Evaluation of Serum Perforin, Caspase-3, sFasL and M-30 Levels as Apoptotic Markers in Children With Crimean-Congo Hemorrhagic Fever

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Background: Apoptosis is a main regulator in responses of cellular immunity throughout systemic viral infections. Perforin, soluble Fas ligand, caspase-3 and caspase-cleaved cytokeratin-18 (M-30) are mediators of apoptosis. The aim of this study is the evaluation of Crimean-Congo hemorrhagic fever (CCHF) disease changes in the levels of these apoptotic markers and the relation of these changes with disease severity.

Methods: Forty-nine hospitalized children with CCHF and 36 healthy controls were enrolled in this prospective study. The CCHF patients were classified into 2 groups based on disease severity (severe group and nonsevere group). Demographic characteristics and clinical and laboratory findings of all patients were recorded on admission.

Results: Serum perforin, caspase-3 and soluble Fas ligand levels were found to be significantly higher both in the severe and nonsevere CCHF groups than the healthy control group ($P < 0.05$), but there was no significant difference in these apoptotic markers between severe and nonsevere CCHF groups ($P > 0.05$). In addition, serum M-30 levels did not differ significantly among all groups ($P > 0.05$). There was a positive correlation between serum values for perforin, caspase-3 and M-30 and the disease's severity criteria such as aspartate aminotransferase and/or alanine aminotransferase. The serum levels of all these markers were negatively correlated with disease severity criteria such as the platelet count.

Conclusions: In this study, we concluded that the interactions of cytolytic granules containing perforin and caspase cascade and Fas-FasL may play an important role in the pathogenesis of CCHF in children.

Key Words: perforin, soluble Fas ligand, caspase-3, M-30, apoptosis, children, Crimean-Congo hemorrhagic fever

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Crimean-Congo hemorrhagic fever (CCHF) is a zoonotic viral disease caused by the CCHF virus (CCHFV). CCHFV is a member of the *Nairovirus* genus, family Bunyaviridae, and is the only one of its genus leading to hemorrhagic symptoms in humans. It is generally transmitted to humans via a tick bite, contact with tissues of infected livestock or exposure to infected blood.[1,2] The specific mechanisms underlying the pathogenesis of the CCHF infection have not been clearly explained. The most important targets are mononuclear phagocytes, hepatocytes and endothelial cells for the CCHFV.[3,4] Burt et al[3] reported the necrotic foci in liver parenchyma due to the direct viral cytopathic effect of CCHFV. Several studies have shown that secondary hemophagocytosis is due to CCHFV.[5–8] Natural killer cells and cytotoxic T lymphocytes (CTLs) play an important role in hemophagocytosis[7–9] and the viral clearance known as apoptosis in CCHF.[10,11] Also, recent in vitro studies have demonstrated that macrophages, dendritic cells[12] and hepatocytes are involved by apoptotic process in CCHF pathogenesis.[13,14] The apoptotic process offers 3 pathways to fulfill this function.

The secretion of cytokines [interferron gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) and chemokines] that either interfere with viral attachment, input and replication or induce apoptosis of the infected cells is the first apoptotic pathway.[15–17] It has been reported that these cytokines [interleukin (IL)-6, IL-10, TNF-α] play an important role in the mortality of CCHF patients.[18] The second pathway is associated with the exocytosis of perforin.[16,17,19] The release of serine proteases and activating caspases that have been divided by their mechanism of action and are either initiator caspases (caspase-8 and -9) or executioner caspases (caspase-3, -6 and -7) initiate apoptosis in the target cells.[20] The induction of apoptosis by a Fas-Fas ligand (FasL)-mediated interaction that also activates caspase-dependent pathways in the infected cell represents the last apoptotic pathway.[16,17,20] Soluble FasL (sFasL) is formed to cleave FasL by a matrix metalloproteinase-like enzyme. Therefore, serum sFasL levels can be a marker for the activation of cytotoxic cells in some diseases.[21] Cytokeratin-18, also known as M-30, is a caspase substrate, the cleavage of which contributes to cellular collapse during programmed death of hepatocytes.[22]

According to our knowledge, changed levels of apoptotic markers such as perforin, sFasL, caspase-3 and M-30—which play an important role in the other viral hemorrhagic fevers of apoptotic pathways—have been reported,[16,23,24] but the changes of the markers in CCHF are limited by in vitro studies which mention that CCHFV induces possible caspase-3-dependent apoptosis.[10,11] There is no study evaluating the relationship between these apoptotic markers and clinical course in children with CCHF, as far as we know. Therefore, we proposed to evaluate the role of apoptosis on the pathogenesis and clinical course of the disease by measuring serum perforin, sFasL, caspase-3 and M-30 levels in children with CCHF.

MATERIALS AND METHODS

This prospective study included all patients with CCHF (n = 49; mean age, 12.3 ± 3.8 years; 30 males) who had been hospitalized in the pediatrics unit. As a healthy control group (HC), age- and gender-matched individuals (n = 36; mean age, 11.0 ± 2.9 years; 20 males) with no history of health problems are involved in the study. Blood samples were obtained in the acute phase before ribavirin therapy, and laboratory parameters were measured. Our study protocol was approved by the local Human Ethics Committee, and informed consent was obtained from the parents.

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The patients had positive serological tests with enzyme-linked immunosorbent assay (ELISA) (anti-CCHF IgM antibodies) and/or CCHFV genomic segments by real-time reverse transcription-polymerase chain reaction (RT-PCR) were recruited for the study. Serum samples of the patients were sent to the Refik Saydam National Public Health Agency (RSNPHA), Virology Reference and Research Laboratory of the Ministry of Health. TaqMan-based one-step real-time RT-PCR was used to reveal and quantify CCHF viral RNA at RSNPHA.[25,26] CCHF IgM in-house ELISA testing was performed at the RSNPHA, Virology Research and Reference Laboratory following the recommendations of the Centers for Disease Control and Prevention (Atlanta, GA). CCHF IgM was identified on MAC-ELISA prepared with inactivated native CCHF viral antigens (Strain IbAr 10200) grown in Vero E6 cells on serum samples.[25,26]

Previous CCHF studies were conducted mostly on adult patients using fatality rate as a marker for disease severity[27–29]; hence, such marker could not be used for disease severity among children at all time. The classification of disease severity in our study was based on the study by Deveci et al[30] due to the fact that there was no fatality in our study. Furthermore, the other studies suffered much less fatality in children[31,32] than in adults with CCHF.[28,29] Deveci et al[30] based their classification of disease severity criteria on several laboratory and clinical findings. In our study, planned in the light of the aforementioned studies, the CCHF patients were classified into 2 groups based on disease severity (severe and nonsevere). Patients with at least one of the following were considered as severe cases: somnolence; melena; activated partial thromboplastin time (aPTT) ≥60 seconds; and thrombocyte count ≤ 20×10^9/L during their hospital stay.[28,30] After samples were obtained, all patients were treated with Ribavirin according to the World Health Organization recommendations.[33]

In this study, serum samples were collected as soon as venous lines were placed in all patients and HC. Blood samples were centrifuged and the serum was stored below −80°C. All clinical chemical measurements were performed at the Clinical Laboratory of Cumhuriyet University Medical Research Hospital. Perforin, sFasL, caspase-3 and M30 were measured as apoptotic markers. Serum perforin levels were measured with the commercial quantitative sandwich enzyme immunoassay (EIA) method (Novatein Biosciences, Cambridge, MA). The intra-assay coefficent of variation (CV) value was <10% and the interassay CV was <10% for perforin. Serum sFasL levels were measured by the EIA kit with instant ELISA technology (eBioscience Campus Vienna Biocenter 2, Vienna, Austria). The intra-assay CV value was 6.1% and the interassay CV was 7.0% for sFasL. Serum caspase-3 levels were measured by the EIA kit with instant ELISA technology (eBioscience Campus Vienna Biocenter 2). The intra-assay CV value was 7.7% and the interassay CV was 5.4% for caspase-3. Serum M-30 (caspase-cleaved part of cytokeratin-18) levels were determined with commercial quantitative sandwich EIA method (M30-Apoptosense ELISA kit, PEVIVA). The intra-assay CV value was <10% and the interassay CV was <10% for M-30.

Statistical Analysis

The data were evaluated using the Statistical Package for the Social Sciences (SPSS) 16.0 Package (SPSS, Chicago, IL) for Windows program. Descriptive statistics were expressed as an arithmetic median (min–max). Nonparametric Mann-Whitney *U* test and Kruskal-wallis test were conducted to determine the significance of independent continuous variables. Depending on the normality of the variables, the Pearson's or Spearman's test was used for bivariate correlations. Differences were assessed by the χ^2 test for categorical variables. *P*-values of less than 0.05 were regarded as significant.

RESULTS

The clinical signs most frequently detected in children with CCHF were tonsillopharyngitis, nausea/vomiting and headache (44.9, 40.8 and 35.4%, respectively). Bleeding (epistaxis or hemoptysis) was seen in only 2 (4.1%) patients. The other significant identified clinical symptoms were facial-conjunctival hyperemia (25.0%) and petechiae-purpura or ecchymosis (6.1%). Hospitalization days were significantly greater in the severe group than in the nonsevere group. In addition, fatigue, weakness, myalgia and arthralgia were significantly more common clinical features in the severe group than in the nonsevere group. Table 1 presents selected demographic and clinical data of the CCHF and HC groups.

Although serum levels of aspartate aminotransferase (AST), lactate dehydrogenase (LDH), ferritin, aPTT and D-dimer were

TABLE 1. Demographic and Clinical Characteristics of the Groups

Characteristics	Healthy Control Group (n = 36)	Severe CCHF Group (n = 22)	Nonsevere CCHF Group (n = 27)	*P* Value
Age (yr)	11.0 ± 2.9	12.8 ± 3.3	11.9 ± 4.2	NS
Gender (male/female)	20/16	12/10	18/9	NS
Days from symptoms to admission	—	5.1 (2.0–12.0)	5.6 (0.0–15.0)	NS
Hospitalization days	—	11.0 ± 3.6	8.4 ± 2.7	**0.017***
Symptoms (n, %) and clinical findings (n, %)				
Fever (>38°C)	—	9 (41%)	7 (26%)	NS
Fatigue/weakness	—	9 (41%)	4 (15%)	**0.036***
Myalgia	—	5 (23%)	0 (0%)	**0.008***
Arthralgia	—	3 (14%)	0 (0%)	**0.041***
Headache	—	10 (45%)	7 (26%)	NS
Abdominal pain	—	6 (27%)	2 (7%)	NS
Diarrhea	—	6 (27%)	3 (11%)	NS
Vomiting	—	17 (77%)	3 (11%)	**<0.001***
Somnolence	—	1 (4%)	0 (0%)	NS
Tonsillopharyngitis	—	12 (54%)	10 (37%)	NS
Facial-conjunctival hyperemia	—	11 (50%)	1 (4%)	**<0.001***
Bleeding (epistaxis, hemoptysis or melena)	—	2 (9%)	1 (4%)	NS
Petechiae, purpura or ecchymosis	—	2 (9%)	1 (4%)	NS
Hepatomegaly	—	1 (4%)	0 (0%)	NS
Splenomegaly	—	1 (4%)	0 (0%)	NS

*$P < 0.05$.
NS indicates nonsignificant ($P > 0.05$).

TABLE 2. Comparison of Laboratory Parameters of the Groups

Laboratory Parameters	Severe CCHF Group (n = 22)	Nonsevere CCHF Group (n = 27)	Healthy Control Group (n = 36)	*P* Value
WBC (×10⁹ cells/L)	3.1 (0.8–14.6)*	3.3 (1.5–7.7)†	7.2 (2.2–4.4)	**<0.001**§
Hb (g/dL)	13.3 (11.8–16.8)	13.4 (10.7–15.6)	13.2 (11.4–15.9)	NS
PLT (×10⁹ cells/μL)	66.7 (8.0–177.0)‡	129.5 (71–221.0)†	327.6 (196.0–503.0)	**<0.001**§
PT (s)	15.1 (9.2–25.9)	13.8 (9.7–22.4)	12.2 (10.1–14.5)	NS
aPTT (s)	43.3 (23.9–66.3)*‡	34.8 (16.0–47.4)†	30.7 (23.7–35.9)	**<0.001**§
INR	1.3 (0.9–2.4)	1.2 (0.9–2.0)	1.0 (0.9–1.5)	NS
Fibrinogen (mg/dL)	211.0 (96.0–261.0)*‡	246.3 (136–303)†	326.4 (216.2–465.6)	**<0.001**§
D-dimer (μg/mL)	7.7 (0.6–39.8)*‡	1.3 (0.1–5.1)†	147.8 (56.0–256.0)	**<0.001**§
AST (IU/L)	175.2 (29.0–578.0)*‡	73.0 (20.0–235.0)†	20.3 (13.0–38.0)	**<0.001**§
ALT (IU/L)	79.7 (11.0–341.0)*‡	38.8 (8.0–218.0)	20.7 (6.0–49.0)	**0.001**§
LDH (IU/L)	584.6 (160.0–1416.0)*‡	289.9 (132.0–533.0)†	205.6 (119.0–283.0)	**<0.001**§
CPK (IU/L)	1350.2 (63.0–17856.0)	375.1 (64.0–2352.0)	127.2 (67.6–202.1)	NS
CRP (mg/L)	18.9 (1.6–78.0)*	9.5 (0.5–59.0)	4.8 (1.0–13.3)	**0.044**§
TG (mg/dL)	108.6 (34.0–242.0)	109.5 (13.0–226.0)	116.4 (16.0–214.0)	NS
Ferritin (ng/mL)	3375 (195.0–7500.0)*‡	958 (42.0–5350.0)†	32.5 (13.0–65.0)	**<0.001**§

ALT indicates alanine aminotransferase; aPTT, activated partial thromboplastin time; AST, aspartate aminotransferase; CPK, creatine phosphokinase; CRP, C-reactive protein; Hb, hemoglobin; HC, healthy control; INR, international normalized ratio; LDH, lactate dehydrogenase; NS, nonsignificant ($P > 0.05$); PLT, platelet count; PT, prothrombin time; TG, triglyceride; WBC, white blood cell.

*$P < 0.05$ vs. HC group.

†$P < 0.05$ vs. HC group.

‡$P < 0.05$ vs. nonsevere group.

§$P < 0.05$ when all groups were compared.

significantly higher, the fibrinogen level and thrombocyte count were lower in the severe and the nonsevere CCHF groups compared with the HC group. The serum levels of AST, alanine aminotransferase (ALT), LDH and ferritin were significantly higher in the severe group than in the nonsevere group. When we compared the nonsevere with the severe group, the serum levels of aPTT and D-dimer were higher and serum levels of the fibrinogen and thrombocyte count were significantly lower in the severe group than those in the nonsevere group (Table 2).

Serum levels of perforin and caspase-3 were found to be significantly higher in both severe and nonsevere CCHF groups than in the HC group ($P < 0.001$). In addition, serum levels of sFasL were found to be significantly higher in severe and nonsevere CCHF groups than in the HC group ($P < 0.05$). Although there were no statistical differences between the severe and nonsevere groups, there were statistical differences between the CCHF groups and the HC group in the serum levels of perforin, caspase-3 and sFasL. In addition, there were no statistical differences among all groups in the serum levels of M-30 (Table 3).

There was a positive correlation between serum levels of perforin and caspase-3 ($R = 0.297$; $P = 0.006$). Although perforin had a positive correlation with AST, LDH and caspase-3, there was a negative correlation between perforin, white blood cell, platelet count (PLT) and fibrinogen. Although sFasL had a positive correlation with prothrombin time (PT), there was a negative correlation between sFasL and AST, ALT and triglyceride. Although caspase-3 had a positive correlation with PT, international normalized ratio, aPTT, D-dimer, AST, ALT, LDH and C-reactive protein, there was a negative correlation between caspase-3 and PLT and triglyceride. Although M-30 had a positive correlation with aPTT, AST, ALT, LDH and ferritin, there was a negative correlation between caspase-3 and PLT (Table 4).

The diagnostic tests of CCHF patients were positive with ELISA (n = 5), RT-PCR (n = 12), and both ELISA and RT-PCR (n = 32). There was no significant association between the apoptotic markers and ELISA and/or PCR positivity (see Table 5).

DISCUSSION

Although knowledge about hemorrhagic fever viruses has increased considerably, the specific mechanisms underlying the pathogenesis of CCHF infection have not been clearly discussed in the literature.[1,3,4] As is the case with other hemorrhagic fevers, lymphocytes, monocyte-macrophage activation, excessive cytokine secretion, delayed induction of interferons, weak antibody response, partial activation of dendritic cells, apoptosis and hemophagocytosis play essential roles in the pathogenesis and prognosis of CCHF.[3,4,7,8] In recent literature about children with CCHF, increased resting heart rate (>96 bpm) was found as independent risk factor for severe disease,[34] and the children with CCHF who had O blood type were shown to be more likely to bleed.[35] In this study, we aimed to evaluate the role of apoptosis on the pathogenesis and clinical course of the disease by measuring serum apoptotic marker levels in children with CCHF.

Hemorrhagic fever viruses have the potential to attack the antiviral-response-initiating-cells; therefore, collapsing the host immune response by manipulating them.[36] The CCHFV provides many different ways of bypassing the immune response, such as apoptosis of hepatocytes[13,14] and lymphocytes and hemophagocytosis, triggering uncontrolled viral replication followed by the viral systemic spreading throughout the body.[4] Rodrigues et al[13] showed that IL-8 levels were increased in response to viral replication in CCHFV-infected hepatocytes. Unlimited CD4-T lymphocyte and monocyte-macrophage activation, causing to massive secretion

TABLE 3. Comparison of the Apoptotic Markers Among the Groups

	Severe CCHF Group	Nonsevere CCHF Group	Healthy Control Group
Perforin (pg/mL)	32.7 ± 14.9*	30.4 ± 14.7*	14.6 ± 15.5
sFasL (ng/mL)	0.15 ± 0.04†	0.14 ± 0.06†	0.19 ± 0.06
Caspase-3 (ng/mL)	0.21 ± 0.06*	0.20 ± 0.04*	0.17 ± 0.01
M-30 (IU/L)	512.9 ± 380.2	379.7 ± 174.0	361.2 ± 136.7

*Significantly different from HC at $P < 0.001$ level.

†Significantly different from HC at $P < 0.05$ level.

TABLE 4. Correlation Analyses Between the Apoptotic Markers and the Routine Parameters in Patients With CCHF

Correlations	Apoptotic Markers							
	Perforin		sFAS-L		Caspase-3		M-30	
Continuous Variables	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
WBC	**–0.294**	**0.011**	0.169	0.149	–0.180	0.126	–0.240	0.050
PLT	**–0.468**	**<0.001**	0.220	0.060	**–0.333**	**0.004**	**–0.246**	**0.045**
PT	0.154	0.260	**0.275**	**0.042**	**0.570**	**<0.001**	0.152	0.283
INR	0.191	0.161	0.240	0.078	**0.605**	**<0.001**	0.162	0.251
aPTT	0.205	0.144	0.018	0.901	**0.300**	**0.031**	**0.365**	**0.010**
Fibrinogen	**–0.347**	**0.033**	0.021	0.901	–0.217	0.192	–0.229	0.172
D-dimer	0.016	0.926	0.051	0.763	**0.416**	**0.009**	–0.022	0.895
AST	**0.273**	**0.021**	**–0.266**	**0.025**	**0.419**	**<0.001**	**0.431**	**<0.001**
ALT	0.187	0.118	**–0.244**	**0.040**	**0.319**	**0.007**	**0.452**	**<0.001**
LDH	**0.286**	**0.027**	–0.003	0.982	**0.342**	**0.008**	0.027	0.140
CRP	–0.012	0.928	0.211	0.111	**0.518**	**<0.001**	0.143	0.302
Triglyceride	–0.011	0.945	**–0.386**	**0.013**	**–0.337**	**0.031**	–0.066	0.689
Ferritin	0.213	0.205	–0.037	0.830	0.231	0.170	**0.378**	**0.027**
Caspase-3	**0.297**	**0.006**	–0.159	0.147	—	—	0.126	0.280

ALT indicates alanine aminotransferase; aPTT, activated partial thromboplastin time; AST, aspartate aminotransferase; CRP, C-reactive protein; INR, international normalized ratio; LDH, lactate dehydrogenase; PLT, platelet count; PT, prothrombin time; WBC, white blood cell.

Bold *r* values indicate significant correlation.

Bold *P* values indicate statistical significance.

TABLE 5. Comparison of the Apoptotic Markers Between CCHF Patients Diagnosed With Only ELISA, Only PCR and Both

Apoptotic Markers	ELISA (n = 5)	PCR (n = 12)	ELISA and PCR (n = 32)	*P* Value
Perforin (pg/mL)	22.7 ± 17.1	31.6 ± 14.3	32.8 ± 14.5	0.165
sFasL (ng/mL)	0.16 ± 0.05	0.15 ± 0.04	0.15 ± 0.05	0.627
Caspase-3 (ng/mL)	0.20 ± 0.02	0.20 ± 0.04	0.21 ± 0.05	0.833
M-30 (IU/L)	385.7 ± 143.2	433.1 ± 265.0	456.8 ± 328.4	0.995

ELISA indicates enzyme-linked immunosorbent assay; PCR, polymerase chain reaction.

of proinflammatory cytokines such as IFN-γ, IL-1, IL-6, TNF-α and a soluble IL-2 receptor, all appear to be the main mechanisms involved in the pathogenesis of hemophagocytic syndrome in CCHF.[4,7,18,30] Other hemophagocytic markers such as soluble CD8, sFas and sFasL are increased in the active stage of the hemophagocytic syndrome and return to normal levels in a remission situation, providing insights for the role of CTLs.[15,21,37] In addition, serum sFas and sFasL levels in hemophagocytic syndrome could not predict a patient's clinical course.[15,37] In a prospective study of children with dengue virus, it was shown that increased plasma levels of sFas were also higher in children with dengue hemorrhagic fever than in those with dengue fever.[24] Emmenegger et al[37] reported a strong correlation between ferritin as hemophagocytosis diagnostic criteria and sFasL levels in the reactive macrophage activation syndrome were evidenced. It has previously been demonstrated that serum ferritin levels are increased in CCHF.[7,29] In our study, serum sFasL and ferritin levels were found to be significantly higher in CCHF groups than in the HC group. Although serum ferritin levels were significantly higher in the severe group than in the nonsevere group, there was no correlation between serum sFasL and ferritin. It should be noted that only the serum M-30—the major intermediate filament protein in the hepatocyte apoptosis—had a moderate correlation with ferritin ($R = 0.378$) and a strong correlation with AST and ALT ($R = 0.431$ and $R = 0.452$, respectively). These results were consistent with those found by Kim et al,[38] who reported that serum M-30 levels in patients with nonalcoholic steatohepatitis had strong correlation with ferritin ($R = 0.432$), AST ($R = 0.609$) and ALT ($R = 0.588$). This study's results supported the theory of the probable role of hemophagocytosis in the pathogenesis of CCHF pediatric patients. In addition, M-30 may play an important role in the apoptosis of hepatocytes in CCHF.

Natural killer cells and CTLs are a component of the native immune system response against viruses and play a role in the detection and analysis of infected cells known as apoptosis.[4,9,16] The granule exocytosis by the perforin and the granzyme B, which together can reproduce all the properties of CTL-induced apoptosis, lead to the start of apoptosis in target cells.[19] Several viral infections such as the HIV-1 infection[39] and the Puumala hantavirus infection[23] have been reported to increase serum levels of perforin. Klingström et al[23] demonstrated increased levels of serum perforin, granzyme B and M-30 during the Puumala hantavirus infection. According to their study, there were significant correlations between the levels of LDH and perforin and the levels of LDH and M-30. In our results, serum perforin levels were found to be significantly higher in CCHF groups than in the HC group ($P < 0.001$); however, there were no significant differences between all groups in M-30 levels. In addition, in our study, similar to their work, we observed a significant correlation between the levels of LDH and perforin but could not determine any correlation between M-30 and LDH. The levels of perforin and LDH correlated significantly; therefore, this may indicate that CCHFV-specific CTLs and/or natural killer cells might be responsible for the cell damage during CCHF.

Activation of T cells by attachment of the T-cell receptor by the suitable major histocompatibility complex-peptide complex induces up-regulation of Fas and FasL in addition to induction of perforin pathways in apoptosis. Their interaction causes the association of adapters such as Fas-associated death domain protein that results in caspase activation and apoptosis.[19,20] The absence

of perforin, FasL or TNF-Receptor-1 has no significant effect on the proliferation and functional inactivation of virus-specific CTLs at the onset of chronic lymphocytic choriomeningitis virus infection.[40] The animal study of Gupta et al[16] reported that protection against the Ebola virus infection is free from Fas-FasL-mediated killing and necessitates the presence of perforin. The study of Valva et al[22] on chronic hepatitis-C in children and adults showed that relationships between sFas, M30 and caspase activity and liver damage impelled them to assess the diagnostic value of apoptotic markers as potential indicators of liver damage. According to the in vitro study of Karlberg et al,[10] CCHFV-induced apoptosis might depend on caspase-3 activation. Furthermore, caspase-3-dependent cleavage of the nucleocapsid protein might indicate a host defense mechanism against lytic CCHFV infection. In our study, serum levels of perforin, caspase-3 and sFasL were found to be significantly higher in severe and nonsevere CCHF groups than in the HC group; however, between severe and nonsevere CCHF groups, there was no significant difference in apoptotic markers (perforin, sFasL, caspase-3, M-30). According to the results of this study, these apoptotic pathways—cytolytic vesicles containing perforin, caspase cascade and Fas-FasL-mediated interactions—in the pathogenesis of CCHF are active. Could these results—there are no differences between these apoptotic markers and disease severity—explain the reasons of disease severity in children with CCHF much less than in the adults?

The criteria of disease severity in CCHF according to the laboratory findings were markedly elevated levels of serum AST, ALT, gamma glutamyl transferase, LDH, creatine phosphokinase, PT, aPTT, international normalized ratio, D-dimer and ferritin and decreased levels of fibrinogen; hemoglobin levels and thrombocyte count had been reported by other studies to date.[27–29] The basic criterion of these studies aimed at identifying disease severity was fatality and included adult patients. The child fatality rate was less frequent than in adults in Turkey.[30–32] Our results, when comparing the severe and nonsevere groups, indicated that levels of serum AST, ALT, LDH, ferritin, aPTT and D-dimer increased and serum fibrinogen levels and thrombocyte count were significantly low in the severe group, so these results were consistent with those of Deveci et al.[30] In addition, there were no changes of levels of serum perforin, caspase-3, sFasL and M-30 in the severe and nonsevere groups. However, there was a positive correlation between serum perforin, caspase-3, M-30 levels and this disease's severity criteria such as AST and/or ALT. The serum levels of all these markers were negatively correlated with the PLT, which is the severity criterion of disease. The results of this study demonstrated that hemophagocytosis and/or apoptosis markers such as perforin, sFasL, caspase-3 and M-30 may play an important role in the pathogenesis of CCHF.

Study Limitations

Apoptotic process has 3 pathways. The first apoptotic pathway includes cytokines such as IFN-γ, TNF-α, IL-6, IL-8 and IL-10 that were not investigated in this study. Therefore, the results of this study do not represent all apoptotic processes. In further studies, investigation of all 3 pathways might reveal more precise results for this issue.

CONCLUSIONS

In this study, serum levels of perforin, caspase-3 and sFasL were increased in children with CCHF. We concluded that the pathogenesis of CCHF in children may play a critical role in the induction of hemophagocytosis and/or apoptosis through cytolytic granules containing perforin, caspase cascade and Fas-FasL-mediated interactions. There are no differences between these apoptotic markers and disease severity; therefore, this may explain the reasons why disease severity in children with CCHF is much less than in the adults, even if the findings of this study alone are in any way valuable. To evaluate the effect of these apoptotic markers on disease severity, there is a need for new studies that endeavor to compare children and adults with CCHF and to show how the fatality rates are different in each case.

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REFERENCES

1. Whitehouse CA. Crimean-Congo haemorrhagic fever. *Antivir Res*. 2004;64:144–160.
2. Ergonul O. Crimean-Congo hemorrhagic fever virus: new outbreaks, new discoveries. *Curr Opin Virol*. 2012;2:215–220.
3. Burt FJ, Swanepoel R, Shieh WJ, et al. Immunohistochemical and in situ localization of Crimean-Congo hemorrhagic fever (CCHF) virus in human tissues and implications for CCHF pathogenesis. *Arch Pathol Lab Med*. 1997;121:839–846.
4. Akıncı E, Bodur H, Leblebicioglu H. Pathogenesis of Crimean-Congo hemorrhagic fever. *Vector Borne Zoonotic Dis*. 2013;13:429–437.
5. Karti SS, Odabasi Z, Korten V, et al. Crimean-Congo hemorrhagic fever in Turkey. *Emerg Infect Dis*. 2004;10:1379–1384.
6. Cagatay A, Kapmaz M, Karadeniz A, et al. Haemophagocytosis in a patient with Crimean Congo haemorrhagic fever. *J Med Microbiol*. 2007;56(pt 8):1126–1128.
7. Tasdelen Fisgin N, Fisgin T, Tanyel E, et al. Crimean-Congo hemorrhagic fever: five patients with hemophagocytic syndrome. *Am J Hematol*. 2008;83:73–76.
8. Bıçakçı Z, Tavil B, Tezer H, et al. Hemophagocytosis in a case with Crimean-Congo hemorrhagic fever and an overview of possible pathogenesis with current evidence. *Turk J Pediatr*. 2013;55:344–348.
9. Yilmaz M, Aydin K, Akdogan E, et al. Peripheral blood natural killer cells in Crimean-Congo hemorrhagic fever. *J Clin Virol*. 2008;42:415–417.
10. Karlberg H, Tan YJ, Mirazimi A. Induction of caspase activation and cleavage of the viral nucleocapsid protein in different cell types during Crimean-Congo hemorrhagic fever virus infection. *J Biol Chem*. 2011;286:3227–3234.
11. Wang Y, Dutta S, Karlberg H, et al. Structure of Crimean-Congo hemorrhagic fever virus nucleoprotein: superhelical homo-oligomers and the role of caspase-3 cleavage. *J Virol*. 2012;86:12294–12303.
12. Peyrefitte CN, Perret M, Garcia S, et al. Differential activation profiles of Crimean-Congo hemorrhagic fever virus- and Dugbe virus-infected antigen-presenting cells. *J Gen Virol*. 2010;91(pt 1):189–198.
13. Rodrigues R, Paranhos-Baccalà G, Vernet G, et al. Crimean-Congo hemorrhagic fever virus-infected hepatocytes induce ER-stress and apoptosis crosstalk. *PLoS One*. 2012;7:e29712.
14. Fraisier C, Rodrigues R, Vu Hai V, et al. Hepatocyte pathway alterations in response to in vitro Crimean Congo hemorrhagic fever virus infection. *Virus Res*. 2014;179:187–203.
15. Larroche C, Mouthon L. Pathogenesis of hemophagocytic syndrome (HPS). *Autoimmun Rev*. 2004;3:69–75.
16. Gupta M, Greer P, Mahanty S, et al. CD8-mediated protection against Ebola virus infection is perforin dependent. *J Immunol*. 2005;174:4198–4202.
17. Harty JT, Tvinnereim AR, White DW. CD8+ T cell effector mechanisms in resistance to infection. *Annu Rev Immunol*. 2000;18:275–308.
18. Ergonul O, Tuncbilek S, Baykam N, et al. Evaluation of serum levels of interleukin (IL)-6, IL-10, and tumor necrosis factor-alpha in patients with Crimean-Congo hemorrhagic fever. *J Infect Dis*. 2006;193:941–944.
19. Kägi D, Odermatt B, Mak TW. Homeostatic regulation of CD8+ T cells by perforin. *Eur J Immunol*. 1999;29:3262–3272.
20. McIlwain DR, Berger T, Mak TW. Caspase functions in cell death and disease. *Cold Spring Harb Perspect Biol*. 2013;5:a008656.

21. Hasegawa D, Kojima S, Tatsumi E, et al. Elevation of the serum Fas ligand in patients with hemophagocytic syndrome and Diamond-Blackfan anemia. *Blood*. 1998;91:2793–2799.
22. Valva P, Casciato P, Lezama C, et al. Serum apoptosis markers related to liver damage in chronic hepatitis C: sFas as a marker of advanced fibrosis in children and adults while M30 of severe steatosis only in children. *PLoS One*. 2013;8:e53519.
23. Klingström J, Hardestam J, Stoltz M, et al. Loss of cell membrane integrity in puumala hantavirus-infected patients correlates with levels of epithelial cell apoptosis and perforin. *J Virol*. 2006;80:8279–8282.
24. Myint KS, Endy TP, Mongkolsirichaikul D, et al. Cellular immune activation in children with acute dengue virus infections is modulated by apoptosis. *J Infect Dis*. 2006;194:600–607.
25. Yapar M, Aydogan H, Pahsa A, et al. Rapid and quantitative detection of Crimean-Congo hemorrhagic fever virus by one-step real-time reverse transcriptase-PCR. *Jpn J Infect Dis*. 2005;58:358–362.
26. Tuygun N, Tanir G, Caglayik DY, et al. Pediatric cases of Crimean-Congo hemorrhagic fever in Turkey. *Pediatr Int*. 2012;54:402–406.
27. Swanepoel R, Gill DE, Shepherd AJ, et al. The clinical pathology of Crimean-Congo hemorrhagic fever. *Rev Infect Dis*. 1989;11(suppl 4):S794–S800.
28. Cevik MA, Erbay A, Bodur H, et al. Clinical and laboratory features of Crimean-Congo hemorrhagic fever: predictors of fatality. *Int J Infect Dis*. 2008;12:374–379.
29. Barut S, Dincer F, Sahin I, et al. Increased serum ferritin levels in patients with Crimean-Congo hemorrhagic fever: can it be a new severity criterion? *Int J Infect Dis*. 2010;14:e50–e54.
30. Deveci K, Oflaz MB, Sancakdar E, et al. Evaluation of the serum levels of soluble IL-2 receptor and endothelin-1 in children with Crimean-Congo hemorrhagic fever. *APMIS*. 2014;122:643–647.
31. Tezer H, Sucakli IA, Sayli TR, et al. Crimean-Congo hemorrhagic fever in children. *J Clin Virol*. 2010;48:184–186.
32. Dilber E, Cakir M, Acar EA, et al. Crimean-Congo haemorrhagic fever among children in north-eastern Turkey. *Ann Trop Paediatr*. 2009;29:23–28.
33. WHO. Crimean-Congo haemorrhagic fever. Available at: http://www.who.int/mediacentre/factsheets/fs208/en/. Accessed April 25, 2014.
34. Oflaz MB, Bolat F, Kaya A, et al. Resting heart rate in children with Crimean-Congo hemorrhagic fever: a tool to identify patients at risk? *Vector Borne Zoonotic Dis*. 2014;14:59–65.
35. Güven AS, Sancakdar E, Kaya A, et al. Value of ABO blood group in predicting the severity of children with Crimean-Congo hemorrhagic fever. *Int J Clin Exp Med*. 2014;7:416–420.
36. Bray M. Pathogenesis of viral hemorrhagic fever. *Curr Opin Immunol*. 2005;17:399–403.
37. Emmenegger U, Zehnder R, Frey U, et al. Elevation of soluble Fas and soluble Fas ligand in reactive macrophage activation syndromes. *Am J Hematol*. 2000;64:116–119.
38. Kim YS, Jung ES, Hur W, et al. Noninvasive predictors of nonalcoholic steatohepatitis in Korean patients with histologically proven nonalcoholic fatty liver disease. *Clin Mol Hepatol*. 2013;19:120–130.
39. Klingström J, Gudmundsdotter L, Zuber B, et al. Elevated levels of serum perforin in chronic HIV-1 and acute SIV/SHIV infection. *AIDS*. 2006;20:125–127.
40. Zhou S, Ou R, Huang L, et al. Critical role for perforin-, Fas/FasL-, and TNFR1-mediated cytotoxic pathways in down-regulation of antigen-specific T cells during persistent viral infection. *J Virol*. 2002;76:829–840.