Up-regulation of the Monocyte Chemotactic Protein-3 in sera from Bone Marrow Transplanted Children with Torquetenovirus infection.

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ABSTRACT

Background: Torquetenovirus (TTV) represents a commensal human virus producing life-long viremia in approximately 80% of healthy individuals of all ages. A potential pathogenic role for TTV has been suggested in immunocompromised patients with hepatitis of unknown aetiology sustained by strong proinflammatory cytokines.

Objectives: The aim of this study was to investigate the sera immunological profile linked to TTV infection in bone marrow transplant (BMT) children with liver injury.

Study design: TTV infection was assessed in sera from 27 BMT patients with altered hepatic parameters and histological features, by the use of quantitative real-time PCR, along with TTV genogroups and coinfection with HEV. The qualitative and quantitative nature of soluble inflammatory factors was evaluated studying a large set of cytokines using the Bioplex platform. As controls, sera from 22 healthy children negative for serological and molecular hepatitis markers including TTV and HEV, and for autoimmune diseases, were selected.

Results and conclusions: TTV was detected in 81.4% of BMT patients with a viral load ranging from 10^5 to 10^9 copies/mL. All samples were HEV-RNA negative. A pattern of cytokines, IFN-γ, TNF-a, FGF-basic (p<0.01) and MCP-3 (p<0.001) was found significantly highly expressed in TTV-positive patients compared to TTV-negative and controls. Of note, MCP-3 chemokine showed the highest sera concentration independently of the amount of TTV load and the status of immune system deregulation (p<0.001). In this pilot study for the first time, a positive association was found between TTV and increased level of MCP-3 suggesting a indirect role of TTV in liver injury.

Abbreviations

TTV: TorqueTenovirus; ARD: Acute respiratory diseases; BMT: Blood marrow transplantation; GVHD: Graft versus host disease; HEV: Chronic hepatitis E virus; EBV: Epstein Barr virus; PBC: Peripheral blood cells.

Background

Torquetenovirus (TTV) is an ubiquitous DNA non-enveloped virus discovered in 1997 in the serum of a patient with post-transfusion hepatitis of unknown aetiology [1]. The infection is acquired early in life since more than 90% of children tested positive at the age of 2 years. TTV produces long-lasting viremia in about 80% of healthy asymptomatic subjects of all ages [2,3] and the virus is readily detectable in several organs and tissues including bone marrow [4,5]. The high prevalence of TTV in the general population seems not exclude a pathological role for this virus, probably exerted at high viral load and causing opportunistic diseases [6].

Although a direct association between TTV and human diseases has not been proven, some studies have revealed interesting positive correlation between specific TTV genotypes and the severity of some neoplastic and autoimmune diseases, underlining a strong chronic inflammatory process [7-13]. The influence of the immunosuppression on TTV replication has been previously discussed [14] in bone marrow transplanted (BMT) patients since the liver inflammation and the reconstitution of the immune system after transplantation, paralleled with viremia and the amount of the viral load [15]. Specifically, the interplay of TTV with the inflammatory network has been recently evidenced by functional model. An active interaction of TTV, of at least one viral genotype, with the endosomic cellular Toll-like-receptor-9 (TLR-9) [16,17] seems to drive the immune cells to produce specific pro-inflammatory cytokines, including interferon (IFN)γ and interleukin (IL) 6, which address the cascade of the innate and adaptive response to the infection [18].

In BMT recipients as expected, the prevalence of TTV is extremely higher than in the healthy population due, basically, to TTV dependence on the immune status of donor (autologous vs allogeneic) and the time elapsed from the transplant [19]. In these patients, although this virus

seems not to be associated with hepatitis [20], TTV-induced hepatitis by the genotype I has been reported, demonstrating how infection correlate to severe complications caused by the strong inflammatory response of the infected tissue [21].

Recent studies highlighted the possibility that HEV hepatocytes coinfection could contributed to the liver damage through the non-specifically impairment of TTV replication by the liver inflammatory activity [22,23,24]. However, the immunological profile of the inflammatory response in BMT patients during TTV infection, has been little explored.

Objectives

Since inflammation is considered an important component of the natural history of TTV, we found of interest to explore retrospectively, the prevalence of TTV infection and the concomitant inflammatory milieu of a large panel of cytokines and cellular growth factors in relation to clinical course and HEV coinfection, in a selected pediatric bone marrow transplant patients with signs and symptoms of hepatic injury.

Study Design

Patients. Twenty-seven BMT patients (12 females and 15 males, median age, 9.7 years [range 1-17 years]) underwent BMT for the following conditions: acute lymphoblastic leukemia (10), acute myeloid leukemia (7), juvenile myelomonocytic leukemia (2), myelodysplastic syndrome (2), severe aplastic anemia (2), sickle cell anemia (1), Fanconi anemia (1) and Ewing sarcoma (2).

All patients performed prophylaxis for GVHD with tacrolimus and mofetil micofenolate while micafungin, acyclovir and levofloxacin were administered prophylactically .Twenty–five out of 27 BMT patients had history of blood transfusion before transplant. Patients received blood from 2 to 25 donated units (median number/patients 15 transfusions) according to patient clinical conditions.

Liver injury was defined on the basis of abnormal and/or persistent values of hepatic markers, including alanine aminotransaminase (ALT), aspartate aminotransferase (AST), y-glutamyltransferase (GGT), biliary salts, total bilirubin, alkaline phosphatase, colinesterasi, NH_3, ferritin, and histology features. Histological features, included the modified hepatitis activity index

(mHAI) to classify active chronic hepatitis [25], were reviewed for 25 patients. Serological and molecular markers for routine hepatitis viruses (IgM-type anti-hepatitis A antibody, hepatitis B surface antigen, anti-hepatitis B core antibody, hepatitis B virus DNA, hepatitis C virus RNA, anti-hepatitis C virus antibody, hepatitis G virus RNA) and usual infections, Cytomegalovirus, Adenovirus, Herpes 1-2 virus were ruled out in these patients, while EBV infection was diagnosed only in one of them.

Control group

Serum samples, selected from 22 children (12 female, 10 males; median age 7.7years [range 2-12 years]) undergone to routine haematological analysis, were included in the study as controls. Eligibility criteria for samples collection included: no history of blood transfusion, no hospitalization within the two past years; no chronic systemic medication, no haematological evidence of autoimmune disorders (negative for Ab anti-nucleus and Ab anti-DNA) and negative for serological and molecular markers for hepatitis viruses including TTV and HEV.

Samples. Whole blood samples were collected in a covered test tube with EDTA and following centrifugation at 1500xg for 10 min, two aliquots of sera (1 mL each) from enrolled subjects were immediately transferred into clean polypropylene tubes and maintained at -80°C until analysis.

Laboratory Analysis

Nucleic acids extraction. DNA and RNA were extracted from freeze–stored sera aliquots (500μl) from each subject, with the automated extractor NucliSENS EasyMAG (BioMerieux, Durham, NC) according to the generic protocol with a final elution of 25 μl.

Detection of TTV infection. TTV DNA was determined by the TaqMan real time PCR method, using sense-TTV1 and antisense-TTV2 primers and TTVTP probe [26]. Primers and probe were designed in the conserved, untranslated region of the viral genome, which allowed annealing to TTV isolates belonging to all genogroups. Amplicon size was of 79 bp (nt positions 178-256 of TTV prototype TA278 isolate). Each reaction was performed in a 7900 Real Time PCR System

(Applied Biosystems, Milan, Italy) for 45 cycles of two-temperature cycling (15s at 95° C and 1 min at 60° C) [26]. A standard DNA curve of 10-fold dilutions was constructed for quantification of viral copies in clinical samples, cloning the PCR TTV1-2 product (TA cloning vector, Invitrogen, San Diego, California). In brief, using strip tubes, 50 µl PCR reaction was prepared containing 900 mM of each primer, 100 nM of TaqMan FAM-MGB probe, 25 µl of the $2\times$ TaqMan Universal PCR Master Mix, and 10µl of DNA sample. In addition, 10 µl of each specific standard scale dilution (from 10 to 10^7 copies), were added after sealing the tubes containing the master mix and negative controls. Negative controls, containing distilled sterile water instead of DNA template and TTV negative DNA genomic samples, were included in each Q-PCR experiment run.

TTV positive DNA extracts were then genotyped using a primers set amplifying a sequence of the ORF1 region, as described previously [3]. Sequence homology was performed using BLAST in accordance to Okamoto H, 2009 [27].

HEV Detection

HEV infection was established for all samples by a qualitative test for the detection of HEV specific RNA based on real-time RT-PCR technology (RealStar HEV RT-PCR Kit, Altona Diagnostics, Germany) following protocol.

Determination of cytokines expression

Fifty ul of serum from BMT and healthy children were assayed for the simultaneous quantification of 48 cytokines, pro-human cytokine 27-Plex M50-0KCAF0Y and 21-Plex MF0-005KMII, (Bio-Rad, Hercules, CA) by using a magnetic bead-based multiplex immunoassays (Bio-Plex®; BIO-RAD Laboratories, Milano, Italy) as recently described [28]. Cytokines assay was performed in duplicate and each well was assayed twice. Data was presented as Median Fluorescence Intensity (MFI) and concentration (pg/mL) by the Bio-Plex Manager® software.

Statistical Analysis

The software Stata (v. 13.1) and GraphPad Prism (v. 5) were used for statistical data analysis. The comparison between two groups was made by means of the Mann-Whitney test. The Kruskall-

Wallis one-way analysis of variance was used to compare the three groups. Differences were considered significant at p<0.05.

Results

Viral investigation

Among BMT children, 22 out of 27 (81.4%) had detectable TTV-DNA with a viral load ranging from 3.1×10^5 copies/mL to 2.3×10^9 copies/mL, reaching a peak during the post-transplant follow-up period with a mean viral load of 2.4×10^6 copies/mL.

Specifically, TTV DNA was detected in 11/27 (40.7%) of the samples before transplant and in 11/27 (40.7%) of the samples during the follow-up period (+1 day to+ 605 day). In the phase of myelosuppression (from +1day to +22 day), 5/11 (45.4%) of the children tested TTV-positive (Tab.1) showing higher viral loads. Sequence analysis of the available TTV-positive samples (15/22) turned out that the genotype 1a was the most frequently strain detected, although some point mutations were observed among analysed strains, according to Ishimura M et al [21]. In contrast, in this series HEV-RNA has not been detected.

TTV infection and clinical course.

Considering the clinical course, the ALT transaminase levels (normal range, 30 IU/L) at the time of TTV investigation were 71 IU/L in the TTV-positive and 34.4 IU/L in the TTV-negative patients, showing no significant relationship between TTV infection, viral load and the regimen-related toxicity of the liver. The overall incidence of GVHD (grade I-II) was of 48% (13/27). Twelve out of 13 GVHD-children showed a concomitant TTV infection of whom 6 before and 6 post-transplantation (p=0.18). Only 1 out of 5 patients, tested TTV negative, developed a GVHD of I grade. Nevertheless, among the 3 patients suffered by active chronic hepatitis of unknown etiology, TTV DNA was detected with a high load. Specifically, TTV viral load of 1.2×10^6 copies/mL was found in patient with FA and 5.3×10^7 copies/mL and 2.3×10^9 copies/mL in two patients with ALL, respectively (Tab.1). Moreover, among blood transfused patients 22/25 (88%) had detectable TTV-

DNA. No significant correlation was found between the number of transfusions and the viral load (p=0.02).

Cytokines

The levels of 48 cytokines were simultaneously measured in serum of BMT patients and controls. The expression profile of the detected cytokines was first analyzed by comparing the two groups. As shown in Fig.1, when compared to the controls, a variety of soluble cytokines were found significantly associated to BMT patients. A pattern of proinflammatory proteins, Eotaxin, IL-3 ($p<0.05$), G-CSF, INFα2, MCP-3, SCF ($p<0.001$) and IP-10, IL-2Ra ($p<0.01$) were present at high concentration while conversely, 4 cytokines including FGF-basic, IL-10, IL-2 and IL-4 ($p<0.01$) were found at low level. To better characterize the immunological profile of the secreted cytokines associated to TTV infection, the analysis of cytokines was performed comparing BMT patients TTV-positive to BMT patients TTV-negative. As shown in Fig.2, a specific pattern of factors including IFN-γ, TNF-a, FGF-basic ($p<0.01$) and MCP-3 ($p<0.001$) were found up-regulated and significantly associated to TTV.

Of note, among the above cited cytokines, the increase in MCP-3 production was more pronounced (mean value:50.11 pg/mL) in all patients with detectable TTV-DNA suggesting a strong association with the virus independently of the amount of viral load measured and the deregulation of the immune status of these patients ($p<0.001$) (Fig.3). Moreover, MCP-3 concentration showed a marked correlation with the liver injury in TTV-positive patients, although the data was not statistically significant (p=0.26) due to the small numbers of TTV-uninfected patients.

Discussion

Hepatic injury is considered an increasingly frequent complication following bone marrow transplant. In some cases, the cause is linked to the toxicity of the drugs adopted for conditioning, to the infiltration of tumor cells or to GVHD. However, TTV highlighted as candidate for causative agent in hepatitis since strongly influenced by the immune and the inflammatory status [19].

In this study and consistent with previous data [19], the overall prevalence of TTV was very high (81.4%). TTV detection neither before nor after BMT significantly affected the clinical course, although patients with unknown chronic hepatitis, were found associated to a high viral load. This finding seemed reinforce the notion that TTV-induced clinically relevant pathological process may be dependent on replication levels rather than on mere TTV infection.

TTV viremia was detected in 45.5% of patients during the phase of myelosuppression. According to recent data, TTV viral load was particularly higher in this period supporting the influence of the impairment of the cellular immune status on virus replication [24] and the fact that TTV replicate mainly in the liver than in other cells [29-32].

In liver disease, chronic inflammation is an integral component leading to continuous hepatocyte damage. Prompted by the report of recent in vitro experiments showing the potential of TTV to stimulate proinflammatory cytokines (e.g IL-6 and IL-10) with a strong impact on development and severity of the disease [33], our study demonstrated that a different profile of proinflammatory cytokines, IFN-γ, TNF-a, FGF-basic ($p<0.01$) and MCP-3 ($p<0.001$), was specifically associated to BMT patients and to TTV infection. Among these, MCP-3 showed a significantly greater level in TTV-positive patients than in TTV-negative and controls. Remarkably, MCP-3 is the unique cytokine found strongly associated with TTV infection independently of the impairment of immune status due to regimen-related toxicity and of the amount of the measured viral load.

Human monocyte chemotactic protein-3 (MCP-3), also known as CCL7, was first described as a protein with potent chemotactic activity for monocytes inducible in most body compartments, playing an important role in various pathologies including cancer, auto-immune and chronic inflammation. Nevertheless, this chemokine occurs in the context of viral infections although the mechanisms by which viruses naturally induce MCP-3 synthesis are not well understood [34-38]. A partial interpretation about MCP-3 up-regulation documented in our patients, it could be drawn by the concomitant detection of elevated level of IFN-γ and TNF-α, that in turn, can synergically

induced the over-expression of MCP-3, a mechanism documented both in human fibroblasts and in mononuclear leukocytes [39].

Macrophages and Kupffer cells are principal source of inflammatory cytokines promoting Toll-like receptor (TLR)-mediated liver injury by inducing the production of chemokines such as MIP-1, MCP-1, MCP-2 and MCP-3, playing an important role in the formation of portal tract lesions and particularly of the bile duct [40]. Among the liver cells, Kupffer cells, hepatocytes, stellate cells, biliary epithelium, and sinusoidal endothelium, constantly express TLR-4 which levels have been found to be up-regulated in hepatitis B-and C-infected cells [41]. Recently, TLR-9, another member of the TLR family, has been found involved in the pathogenesis of HCV hepatitis and as indirect marker for viral replication [41]. To note, TTV can also activate TLR-9 which seems to act as intermediate in the production of inflammatory cytokines network [33].

Although functional studies are necessary to better define the relationship among TTV infection, TLR and MCP-3 serum released, this data supported evidence that TTV viremia in BMT children paralleled with elevated concentration of this pluripotent chemokine. Increased level of MCP-3 have been reported in inflammatory diseases, including biliary cirrhosis and microbial infection in the PBC liver, supporting the crucial role of the immune-regulation in the recruitment of mononuclear cells in pathological lesions [42].

The findings described in this pilot study, despite the limitation of the small number of patients with suitable samples for viral and immunological analysis, demonstrated for the first time a significantly association between sera high level of MCP-3 and TTV infection in BMT children. Likewise, liver injury complication seems to correlate with the soluble level of this chemokine. However, it remains to be determined whether MCP-3 may be a useful prognostic biomarker of liver injury through a further prospective study including a large cohort BMT patients.

Funding

Competing interests

None declared.

Ethical approval

This study was approved by the local Ethic Committee of the Institute for Maternal and Child Health-IRCCS "Burlo Garofolo"-Trieste, Italy, and informed consent was obtained from each participant in accordance with the principles outlined in the Declaration of Helsinki.

Authors' contributions: ZN performed experiments and drafting the manuscript; MN, BE and PA provided clinical samples and clinical records; GC and RDS supported technical assistance; MC design the study, analysed the data and revised the manuscript.

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