Elevated SAP/SLAM expression in hepatitis B vaccine responders, but not in non-responders among chronic hepatitis C patients.

Jian Fu[1], Xuejiao Pan[1], Jun Yao[1], Shuyun Xie[1], Huakun Lv[1], Zhifang Wang[2,3]*, Chonggao Hu[3]*

[1]Department of Immunization Programme, Zhejiang Provincial Center for Disease Control and Prevention, 3399 Binsheng Road, Hangzhou, China

[2]Department of Environmental and Occupational Health, Zhejiang Provincial Center for Disease Control and Prevention, 3399 Binsheng Road, Hangzhou, China

[3]Key Medical Research Center, Zhejiang Provincial Center for Disease Control and Prevention, 3399 Binsheng Road, Hangzhou, China

Abstract

About 5%-10% of vaccinated individuals fail to produce a protective level of anti-hepatitis B surface antibody (anti-HBs) though hepatitis B vaccines show excellent immunogenicity in the general population. The percentage of humoral immunity failure to hepatitis B vaccination is about 30% in individuals infected with hepatitis C virus (HCV). However, mechanisms on the higher non-responsive rate in HCV patients are still unknown. Our study was conducted to describe the potential mechanisms of the Singling Lymphocytic Activation Molecule (SLAM) and SLAM-Associated Protein (SAP) between T cells and B cells borders during humoral immunity response. Based on anti-HBs Ab titers post immunization, we designated three groups: healthy controls (HC), vaccine responders in HCV-infected patients (VR), and vaccine non-responders in HCV-infected patients (VNR), respectively. Protein and mRNA expression levels of SAP and SLAM were determined among three groups at two time points, pre-vaccination and post-vaccination, respectively. For VR and HC, mRNA and protein expression levels of SAP and SLAM were elevated post-vaccination. However, these changes were not seen in VNR. Increased expression levels of SAP and SLAM may be associated with the development of a protective anti-HBs Ab response to hepatitis B vaccination. Therefore, modulation of SAP and SLAM may be considered as a strategy to improve vaccination for HCV-infected patients.

Introduction

Globally, Hepatitis B Virus (HBV) infection affects more than one third of the population, with an incidence rate of 1.26 million deaths per year [1]. Immunization with hepatitis B vaccine containing the major surface antigen of HBV (HBs Ag) is considered as the economic, efficient and safe strategy to prevent and control this disease. Patients infected with hepatitis C virus (HCV) are suggested to be immunized with hepatitis B vaccine due to co-infections with HBV and HCV can increase severity of hepatitis and serious complications. Though hepatitis B vaccine show excellent immunogenicity in the general population with normal immune status, approximately 30% of chronic hepatitis C patients fail to develop protective hepatitis B surface antibody (defined as anti-HBs titers are less than 10 mIU/ml) after a series of standard vaccination schedule (involving 3 doses of 20 µg HBs Ag vaccine at 0, 1, 6 months) with hepatitis B vaccine [2-4].

Many factors have been recognized as the potential causes for failure to induce a protective anti-HBs response to hepatitis B vaccination, such as host-related factors (increasing age, obesity, smoking cigarettes, as well as male gender) and genetic factors (specific human leukocyte antigen genes) [5,6]. Recent studies show that the importance of signaling lymphocytic activation molecule (SLAM)-associate protein (SAP) as an intracellular signal molecular in humoral immunity [7,8]. SAP-deficient mice lack long-term humoral immunity after immunization by their paucity of antigen (Ag)-specific memory B cells and long-lived Ab-secreting plasma cells [9-12]. SAP-mutated patients in X-linked lympho-proliferative disease character a dysregulated immune response [13]. Although defects in several immune cell types associated with SAP were found to relate with the humoral immunity response, these studies were usually implemented on the patients and the mice models. The patients with immunodeficiency disorders may complicate the interpretation of the data; the immune system in mice distinguishes from that

in human beings due to different species. In addition, analysis on the association SAP/SLAM family adhesions and immune response to hepatitis B vaccine is crucial to shed light on the underlying mechanisms on vaccination failure. However, to date, the underlying mechanisms on how SAP regulates lymphocyte functions are not well understood in humans with normal immune status. In this study, we undertook to address this issue between hepatitis B vaccine responders and non-responders in healthy individuals and chronic hepatitis C patients.

Materials and Methods

Vaccine schedule and study subjects

Between February 2013 and June 2016, vaccines were voluntarily immunized with 3-dose 20 µg recombinant Chinese hamster ovary cell HB vaccine (200802A21, North China Pharmaceutical Group Corp GeneTech Biotechnology Development Co., Ltd.) at 0, 1 and 6 months. Peripheral venous blood (10 ml) was collected in plastic tubes (BD Biosciences) under sterile condition at two time points: before the first dose vaccine (T0) and 4 weeks after 3 doses vaccine (T1). Anti-HBs titers were measured by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (ABIN509703, antibodies-online Inc.).

Vaccine non-responders were defined as participants whose anti-HBs titers were lower than 10 mIU/ml. Vaccine responders were designated as subjects whose anti-HBs titers were greater than 10 mIU/ml; In this study, total of 8 vaccine responders without hepatitis C virus infection were selected as healthy controls (HC); 17 participants infected with hepatitis C virus were enrolled, including 8 vaccine responders (VR) and 9 vaccine non-responders (VNR). Characteristics of study participants were shown in Table 1.

Table 1. Characteristics of study participants and their anti-HBs responses.

Groups	Age (Mean ± SD)	Gender	HCV RNA (log$_{10}$ copies/ml)	Anti-HBs titers (mIU/ml) T0	T1
HC	30.9 ± 15.6 y	M=11; F=9	NA	1.5 ± 0.4	205.3 ± 57.0
VR	48.1 ± 18.4 y	M=6; F=10	6.1 ± 3.2	0.7 ± 0.2	197.2 ± 34.3
VNR	60.5 ± 20.4 y	M=11; F=7	5.2 ± 2.1	1.2 ± 0.7	8.5 ± 0.4

SD: Standard Deviation; NA: Not Applicable; HC: Healthy Controls; VR: Vaccine Responders among chronic hepatitis C patients; VNR: Vaccine Non-Responders among chronic hepatitis C patients.

RNA preparation, reverse transcription and qRT-PCR

Total RNA from 106 PBMC was extracted using TRI Reagent RNA Isolation Kit (T9424, Sigma Molecular Research Center, Inc.) following the manufacturer's instructions. The concentration and purity of the RNA was quantified by a spectrophotometer (NanoDrop 8000; Thermo Fisher Scientific) using A260/A280 ratio and A230/A260 ratio. Samples with RNA concentration (A260/A280 ≥ 1.7 ng/µl) and purity (1.5 ng/µl ≤ A230/A260 ≤ 2.4 ng/µl) were chosen for further analysis.

Extracted RNA (3.0 µg) from each sample was used as template for cDNA generation with 3.6 µM oligo-dT primers in the synthesis solution (20 µL), using Thermo Scientific Verso cDNA Synthesis kit (AB-1453/A). Reactions were incubated at 70°C for 5 min and then place immediately on ice. In addition, a total of 40 units of RNaseOUT Recombinant RNase inhibitor (Verso cDNA Kit, AB1453A, Thermo Fisher Scientific) and 14 mM dithiothreitol were added to the mixed reaction. Next, reverse transcriptase (1 µL) was added and the reactions started at 42°C for 30 min and 95°C for 2 min to inactivate reverse transcriptase. cDNA samples were diluted to a RNA equivalent of 1 ng/µL with DEPC-treated water for downstream processing. Each sample in the test was assayed in triplicate.

We performed quantitative real-time quantitative PCR assays (qRT-PCR) in Bio-Rad DNA Engine Opticon 2 System instrument (Bio-Rad Laboratories, Hercules, CA). Reactions (20 µL/well) were performed in skirted PCR plates consisting of 3 µL cDNA sample, 10 µL iQ SYBR green super mix (170-8882; Bio-Rad Laboratories) and 300 nM the following primers: SLAM-forward: 5'-AGC AGG TCT CCA CTC CAGAA-3'; SLAM-reverse: 5'-GCT CAC GGT GCA GAT GTAGA-3'; SAP-forward:5'-AAC TGG GAG TCA GGT GGTTG-3'; SAP-reverse: 5'-GCT GTC CCT CAG CAA ATAGC-3'). Cycling conditions for each plate included a 95°C for incubation for 2 min followed by 40 cycles of 95°C for 10 s and 55°C for 30 s. Melting curve analysis was performed between 55°C and 95°C. We used β-actin housekeeping gene as the internal control under the same PCR conditions described for SLAM and SAP.

Protein extraction and Western blotting analysis

Peripheral Blood Mononuclear Cells (PBMC) was isolated by density gradient centrifugation on Ficoll-Paque (Sigma, USA). We extracted total cell protein from each sample by lysing cell in the cold radioimmuno-precipitation assay buffer in the presence of protease inhibitors. The amount of protein was determined by Coomassie Protein Assay Reagent Kit (23200, Thermo Fisher Scientific) according to the manufacturer's instructions. Protein was resolved by sodium dodecyl sulfate

polyacrylamide gel electrophoresis in 10% polyacrylamide gels and blotted onto PVDF membrane. Blots were blocked in PBS with 0.5% skim milk for 2 h at room temperature and incubated with primary and secondary antibodies. Monoclonal antibodies against CD150 (sc-166939, Santa Cruz Biotechnology) and SH2D1A (sc-398118, Santa Cruz Biotechnology) were used at a dilution of 1:500 and 1:200, respectively. Secondary antibody (sc-2005, Santa Cruz Biotechnology) was used at dilution of 1:1000. The super signal chemiluminescent substrate detection kit (34094, Thermo Fisher Scientific) and ECL film (Kodak, China) were used to visualize the presence of specific protein in the PVDF blots. Immunoreactive bands were quantified by volume densitometry using Image J Basics Version 1.49 (http://rsb.info.nih.gov/ij/download.html) and normalized to β-actin (sc-47778, Santa Cruz Biotechnology). All data represent the results of at least three independent assays.

Statistical analysis

SAP/SLAM mRNA and protein levels were quantified in each study participant at two time points: T0 and T1. Results were expressed as ratios and normalized by the internal control (β-actin). Statistical analysis was performed using SPSS version 23.0 (SPSS Inc. Chicago, IL, USA). Statistically significant differences were determined using paired t-tests. A P value less than 0.05 were considered as a statistical significance.

Ethical statement

Written informed consent was obtained from each participant. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by Zhejiang Provincial Center for Disease Control and Prevention Ethics Committee.

Results

Increased SAP/SLAM mRNA levels in producing protective anti-HBs response among HC and VR, but not among VNR

SAP/SLAM mRNA levels were determined by qRT-PCR before and after vaccination with recombinant hepatitis B vaccine (Table 2). Compared with the SAP mRNA levels at T0, they were significantly elevated for persons developing a protective antibody response among HC and VR at T1. However, the similarly increased SAP mRNA levels were not observed for VNR. Post immunization, the relative SLAM mRNA levels were significantly increased for HC and VR, but not for VNR.

Table 2. mRNA levels in HC, VR and VNR to hepatitis B vaccine.

mRNA	Groups	T0	T1	P value
SAP	HC	10.7 ± 4.19	14.48 ± 6.35	0.001*
	VR	7.98 ± 5.24	10.08 ± 4.28	0.005*
	VNR	10.20 ± 3.44	11.15 ± 5.4	0.186
SLAM	HC	7.86 ± 5.02	10.53 ± 6.32	0.034*
	VR	6.69 ± 5.32	12.24 ± 6.78	0.039*
	VNR	7.89 ± 4.81	6.92 ± 5.89	0.981

mRNA: message RNA; HC: Healthy Controls; VR: Vaccine Responders; VNR: Vaccine Non-Responders; *P<0.05.

SAP mRNA levels are positively correlated with SLAM in three groups

SAP is a SRC homology 2 (SH2) domain-containing molecular. *In vivo*, expression of SAP is regulated following immune activation [14]. After vaccination, SAP functions as a transduced signal between T cells and B cells by binding of the cytoplasmic domain of SLAM. To establish the relationship between SAP and SLAM mRNA levels in three different groups before and after vaccination with recombined hepatitis B vaccine, we calculated the correlation coefficients by correlation analysis (Table 3). It was shown that SAP mRNA levels in peripheral blood among HC, VR, and VNR were significantly positively related with SLAM mRNA levels.

Table 3. Correlation analysis between SAP and SLAM based on mRNA levels.

Groups	Time points	Correlation coefficient	P value
HC	T0	0.81	0.0006**
	T1	0.83	0.0086*
VR	T0	0.76	0.0001**
	T1	0.91	0.03*
VNR	T0	0.79	0.01*
	T1	0.9	0.0001**

HC: Healthy Controls; VR: Vaccine Responders; VNR: Vaccine Non-Responders; *P<0.05; **P<0.001.

Increased SAP/SLAM protein levels post vaccination for HC and VR but not for VNR

To show whether SAP/SLAM protein levels increased after vaccination with recombined hepatitis B vaccine, we further determined each protein expression level by Western blot (Figure 1).

The results showed that SAP protein levels were significantly higher post vaccination (T1) than pre-vaccination (T0) among HC and VR, however, SAP protein level was not increased among VNR post immunization (T1) (Figures 1A and Figure 1B).

Similarly, SLAM proteins levels were significantly enhanced post vaccination (T1) among HC and VR not among VNR, compared with the level before the day of vaccination (T0) (Figures 1C and Figure 1D).
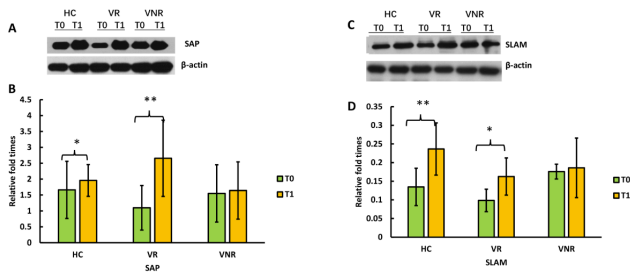
Figure 1. SAP/SLAM protein levels post vaccination.

Discussion

To our knowledge, this is the first time to determine, post-immunization with recombined hepatitis B vaccine, the expression levels of both SAP and SLAM were increased among HC and VR who had a protective anti-HBs response but not among VNR who failed to produce a protective humoral immunity response.

Post immunization with recombined hepatitis B vaccine, CD4 T cells interact with antigen-presenting cells, proliferate, activate, and migrate toward the T-B cell border in germinal centers (GCs). This cooperation between T cells and B cells require direct physical interactions, which induce maturation of HBs antigen-specific B cells and production of long-lived of plasma cell and generate long-term anti-HBs.

For humoral immunity response, SAP family adaptors have been found to be expressed in B cells and T cells [12,15]. Previous studies in SAP-deficient mice show that, SAP play important roles for T cell functions and B cells differentiation, maturation and antibody production [16,17]. Therefore, SAP-deficient mice have flaws in humoral immunity to reproduce the antibody by severely decreasing to form GCs, losing of long-term antibody-secreting cells and reducing serum IgG. In this study, we show in the unique design of recombined hepatitis B vaccination-induced antibody responses in chronic hepatitis C virus-infected patients and healthy controls, SAP expression levels of vaccine responders were quantitatively superior to those of vaccine non-responders despite being similar levels before vaccination in different research groups. This result is in agreement with existing evidence that SAP is needed to mediate signal transduction as cell adhesions to produce humoral immune response [18,19]. This result is also agreement with the studies in SAP-deficient CD4 T cells in vitro and in SAP-deficient mice *in vivo* [20,21]. These phenomena imply that SAP up-regulation is necessary for T-B cell adhesions to produce efficient antigen-specific antibody post immunization.

SLAM family of receptors express on the surface of a wide range of immune cells, including CD4 T cells, CD8 T cells, macrophages, dendritic cells, natural killer cells and B cells.

There is accumulating evidence that SLAM family receptors are necessary to form stable conjugates between T cells and antigen-specific B cells [18,21]. In the present study, we also observed that mRNA expression levels of SLAM were up

regulated post-immunization among HC and VR but not among VNR. This phenomenon implies that SLAM is also crucial for a protective humoral immunity to have efficient anti-HBs levels.

The global researches on function of the SAP and SLAM are not always identical. Some data showed that up-regulation of SLAM levels occurred on activated lymphocytes during an immune response [21-23]. It has been presumed that SLAM may play a critical role on controlling humoral immunity. Nevertheless, SLAM-deficient mice show the normal germinal center and may not be essential for T-B interaction and humoral immunity response [24]. In SAP-deficient mice model, one group observed that SAP is indispensable in CD4 T cells for germinal centers development and humoral immunity response [25]. In this current study, we observed elevated levels of both SAP and SLAM for participants who a produce efficient humoral immunity while none were changed on expression level for non-responsive subjects. It indicates that SAP may couple SLAM to protective humoral immunity response. Both of them are important for a successful humoral immune response. Based on our research result, we recognized that the modulation of SAP and SLAM expression may represent an axis by which the quality and duration of anti-HBs in humoral immunity response is regulated post immunization.

Declaration of Conflicting Interest

The authors declare that there are no conflicts of interest.

Acknowledgement

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*Correspondence to

Zhifang Wang

Department of Environmental and Occupational Health

Zhejiang Provincial Center for Disease Control and Prevention

3399 Binsheng Road

Hangzhou

China

Chonggao Hu

Key Medical Research Center

Zhejiang Provincial Center for Disease Control and Prevention

3399 Binsheng Road

Hangzhou

China