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Lymphocyte subset characterization associated with persistent hepatitis C virus infection and subsequent progression of liver fibrosis

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ABSTRACT

This study aims to deepen the understanding of lymphocyte phenotypes related to the course of hepatitis C virus (HCV) infection and progression of liver fibrosis in a cohort of atomic bomb survivors. The study subjects comprise 3 groups: 162 HCV persistently infected, 145 spontaneously cleared, and 3,511 uninfected individuals. We observed increased percentages of peripheral blood T_H1 and total CD8 T cells and decreased percentages of natural killer (NK) cells in the HCV persistence group compared with the other 2 groups after adjustment for age, gender, and radiation exposure dose. Subsequently, we determined that increased T_H1 cell percentages in the HCV persistence group were significantly associated with an accelerated time-course reduction in platelet counts—accelerated progression of liver fibrosis—whereas T_C1 and NK cell percentages were inversely associated with progression. This study suggests that T_H1 immunity is enhanced by persistent HCV infection and that percentages of peripheral T_H1, T_C1, and NK cells may help predict progression of liver fibrosis.

1. Introduction

Hepatitis C virus (HCV) infects some 120 to 170 million people worldwide, and persistent HCV infection is a major cause of liver diseases, including chronic hepatitis, cirrhosis, and hepatocellular carcinomas [1,2]. Both innate and adaptive arms of the host immune system are closely involved in persistent infection, liver injury, and virus clearance [3,4]. For instance, cytotoxic granule release and cytokine production of natural killer (NK) cells are inhibited by direct binding of HCV envelope protein E2 to CD81 on NK cells or stabilizing the human leukocyte antigen (HLA)-E expressions on hepatocytes in HCV-infected patients [5,6]. However, the comprehensive understanding of interactions between HCV and the immune system remains incomplete [3,4]. Moreover, aging, gender, and several environmental factors, such as alcohol drinking, smoking, and ionizing radiation, have been reported to influence host immune functions as well as HCV spontaneous clearance [7–9], which may increase the complexity of virus–host interactions. Therefore, a comprehensive characterization of host immunologic phenotypes in HCV infection is needed, especially with a cohort-based study design without conceivable selection bias [10]. Nevertheless, few studies along those lines have been carried out. One prospective cohort study (the Adult Health Study [AHS]) of atomic bomb survivors—a longevity cohort with biennial health examinations—has been conducted at the Radiation Effects Research Foundation (RERF) and provides clinicoepidemiological data related to HCV infection and immunologic status [11,12]. Within the cohort study, we conducted a cross-sectional analysis for peripheral blood lymphocyte subsets among HCV persistently infected, spontaneously cleared, and uninfected groups, aiming to delineate immunologic distinctions among these 3 groups. We also aimed to identify the lymphocyte subsets that can predict hepatitis progression in HCV-persistent individuals on the basis of a longitudinal analysis of time-course changes of platelet counts.

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2. Subjects and methods

2.1. Study population

The Atomic Bomb Casualty Commission, subsequently the RERF, established the AHS cohort in 1958. This cohort study enrolled a total of 23,000 atomic bomb survivors in Hiroshima and Nagasaki who biennially received health examinations in outpatient clinics [11]. Hepatitis screening (HBsAg, anti-HBc antibody (Ab), anti-HBs Ab, and anti-HCV Ab tests, as well as HCV RNA test if the anti-HCV Ab was positive) was conducted among 6,121 AHS participants in 1993–1995 [12]. Anti-HCV Ab negative subjects were categorized as the HCV-uninfected group in this study, whereas a "persistence" group was identified by anti-HCV Ab positive with detected HCV RNA, and a "spontaneous clearance" group was identified by anti-HCV Ab positive and undetectable HCV RNA. Subjects who were hepatitis B virus surface antigen positive were excluded from this study. From the 6,121 AHS subjects, lymphocyte subsets in the peripheral blood were then examined in 162 HCV persistence, 145 virus clearance, and 3,511 uninfected subjects in 2000–2002. Most subjects ($n = 120$, 74%) in the persistence group ($n = 162$, including those with cancer history) were confirmed by a second RNA test at least 2 years after the first RNA test performed in 1993–1995. Although the remaining 42 subjects in the group did not undergo the second RNA test, these subjects were confirmed to have developed type C chronic liver disease based on medical chart review (*e.g.*, treatment history, abdominal sonographic observation, changes in platelet counts, zinc sulfate turbidity, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) between 1993–1995 and 2000–2002) by a hepatologist (one of the authors, WO). Subsequent treatment data of hepatitis C from attending physicians were also taken into account. No subjects in the persistence group underwent interferon (IFN) therapy in 2000–2002.

This study was approved by the RERF Human Investigation Committee, and all subjects gave written informed consent before each examination.

2.2. Assays in hepatitis screening and clinical examinations

In 1993–1995, anti-HCV Ab and hepatitis B virus surface antigen were examined using a second-generation passive hemagglutination kit and a reverse passive hemagglutination kit (Dynabott, Tokyo), as described previously [12]. Subjects were diagnosed as having Ab when agglutination was reported in a serum diluted 2^5. Qualitative and quantitative detection of HCV RNA was carried out using the Amplicor HCV ver. 2.0 and the Amplicor HCV monitor test ver. 1.0 and/or ver. 2.0 (Roche Diagnostics Systems, Tokyo, Japan).

Platelet count decreases with progression of liver fibrosis, and this marker has widely been used as a reliable diagnostic tool for liver fibrosis/cirrhosis in patients with chronic HCV infection [13–16]. Postulated mechanisms for such platelet reduction include decreased secretion of the hematopoietic growth factor thrombopoietin from the liver and increased destruction of platelets by antiplatelet antibodies [17,18]. Platelet count was routinely measured in the AHS health examination, and an automatic blood cell counter (Coulter MAXM, Beckman Coulter, Inc, Tokyo, Japan) was used in 2000–2002. AST, ALT, γ-glutamyltransferase (γ-GTP), and total cholesterol were also routinely measured, and an autoanalyzer (Hitachi 7180, Hitachi, Ltd., Tokyo, Japan) was used in 2000–2002.

2.3. Information on lifestyle/environmental factors and clinical data

Information on alcohol drinking and smoking was obtained from questionnaires at the time of the AHS health examination in 1993–1995 and 2000–2002, respectively. Body mass index (BMI) was measured at the AHS health examination in 2000–2002. Radiation dose was estimated by the DS02 dosimetry system [19], based on the weighted skin dose computed as the γ dose plus 10 times the neutron dose. No subjects were diagnosed with human immunodeficiency virus infection. No subjects underwent organ transplantation or immunosuppressive therapy. Clinical information was obtained at the AHS examination in 2000–2002 as well as medical chart review and classified according to the International Classification of Diseases code.

2.4. Lymphocyte subset analysis

Circulating T_H1 and T_H2 cells can be straightforwardly enumerated by flow cytometry, using cell surface markers for chemokine receptor, CXCR3, and prostaglandin D receptor, CRTH2, respectively [20,21]. CD8 T cells expressing CXCR3, known as T_C1, are also involved in viral control during HCV infection [22]. We thus focused on T_H1, T_H2, T_C1, and T_C2 cell subsets, as well as total CD4 T and CD8 T, NK, and B-cell subsets in relation to HCV infection status.

Analytical flow cytometry was conducted in a FACScan machine (BD Biosciences, San Jose, CA) as described previously [23]. Monoclonal antibodies as specific cell surface markers were purchased from BD Pharmingen (San Diego, CA), unless otherwise noted. CD4 or CD8 T cells were enumerated as PerCP-labeled CD3 positive and PE–CD4 or FITC–CD8 positive cells; CD16 or CD20 cells were enumerated as PerCP–CD3 negative and FITC–CD16 (Beckman Coulter, Brea, CA) or PE–CD20 positive cells. We used CXCR3 as a marker for T_H1 and T_C1 cells [20,22] and CRTH2 for T_H2 and T_C2 cells [21]. Namely, T_H1 and T_H2 cells were identified with PerCP–CD4, FITC–CXCR3 (R&D Systems, Minneapolis, MN), and biotinylated CRTH2 (kindly provided by Dr K. Nagata, BML, Kawagoe, Japan) plus PE-streptavidin; T_C1 and T_C2 cells were identified with PerCP–CD8, FITC–CXCR3, and biotinylated CRTH2 plus PE–streptavidin. In every measurement, approximately 20,000 cells were analyzed.

2.5. Statistical analysis

Two-sample Wilcoxon or Pearson χ^2 tests were performed to compare distributions of age, gender, city, radiation dose (Gy), smoking (packs/day), alcohol drinking (converted to grams of ethanol/day), BMI (kg/m^2), AST (IU/L), ALT (IU/L), γ-GTP (U/L), total cholesterol (mg/dL), and platelet count ($\times 10^4/\mu$L) among all combinations of the 3 groups.

Because aging and past radiation exposure likely influenced various immunologic markers [23], these events were also evaluated in this study. In each study group, the associations of lymphocyte subsets with age (at the time of examination), gender, radiation dose, and city were evaluated based on the multiple regression model [24]:

$$\log(\textit{subset percentages or ratios}) = \alpha + \beta_1 \times \textit{age} + \beta_2 \times \text{gender} + \beta_3 \times \text{dose} + \beta_4 \times \text{city} + \beta_5 \times \text{alcohol} + \beta_6 \times \text{smoking} + \beta_7 \times \text{BMI} + \beta_8 \times \text{autoimmune disease} + \beta_9 \times \text{allergic disease} + \beta_{10} + \textit{cancer} + \beta_{11} \times \text{other noncancer diseases},$$

where log is the logarithm at base 10, gender = 0 for male and 1 for female, and city = 1 for Hiroshima and 2 for Nagasaki. Smoking, alcohol drinking, BMI, autoimmune disease (1 if diagnosed, otherwise 0), allergic disease (1 or 0), cancer (1 or 0), and other noncancer diseases (*i.e.*, hypergammaglobulinemia and sarcoidosis, 1 or 0) were also used as additional explanatory variables.

We compared lymphocyte subset percentages or ratios among all combinations of the 3 groups in normal regression analysis with adjustment for age, gender, radiation dose, city, alcohol, smoking, BMI, autoimmune diseases, allergic diseases, and other noncancer diseases: In the regression analysis, an explanatory variable regarding a group (1 group = 0, another group = 1) was used.

Regression analysis was also performed to investigate whether any association existed between subset percentages or ratios and