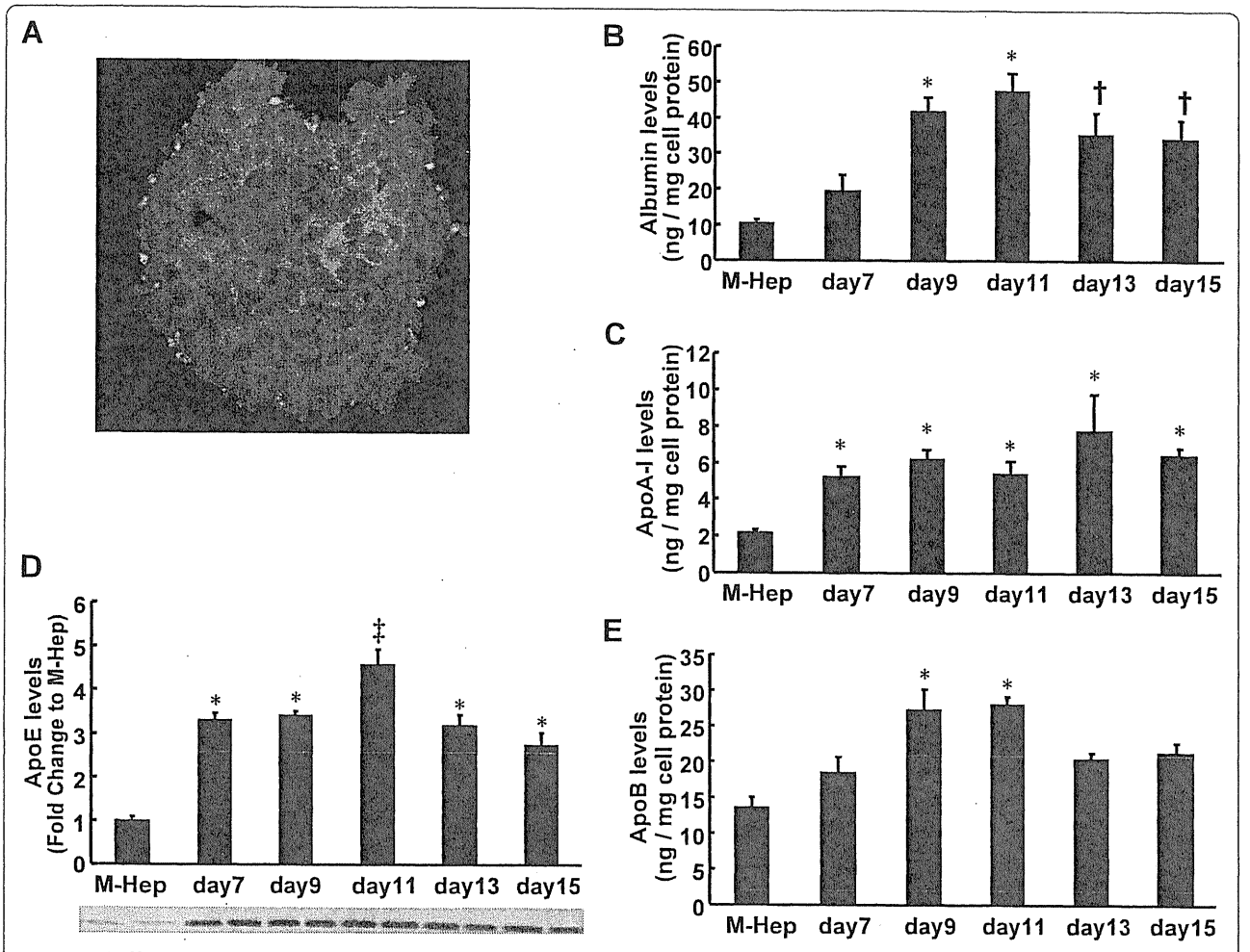

Figure 1 Differences in albumin and apolipoproteins secretion between monolayer and spheroid HepG2 cells. (A) Representative appearance of HepG2 spheroids on Day 11. HepG2 spheroids were fixed with 10% formalin solution, and examined with confocal microscopy. Briefly, from a single HepG2 cell, cells proliferate and form a spheroidal cell-cluster in an aliginate bead. (B-E) Time-course changes in the secretion of albumin (B), apoA-I (C), apoE (D), and apoB (E) from S-Hep cells. S-Hep (spheroid HepG2 cells) were prepared and grown in alginate beads as described in Materials and Methods, and the protein levels in the media on different time points after the beginning of the culture were measured. X axes represent the days after the beginning of S-Hep culture. Data are mean ± SEM (n = 4). *: $P < 0.01$ compared with M-Hep; †: $P < 0.03$ compared with M-Hep; ‡: $P < 0.01$ compared with M-Hep and $P < 0.03$ with S-Hep on the other time points.

not only in S-Hep but also in M-Hep with the incubation of cells with TO (Figure 3C). The induction of apoE in M-Hep plateaued at 0.02 μM TO, while the dose-dependent increase in apoE secretion from S-Hep was observed up to 0.2 μM. In addition, this incremental apoE secretion was more prominent in S-Hep, reaching almost twice the level of cells without TO.

TO901317 increased apoE and ABCA1 mRNA levels in HepG2 spheroids

In order to evaluate whether the increased secretion of apoE from S-Hep treated with TO was associated with the upregulation of the apoE gene, we examined the levels of apoE mRNA as well as ATP binding cassette transporter (ABC) A1 mRNA with quantitative real time PCR analyses. As shown in Figure 3D, the mRNA levels of apoE were significantly elevated by TO treatment, indicating that LXRα activated with TO would have increased the transcription of apoE in S-Hep. ABCA1, which is regulated by LXR, was also upregulated by TO901317, although the levels of the induction were less than those observed with apoE (Figure 3E).

Distribution of apoE secreted from S-Hep among lipoproteins

As shown in the above experiments, we confirmed that the secretion of apoE was enhanced in S-Hep compared to M-Hep, and treatment of HepG2 cells with TO resulted in the augmentation of apoE secretion from HepG2 cells. We next analyzed the distributions of

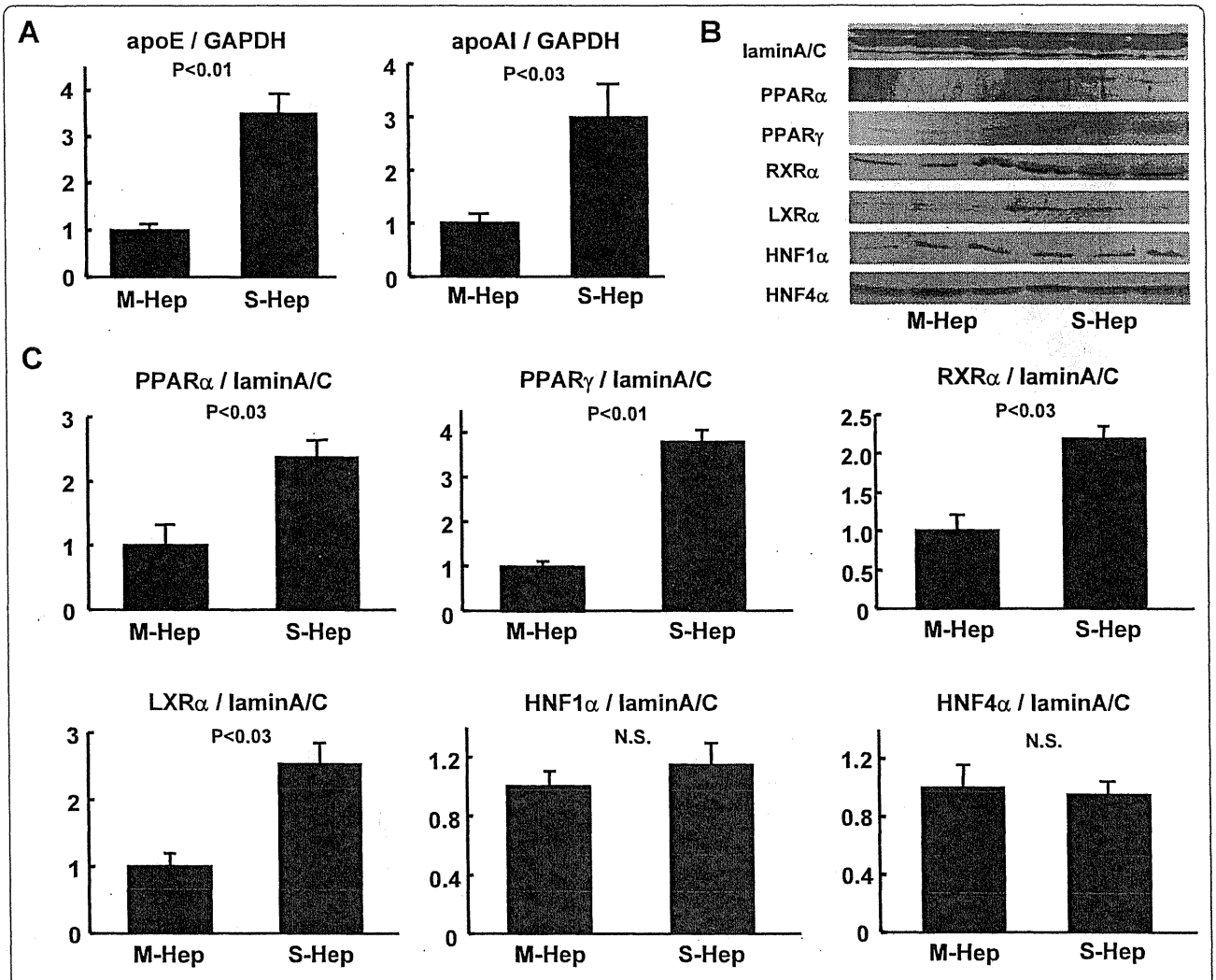

Figure 2 Differences in apolipoprotein mRNA and nuclear receptor protein levels among M-Hep and S-Hep. (A) Analyses of mRNA levels of apoE and apoA-I with quantitative RT-PCR (n = 4). GAPDH mRNA was used as the endogenous control for the expression levels of the gene of interest. (B, C) Western blot analyses were performed with the proteins prepared from nuclear fractions (Panel B), and nuclear protein levels were quantified with Image J utilizing lamin A/C as the internal control (Panel C) (n = 3). Y-axes in panels A and C represent ratios to the data of M-Hep.

apoE, together with those of apoB and apoA-I, among lipoproteins after fractionating the media with fast protein liquid chromatography (FPLC) which separated lipoproteins depending on their sizes. The results are shown in Figure 4 and 5: the fractions 21 - 26 corresponded to VLDL, fractions 29 - 37 to LDL, and fractions 38 - 48 to HDL based on the analysis of human plasma (data not shown). Both in S-Hep and M-Hep, apoA-I distribution was noted almost exclusively on fractions corresponding to HDL fractions, especially small HDL fractions; this distribution was not altered even with TO treatment. ApoB proteins were detected in fractions relevant to VLDL and LDL fractions, although the apoBs found in VLDL were scarce. In addition, no difference was observed in the distribution patterns of apoB between S-Hep and M-Hep, and treatment with TO did not alter these patterns. In contrast, unlike the findings of apoA-I and apoB, the distribution of apoE on lipoproteins was affected not only by the methods of the culture but also by the treatment with TO. ApoE secreted from M-Hep, regardless of the treatment with TO, were detected in the fractions spanning between those of LDL and HDL, suggesting its distribution on large HDL fractions; in addition, no apoE band was found in VLDL fractions even by the treatment with TO. On the other hand, the culture of HepG2 cells in spheroidal form rendered the apoE protein to reside on normal-sized HDL particles. Interestingly, treatment of S-Hep with TO not only increased the amount of secreted apoE incrementally with the increment of the

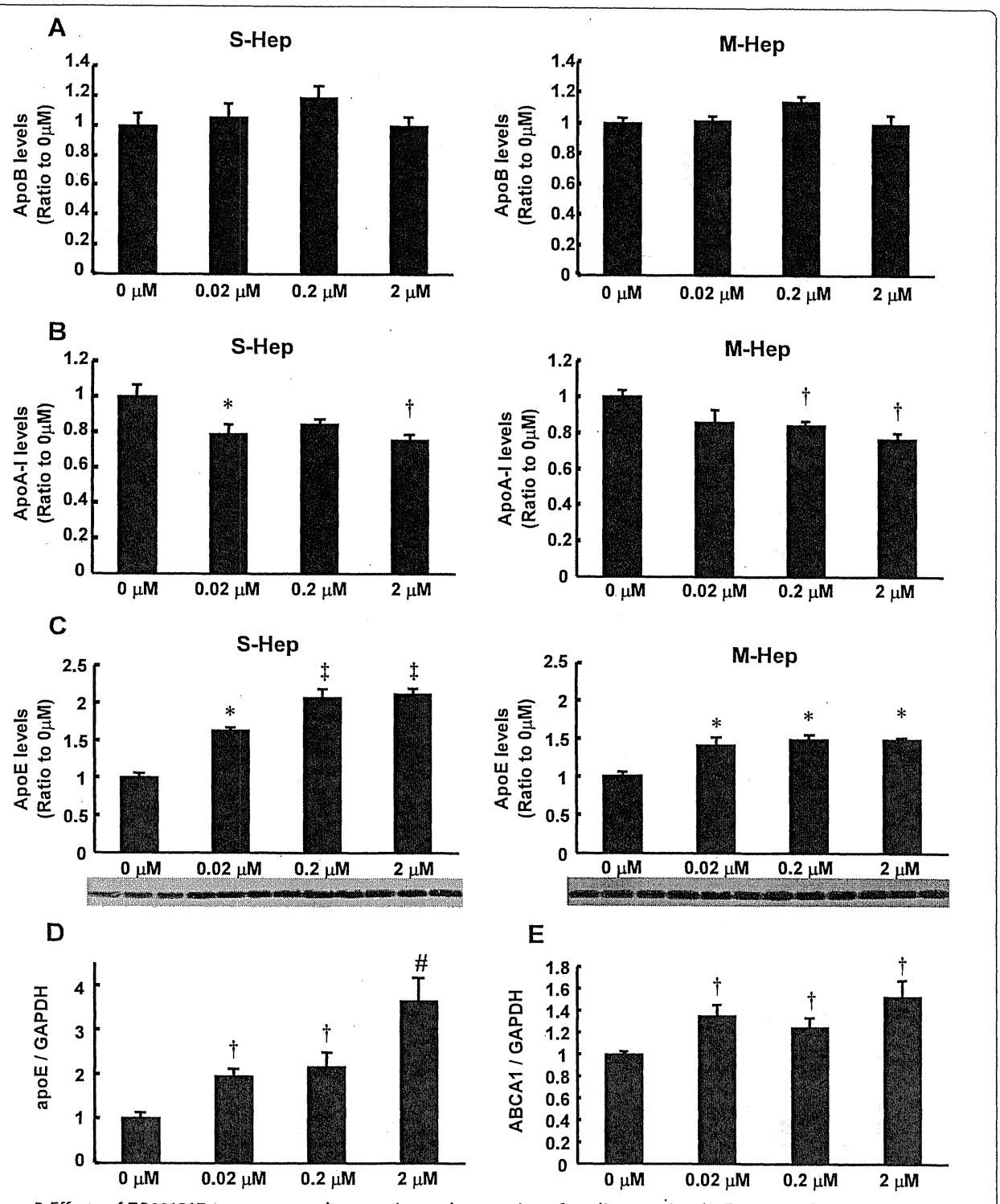

Figure 3 Effects of TO901317 treatment on the secretion and expression of apolipoproteins. (A-C) S-Hep and M-Hep were incubated with different concentration of TO901317, and the levels of apoipoproteins secreted in the medium were analyzed. Panel A, B and C represented apoB, apoA-I and apoE levels, respectively. (D, E) S-Hep were incubated with different concentration of TO901317, and mRNA levels of apoE (D) and ABCA1 (E) were analyzed. Data are mean ± SEM (n = 4). X-axes represent the concentration of TO901317. *: $P < 0.01$ compared with 0 μM, †: $P < 0.05$ with 0 μM, ‡: $P < 0.01$ with 0 and 0.02 μM, #: $P < 0.03$ with 0 and 0.02 μM.

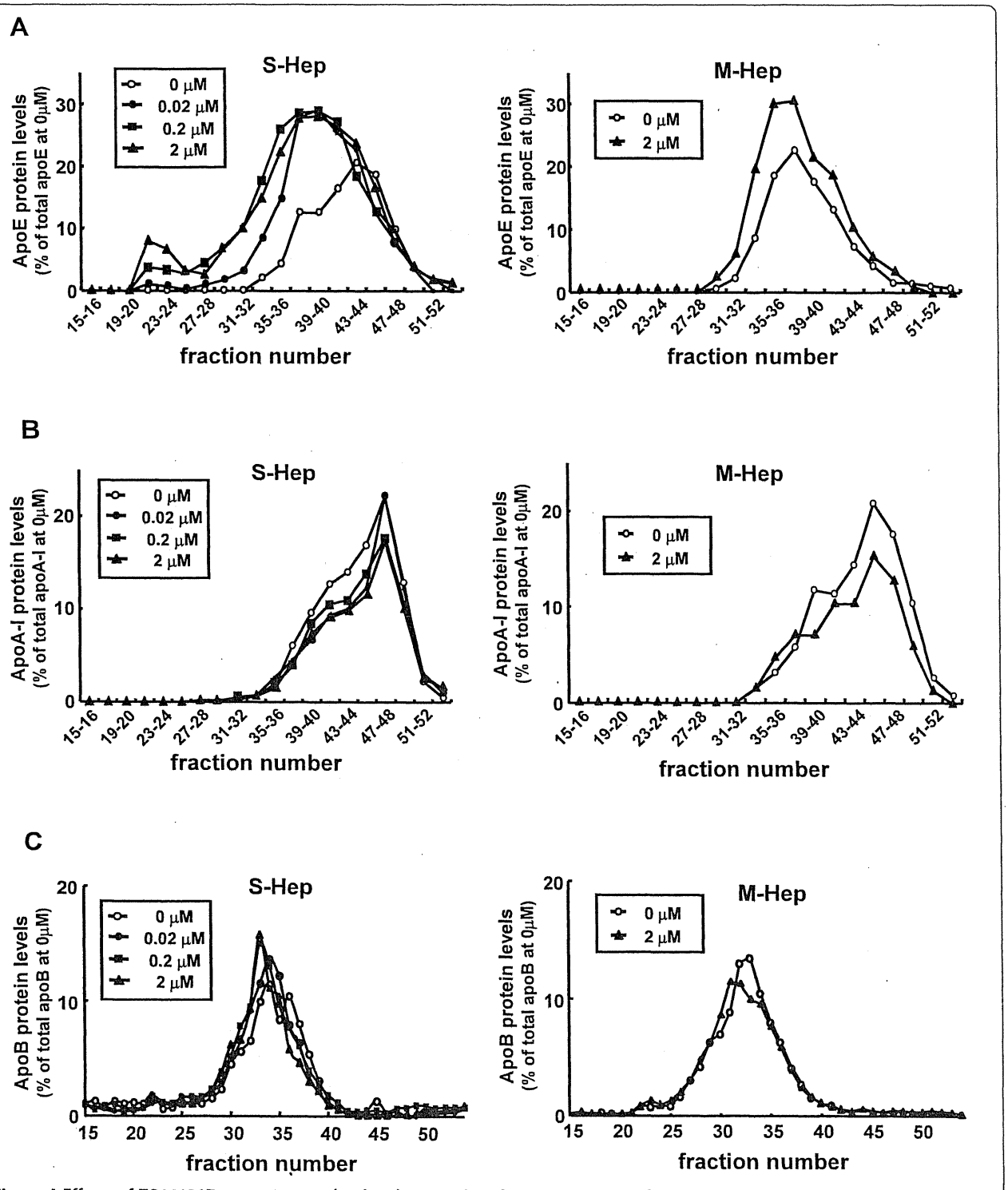

Figure 4 Effects of TO901317 treatment on the distribution of apolipoprotein among lipoproteins. Distribution of apoE (A), apoA-I (B), and apoB (C) on lipoprotein particles in the medium harvested from S-Hep (left) and M-Hep (right). Following the fractionation of the media with FPLC depending on the size of lipoproteins, fractionated samples were subjected to Western blot analyses or protein measurement with ELISA kits. White circles, black circles, squares and triangles represent the results from the medium of cells incubated with 0 μM, 0.02 μM, 0.2 μM and 2 μM of TO901317, respectively.

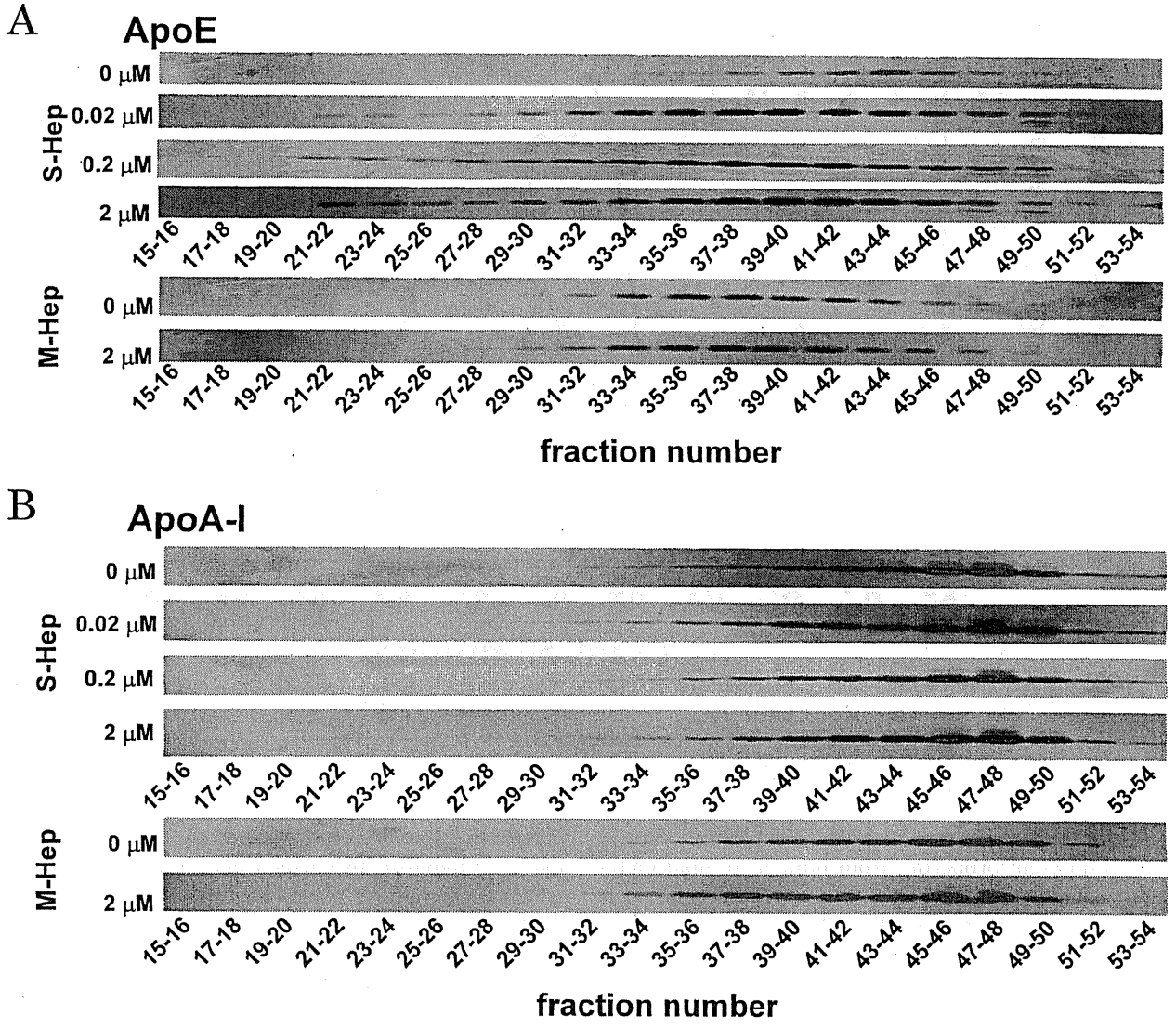

Figure 5 Western blot analyses on the distribution of apoE and apoA-I proteins among lipoprotein classes. After the incubation of S-Hep and M-Hep with TO901317 at the concentrations indicated, the media were fractionated with FPLC and the fractionated samples were subjected to Western blot analyses. Panel A and B represent the distributions of apoE and apoA-I, respectively.

dose of TO, but also rendered apoE to distribute on fractions larger than normal HDL. Based on the observation that neither apoA-I nor apoB was detected in these lipoprotein fractions, these fractions were assumed to be large apoE rich HDL. Furthermore, TO treatment of S-Hep resulted in the appearance of apoE on VLDL fractions; the amount of apoE on VLDL increased incrementally when the concentration of TO was increased up to 2 μM. In addition, as shown in Figure 6, treatment of S-Hep with 2 μM TO resulted in the increased triglycerides levels in the fractions where apoE protein increased with TO treatment. These results indicated that the TO treatment not merely resulted in the increased apoE protein levels in VLDL and large HDL fractions, but also resulted in the increased particle numbers and/or the enrichment of lipid content of VLDL and large HDL particles.

Discussion

Utilization of primary hepatocytes or hepatocyte-derived cell lines for in vitro experiments has helped us understand the lipid metabolism in the liver. However, it has been known that even the primary hepatocytes, when cultured in monolayer form, lose their differentiated, physiological functions quickly probably due to the loss of its three dimensional in vivo conformation in the experimental setting. In vivo experiments with rodents have also enabled us to elucidate the lipid metabolism

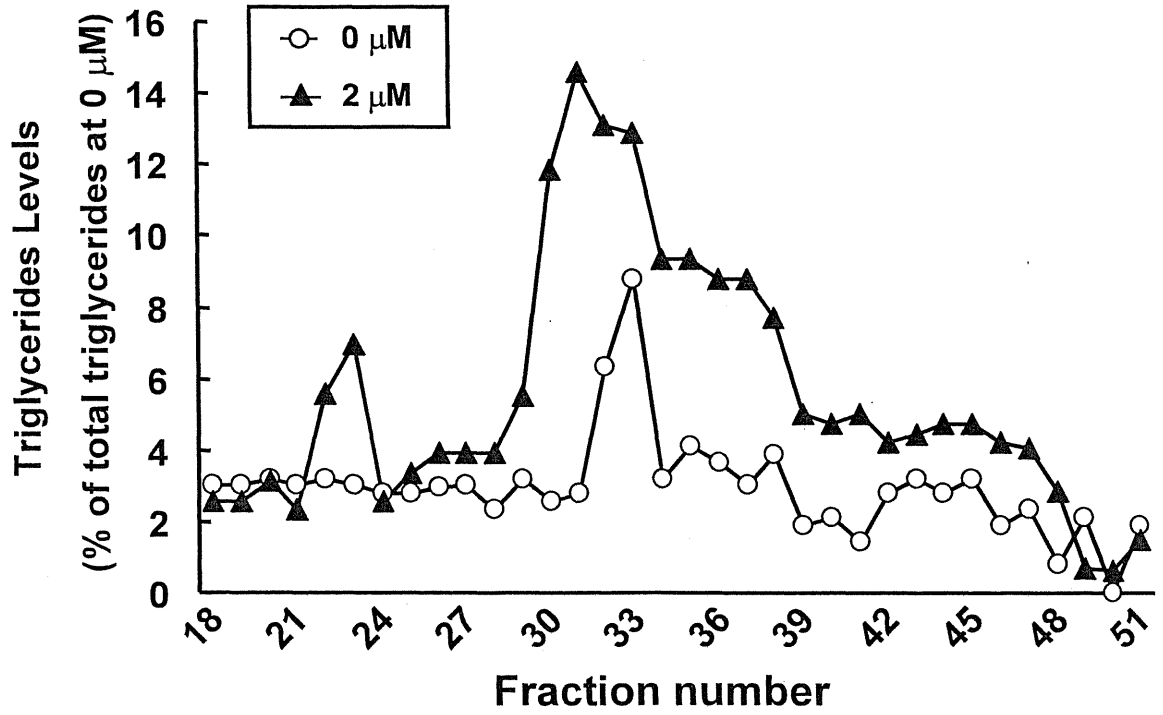

Figure 6 Analyses of triglyceride levels in the FPLC-fractionated samples. FPLC fractions obtained from S-Hep with or without 2 μM TO were subjected to the measurement of triglyceride (TG) levels with enzymatic method. White circles and triangles represent the results from the medium of cells incubated with 0 μM and 2 μM of TO901317, respectively.

in the liver; however, rodents might have been known to possess different properties from humans in lipid metabolism. Thus, in this study, in order to overcome these obstacles, we utilized 3-D spheroid culture system utilizing alginate beads, which has proven to be an easily-manipulative tactic to reproduce hepatocytes or hepatocytes-derived cells similar to their natural, differentiated in vivo counterparts.

Concordant with the previous observation by Khalil et al. [11], we were able to demonstrate that the culture of HepG2 cells in spheroid form resulted in enhanced albumin secretion compared to that in monolayer form, indicating that S-Hep utilized in this study had been more differentiated than M-Hep. The secretions of apoE, apoA-I, and apoB were also enhanced in S-Hep, and the nuclear protein contents of PPAR-α, PPAR-γ, LXR-α and RXR-α were increased in S-Hep, indicating that both of which are the features of differentiated hepatocytes or hepatocyte-derived cells. Furthermore, activation of LXR with TO901317 treatment of S-Hep resulted in the increased secretion of apoE protein which was accompanied by the up-regulation of apoE mRNA levels. Taking into account the previous study not showing a significant up-regulation of apoE gene with M-Hep treated with LXR activation [7], we assume that the differentiation of cells would be important to clearly examine the regulation of apoE gene in hepatocytes or hepatocyte-derived cells with LXR agonist, which is the case for macrophages and adipocytes [7].

The physiological regulation of apoE gene in the liver has so far not been clarified, although the baseline expression has been known to be controlled by distal hepatic enhancer elements [4,5] as well as the proximal promoter region to which TR4 orphan nuclear receptor binds [14]. Laffitte et al suggested that apoE enhancers and promoters containing LXRE would be important for the activation of apoE promoter in M-Hep; however, administration of LXR agonist in mice revealed a slight but non-significant role of LXR in the regulation of apoE in vivo [7]. Their observation does not depart at all from our present study, considering that murine lipid metabolism differs from that of humans in several respects. It is also plausible that factors other than LXR would regulate hepatic apoE gene in mice and probably in humans, considering that the degree of up-regulation of apoE gene observed in our study is less than those found in adipocytes and macrophages [7]. Further studies are needed to clarify these factors, and we believe that utilization of S-Hep would enable us to elucidate these factors.

In this study, we also found that the increased apoE secretion from S-Hep resulted in the alteration of

lipoprotein classes produced from the cells. One of the aspects of this alteration is the increased production of VLDL particles. In the state of hepatic steatosis, not only triglycerides but also cholesterol accumulate in hepatocytes [15], and the oxidative stress which increases in hepatic steatosis [16] transforms the cholesterol to oxysterol, which is a natural ligand for LXR. Because the production of apoE is one of the important determinants for the secretion of VLDL or VLDL-TG from the liver [3], the upregulation of apoE gene together with the increased production of triglycerides [17,18] by LXR activation facilitates the production of VLDL particles, resulting in the atherogenic lipid profile of the metabolic syndrome.

The other interesting finding in the alteration of lipoprotein production from S-Hep with TO901317 treatment was the increased production of large HDL particles containing apoE. Treatment with TO901317 prevented atherosclerosis in various mouse models [19-21], and increased apoE-rich HDL particles in C57BL6 mice [22,23]. These reagent effects have been attributed to the enhanced reverse cholesterol transport from macrophages [24] through the up-regulation of several key macrophagic proteins such as ABCA1 and apoE [25,26]. However, in this study, we did indicate that apoE-rich large HDL particles were also produced from the differentiated hepatocyte-derived cells, and that the induction was more pronounced with increasing increments of LXR activation. Although apoE-rich HDL has been speculated to play a role in delivering cholesterol to hormone-producing tissues such as adrenal tissues [27,28], several lines of study have indicated that apoE-rich HDL would also play an important role in reverse cholesterol transport; apoE-rich HDL is mainly contained in large HDL and large HDL has been demonstrated to extract cholesterol from macrophages [29]. It was also suggested that apoE-containing HDL efficiently enhanced cholesterol efflux [2,30]. Thus the increased production of apoE-rich large HDL particles from differentiated hepatocytes induced by LXR activation might have a role in the protection against atherosclerosis.

Conclusions

In summary, by utilizing the differentiated spheroid HepG2 cells, for the first time we were able to clearly demonstrate that LXR activation resulted in the up-regulation of human hepatic apoE, which also enhanced the production of VLDL particles and large apoE rich HDL particles. In future studies, investigation using HepG2 spheroids as surrogates to well-differentiated human hepatocytes would serve well as a model to precisely understand lipid metabolism in the liver.

Methods

Cell Culture and Experimental Protocol

HepG2 cells, purchased from American Type Culture Collection (ATCC, Manassas, VA), were cultured and maintained in DMEM (Sigma-Aldrich Co. St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Eggstein, Germany) and 1% penicillin/streptomycin (Gibco). For the experiment with M-Hep without TO (Sigma-Aldrich), 24 hours prior to the harvest of medium and cells, the medium was replaced with FBS-free medium to eliminate the plasma proteins derived from FBS in the medium. In the experimentation of M-Hep with TO, the medium was exchanged for that containing various concentrations of TO dissolved in DMSO at the cell confluency of around 70%. Two days later, the medium was replaced with the FBS-free medium containing the same concentration of TO, and cells were incubated for another 24 hours prior to the analysis. The collected cells were suspended in RIPA buffer (Santa Cruz Biotechnology, Santa Cruz, CA) for further analysis. The protein levels of the cell lysates were measured with Lowry methods (BioRad, Hercules, CA.) according to the manufacturer's protocol.

S-Hep were prepared following the methods described previously [11,12] with some modification. Briefly, HepG2 cells cultured in monolayer were detached completely with Trypsin-EDTA (Gibco) and suspended in α-MEM (Gibco) containing 10% FBS at the concentration of 0.5×10^6/mL. The medium containing HepG2 cells was mixed with the same amount of 2% alginate (Sigma). The mixed solution was dropped into 0.102 M $CaCl_2$/0.15 M NaCl (pH 7.4) solution at the speed of 1.5 ml/min through a 23 G cannula equipped inside another 19 G cannula from which the air was ejected at the speed of 1.2 L/min. This procedure yielded alginate-beads containing HepG2 cells, whose diameters ranged from 300 to 500 μm. The alginate beads were washed with DMEM twice and cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Prior to the harvest of cells and medium, beads containing S-Hep were washed twice with DMEM and cultured in FBS-containing DMEM with or without TO for two days. Thereafter, the medium was exchanged with FBS-free medium containing the same concentration of TO, and the cells were incubated for another 24 hours. Then the media were collected, and the cells were dissolved in RIPA buffer after releasing them from alginate beads with the incubation in PBS containing 4 mM EGTA (pH 7.4) for 10 minutes.

Quantification of Secreted Proteins in Medium

The concentrations of albumin, apoA-I and apoB in the media were measured by indirect sandwich enzyme-

linked immunosorbent assay (ELISA) with human albumin ELISA quantification kit (Bethyl laboratories, Inc. Montgomery, TX.) and ELISA kits for human apoA-I and apoB (Mabtech Inc. Nacka Strand, Sweden). For the quantification of apoE levels, the media, the volumes of which were adjusted according to cell protein levels, were subjected to 10% SDS-PAGE followed by Western-blot analysis with anti-apoE antibody (Chemicon International Inc, Temecula, CA), and the intensities of the bands were measured by Image J (from the NIH).

Preparation and Analysis of Nuclear Fraction

The nuclear fractions of HepG2 cells were obtained as follows: cells were dissolved in Buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.05% NP40, protease inhibitor cocktail (Roche, Mannheim, Germany), pH7.9) and incubated on ice for 10 minutes, centrifuged at 900 g for 10 minutes. The pellets were homogenized in Buffer B (5 mM HEPES. 1.5 mM $MgCl_2$, 0.2 M EDTA, 0.5 mM DTT, 26% glycerol, protease inhibitor cocktail, pH 7.9) supplemented with NaCl to the final concentration of 300 mM. Then, the solutions were centrifuged at 24,000 g for 20 minutes, and the supernatants were analyzed as the nuclear fractions of the cells. To quantify the levels of each nuclear protein, 30 μg of nuclear proteins extracted as above were subjected to 8% SDS-PGE followed by Western blot analyses with anti-Lamin A/C, anti-PPAR-α, anti-PPAR-γ, anti-RXR-α, anti-LXR-α, anti-HNF-1α, or anti-HNF-4α antibody (Santa Cruz Biotechnology).

Quantitative Real Time PCR

Total RNAs extracted from M-Hep and S-Hep with GenElute mammalian total RNA miniprep kit (Sigma-Aldrich) were subjected to reverse transcription with Superscript II enzyme (Invitrogen Co. Carlsbad, CA). Real-time quantitative PCR was performed with Light-Cycler system (Roche Diagnostics Basel, Switzerland). The expression levels of the gene of interest were normalized to those of the endogenous control GAPDH mRNA, and the amounts of target gene expressions were expressed as a ratio to those of control cells. The following primers were used: for GAPDH, forward 5' CCACTCCTCCACCTTTGA 3' and reverse 5' GTG GTCCAGGGGTCTTAC 3'; for apoA-I, forward 5' TGTCCCAGTTTGAAGGCT 3' and reverse 5' ATCC TTGCTCATCTCCTGC 3'; for apoE, forward 5' GGGT CGCTTTTGGGATTAC 3' and reverse 5' CAACT CCTTCATGGTCTCG 3'; for ABCA1, forward 5' AAATCCATTGTGGCTGC 3' and reverse 5' GGGA-GAGAGAGGTTGTGATAC 3'.

FPLC Analysis

The media of S-Hep or M-Hep, the total volumes of which were 12 mL, were concentrated to about 500 μl by centrifugation through Amicon Ultara-15 (Millipore Co., Bedford, MA). Then 200 μl of concentrated medium was separated by FPLC utilizing Superose 6 column. The levels of apoB in the separated fractions were analysed with ELISA method. For the analyses of apoE and apoA-I, the separated fractions were subjected to Western blots utilizing anti-apoE antibody and anti-apoA-I antibody (Chemicon). To raise the sensitivity of western blot analysis, after the incubation with primary antibodies, the membranes were incubated in biotin-conjugated anti-goat IgG antibody (Sigma) and then detected by Vecstatin ABC kit (Vector laboratories, Inc, Burlingame, CA). FPLC fractions obtained from S-Hep with or without 2 μM TO were subjected to the measurement of triglycerides (TG) levels with enzymatic method (WAKO Pure Chemical Industries, Osaka, Japan). To standardize the TG values among samples from with or without TO, the values obtained were adjusted utilizing the TG levels of the media which was corrected with cellular protein levels.

Statistical analysis

The results were expressed as mean ± SEM. Differences between two groups were evaluated with student's *t*-test, and the differences among more than assessed with one-way ANOVA, followed by multiple comparison tests. the *P* value less than 0.05 was deemed as statistically significant.

List of Abbreviations

apoA-I: apolipoprotein A-I; apoB: apolipoprotein B; apoE: apolipoprotein E; TO: TO901317; M-Hep: HepG2 cells cultured in monolayer; S-Hep: HepG2 cells cultured in spheroidal form; HNF: hepatocyte nuclear factor; LXR: liver × receptor; PPAR: peroxisome proliferator-activated receptor; RXR: retinoid × receptor; ABC: ATP-binding cassette transporter; FPLC: fast protein liquid chromatography; FBS: fetal bovine serum; ELISA: enzyme-linked immunosorbent assay.

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