Cytokines are early diagnostic biomarkers of graft-versus-host disease in liver recipients

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BACKGROUND: Graft-versus-host disease (GVHD) is associated with high mortality. Early diagnosis is essential to start treatment and to improve outcomes. Because of the inflammatory nature, we hypothesize that cytokine profile of patients with GVHD may serve as diagnostic markers. The present study was to evaluate the role of cytokine profile in the diagnosis of GVHD.

METHODS: An immunoassay was used to detect 29 cytokines simultaneously in the serum; the measuring sensitivity of all cytokines was pg/mL. Healthy subjects undergoing annual routine physical examinations served as negative controls; 23 patients with hepatocellular carcinoma (HCC) who had undergone liver transplantation (the LT group) comprised the test subjects. A total of 22 kidney recipients with biopsy-confirmed GVHD (the RT group) were included for comparison. HCC patients with radical surgery (the HCC group, n=22) served as positive control. The liver contents of the three cytokines, IL-2, IL-18, and IFN-γ, were detected with immunohistochemistry. Serum granzyme B and perforin were measured by flow cytometry.

RESULTS: Of the 29 cytokines, the levels of IL-2 and IL-18 were increased significantly in liver recipients with GVHD compared with healthy controls (P<0.05). The serum levels of these three cytokines in the healthy, HCC, IT, and RT groups were IL-2: 0.90±0.02, 4.14±0.61, 5.10±0.89, and 1.48±0.09 pg/mL; IL-18: 80.61±9.35, 109.51±10.93, 230.11±12.92, and 61.98±7.88 pg/mL; IFN-γ: 24.06±3.88, 24.84±3.21, 40.37±5.88, and 15.33±4.72 pg/mL, respectively. Immunohistochemistry showed that these 3 cytokines expressions in the liver were parallel to the serum cytokine. After standard anti-GVHD treatment, the expressions of IL-2, IL-18, and IFN-γ were decreased in the liver (P<0.05). Serum granzyme B and perforin were significantly increased in GVHD patients (P<0.05).

CONCLUSIONS: IL-2, IL-18 and IFN-γ were from liver and might serve as biomarkers for monitoring GVHD development and the effects of anti-GVHD treatment. Granzyme B and perforin may play a role in increasing IL-2, IL-18, and IFN-γ levels in GVHD patients.

KEY WORDS: cytokines; graft-versus-host disease; transplantation; multiplex immunoassay; high-throughput

Introduction

Graft-versus-host disease (GVHD) is a medical complication following the receipt of transplant ed tissue from a genetically different person. It is the fifth cause of death following liver transplantation. Data from liver transplant registries show that China has the largest number of patients with HCC on transplant waiting lists; almost half of all liver transplants are performed in patients with HCC. Previously, we reviewed follow-up data on 6012 Chinese patients with HCC who underwent liver transplantation and found that the major causes of death post-transplantation were hemorrhage, infection, graft failure, multiple organ
dysfunction syndrome, and GVHD. Of these conditions, GVHD is very challenging for clinicians, as it is difficult to diagnose due to similarities between the clinical presentations of drug reactions or viral infections, especially cytomegalovirus (CMV) disease. Early non-invasive diagnostic biomarkers for GVHD are urgently needed.

GVHD after orthotopic liver transplantation (OLT) is a severe complication. The incidence is 1%-2% and the mortality rate 85%-90%. Recent studies have found that cytokines modulate both T-helper type 1 (Th1) and Th2 responses. Cytokine storms are evident in patients with GVHD. The serum concentrations of many cytokines (e.g., IL-18) are elevated in experimental models of GVHD. However, the cytokine profile of the cytokine storm has not been defined. Most relevant studies have used dedicated sample processing, time-consuming flow cytometry, and complicated data analysis; hindering early diagnosis and treatment. In this study, we established a cytokine examination protocol using routine blood samples. We described a high-throughput method that simultaneously measures the levels of multiple cytokines. This is especially valuable for early non-invasive diagnosis of GVHD in patients after transplantation. We also analyzed an HCC cohort that had not undergone transplantation, and healthy controls. We describe serum diagnostic platform for GVHD after organ transplantation; this is not organ-specific and is not compromised by any underlying disease.

Methods

Patients

We screened the medical records of patients who underwent organ transplantation in our center and included 87 patients for whom long-term follow-up data were available. Forty-five transplantation patients (23 HCC patients who underwent liver transplantation (the LT group) and 22 patients who underwent renal transplantation (the RT group)) with biopsy-confirmed GVHD were included. HCC patients who had not undergone transplantation (HCC group, n=22) and healthy subjects (healthy control, n=20) served as positive and negative controls, respectively. GVHD was confirmed histologically after physical examination and virus and microbiology screening to exclude differential diseases such as continuing bacterial and fungal infections. Treatment for GVHD: methylprednisolone (0.5 g/d) for 3 days and 2 doses of Zenapax (50 mg).

This study was approved by the Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine. Written informed consent for blood sample collection was obtained in advance. All clinical information was retrieved from the Transplant Recipients Database described in previous publications.

Cytokine measurements by multiplex immunoassay

Cytokine levels were measured using the Procarta Plex Multplex Immunoassay Kit (Affymetrix; Foster City, CA, USA) as described in the Procarta manual and previous publications. In this assay, multianalyte-profiling beads are used to detect multiple cytokines employing fluorescent dye technology. The use of two lasers and digital signal processing effectively allows multiplexing of up to 50 unique assays within a single sample. Blood samples were centrifuged at 1000 g at 4 °C for 10 minutes, and the sera were stored below -20 °C prior to simultaneous one-time cytokine measurements. Twenty-five µL of serum from each patient were incubated with antibodies against human cytokines in 96-well plates. After rinsing, the plates were incubated with multiple antibodies and the reactions detected using a streptavidin-phyceroerythrin combination; data were quantified using the Luminex system (Bio-Rad; Shanghai, China). Quality control featured plotting standard curves plotted using dilutions of the reconstituted antigens; the curves were constructed using Certificate Analysis Software.

Pathological confirmation and in-situ immunocytochemistry

Pathological slides containing biopsy material collected at the time of the initial diagnosis (GVHD onset) were compared with those containing material collected at the time of the second biopsy (after standard anti-GVHD therapy). Tissues were fixed in 10% (v/v) buffered formalin overnight and embedded in paraffin. Antibodies against IL-2, IL-18, and IFN-γ (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used for immunohistochemistry. The quality control was samples from patients with inflammatory bowel disease, in which IL-2, IL-18, and IFN-γ are expressed at stable levels. Staining was quantified using Image J software (the Chinese version developed by the National Institutes of Health).

GVHD diagnosis and blood sample collection

After liver or renal transplantation, GVHD generally develops when donor lymphocytes mount an alloreactive response against host histocompatibility antigens. Patients develop fever, rash, diarrhea, and pancytopenia. Blood tests are run and biopsies are performed when GVHD is suspected. Blood samples were collected from patients who developed fever or a rash after liver or renal transplantation; GVHD was confirmed by biopsy.