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Original Article

Alteration in CD8$^+$ T cell subsets in enterovirus-infected patients: An alarming factor for type 1 diabetes mellitus

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Abstract Type 1 diabetes is a multi-factorial disease that can develop due to the combination of genetic and environmental factors. Viruses, particularly enteroviruses, are major environmental candidates in the pathogenesis of type 1 diabetes, even though the mechanisms of pathogenicity of these viruses and their effects on the immune system have not been understood very well yet. Previous studies show that any imbalance in the population of different lymphocyte subsets could develop autoimmune diseases. Our theory is that enteroviral infection causes an impairment in the distribution of lymphocyte subtypes and consequently results in the diabetes onset in some individuals. Therefore, in this project, we evaluated the distribution of T CD8+ lymphocytes and their subsets in type 1 diabetes patients. This study was conducted to investigate the relationship between enteroviral infection and type 1 diabetes mellitus in an Iranian population, and suggestion a predicting approach for susceptible subjects.

Conflicts of interest: All authors declare no conflicts of interests.

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Introduction

Autoimmunity refers to the system of immune responses in which immune cells and involved proteins in this system attack their cells and tissues [1]. Causes of autoimmunity are complex and sometimes unknown but generally, the genetic susceptibility and presence of environmental risk factors can play a role in the development of autoimmunity [1]. Growing incidence of these diseases has attracted much attention. Type 1 diabetes is a cell-mediated autoimmune disease [2] in which pancreatic islet beta cells are destroyed [2—4]. This autoimmune disease is due to the combination of various genetic and environmental factors [5]. In the recent years, having determined genetic factors involved in the development of type 1 diabetes [6,7], efforts have sought to determine the environmental factors of this disease.

Viruses are considered the main environmental causes of autoimmune diseases [8]. Among the viral families, enteroviruses are considered to be involved in the pathogenesis of type 1 diabetes [9,10], although the mechanism(s) of pathogenicity of these viruses has (have) not yet been sufficiently understood. Iran has a low rate of type 1 diabetes incidence (3.7 per 100,000 people) [11], but the frequency of enteroviral infection in type 1 diabetes patients, and their potential effects on the immune system have not been adequately studied in this country. In this study, we investigated enteroviruses genome in peripheral blood mononuclear cells (PBMCs) using a sensitive assay, RT-PCR, and the primers for 5′-untranslated region which are shared by all enteroviruses, to determine the prevalence of infection in Iranian patients with type 1 diabetes.

Previous studies suggested that imbalance in the population of naive, effector and memory lymphocyte subsets could cause autoimmune diseases [12]. It has been shown that enteroviral infections mainly have an immunomodulatory effect and, in rare cases, directly lyse beta cells [5,13]; in other words, these viruses serve as immune modifiers, not just simple triggers. Therefore, we evaluated the potential effects of enterovirus on the distribution of T $CD8^+$ lymphocyte, as main cells involved in the destruction of the beta islet cells, and its subsets in type 1 diabetes patients. First, we looked for the presence of enteroviral infection in patients with diabetes. In the next step, the patient group was divided into two groups consisting of the enterovirus-infected group and non-enterovirus-infected group. Then, we investigated alteration in the distribution of T $CD8^+$ lymphocytes population and their subsets in these groups compared with a control group.

Materials and methods

Patients and clinical specimens

In this case—control study, samples were taken from 35 patients with type 1 diabetes aged 2—27 [mean: 13.7 ± 6.6] years. The patients were followed up by the Department of Internal Medicine of the Shahrekord University of Medical Sciences. After research purposes were explained, the people who volunteered to participate in the study provided informed consent for their voluntary participation. In case of collecting samples from under 18 years-old patients and volunteers, we gained informed consents from their parents or legal guardian after explanations the aims of the research. It should be noted that patients were selected based on their diagnostic criteria by a specialist for this study. We did not sample from diabetic patients with any autoimmune background in their family since we wanted to study on enteroviruses, as an external factor involved in type 1 diabetes diseases, and their probable function on alteration of CTLs subsets.

Then, suitable samples were taken from the patients. Moreover, appropriate samples of 35 healthy people (1—27 (mean: 13.6 ± 7.3) years), who were age and sex-matched with patients, were taken in the same year with controls. The serum samples were kept at −80 °C until assays with anti-enterovirus VP1 protein and autoantibody ELISA kits. RNA was extracted immediately from a portion of freshly PBMCs for RT-PCR enterovirus genome detection. Another part of the collected PBMCs was used to identify phenotypes of T $CD8^+$ subsets using flow cytometry.

RNA extraction and RT-PCR

PBMCs of the patients and controls were isolated from whole blood by Ficoll density gradient centrifugation. We extracted total RNA from PMBCs using QIAamp Viral RNA Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. Total RNA was transferred immediately to and kept in a freezer at −80 °C till cDNA synthesis. cDNA was synthesized with reverse transcriptase kit (Takara, Japan).

Two μl PrimeScript Buffer 5X (containing $MgCl_2$ and dNTP), 0.5 μl PrimeScript RT Enzyme Mix I (containing RNase Inhibitor), 50 pmol Random Hexamer, 1 pmol Gene-specific primer F1: 5′-CACCGGATGGCCAATCCA-3′, and 1.5 μl nuclease free water were added to 5 μl extracted total RNA. This mixture was incubated for 15 min at 42 °C in one cycle for reverse transcription. Then, it was incubated for 5 s at 85 °C for inactivation of reverse transcriptase with heat treatment.

Produced cDNA was used for amplification in nested RT-PCR. We used two-step RT-PCR to detect enteroviral RNA. At the first step, 22.5 μl master mix solution containing 0.5 μl F1 primer, 0.5 μl R1 primer: 5′-CCTCCGGCC CCTGAATG-3′ [9], ready-to-use PCR Buffer (containing Tris—HCl, NH_4SO_4, EDTA and 2-mercaptoethanol in pH = 8.8), 0.5 μl $MgCl_2$, 0.5 μl dNTP, 0.1 μl Taq DNA polymerase 1.2 U and 17.9 μl nuclease-free water was added to 2.5 μl cDNA. The first step of PCR was performed on a total volume of 25 μl of the mixture in the following thermal cycle program: At the first step, we performed initial denaturation at 94 °C for 5 min. Then, five cycles were conducted at 90 °C for 30 s, 59 °C for 25 s and 73 °C for 40 s. These five cycles were followed by 30 cycles in the following thermal cycle program: 94 °C for 45 s, 57.7 °C for 25 s and 73 °C for 45 s. At the end of the first step of PCR, a final extension was conducted at 73 °C for 10 min.

In the second step of the nested-PCR test, 1.6 μl of the first PCR product was diluted at a ratio of 1:20 by nuclease free water. Two and a half μl diluted DNA was mixed in the

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