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Activation of extrinsic apoptosis pathway in HCV monoinfected and HIV–HCV coinfecting patients, irrespective of liver disease severity

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Abstract Chronic hepatitis C virus (HCV) infection is associated with increased levels of peripheral T cell apoptosis. We aimed to study whether T cell apoptosis markers indicate pathways that may contribute to clinical progression in HCV monoinfected and HIV–HCV coinfecting patients. Activation of the extrinsic apoptosis pathways was measured by levels of death receptor Fas, initiator caspase 8 and effector caspases 3 and 7 activity and Annexin V binding on peripheral CD4 and CD8 T cells of HCV monoinfected and HIV/HCV coinfecting patients, as well as healthy controls and HIV-infected, hepatitis B virus-infected and primary biliary cirrhosis disease controls. Association with liver fibrosis was assessed by biopsy or by transient elastography. HCV monoinfected and HIV–HCV coinfecting patients displayed enhanced peripheral CD4 and CD8 T cell apoptosis. Caspase 8 activity was highest in HIV–HCV coinfection, without enhanced downstream activity of caspases 3 and 7. Level of peripheral T cell apoptosis was independent of liver fibrosis or other disease parameters in all disease groups. The extrinsic apoptosis pathway is upregulated in HCV monoinfection and HIV–HCV coinfection, but this is independent of liver disease severity.

Keywords Apoptosis · T lymphocytes · Liver fibrosis · HCV · HIV

Introduction

Coinfection with human immunodeficiency virus is relatively common in hepatitis C virus (HCV)-infected patients due to shared routes of viral transmission [1]. HIV–HCV coinfection is associated with accelerated progression of liver fibrosis and with poor response to HCV treatment [1–3]. Several factors may contribute to this poor prognosis in coinfecting patients, including T cell apoptosis.

Cellular apoptosis is a major pathway of cell death, and may be initiated either by stimulation of death receptors (extrinsic pathway) or by mitochondrial damage (intrinsic pathway), which are both affected in hepatocytes and peripheral blood mononuclear cells (PBMCs) of HCV- and HIV-infected patients [4–6]. The extrinsic pathway involves stimulation of a death receptor (e.g. Fas) by its receptor (e.g. Fas ligand, FasL), which results in the formation of a death-inducing signaling complex (DISC). In the DISC, Fas-associated death domain (FADD) interacts with the intracellular tail of a death receptor and with procaspase 8 [7–11]. Cleavage of procaspase 8 by FADD leads to activated initiator caspase 8, which in turn activates effector caspases 3 and 7 to initiate apoptosis [7].

HCV leads to chronic infection in the majority of patients [12]. Ongoing viral replication in hepatocytes triggers chronic hepatic inflammation, which may over time result in liver fibrosis and finally cirrhosis [12, 13]. Vigorous CD4 and CD8 T cell responses are essential in viral clearance as can be observed in the first months after infection, but viral persistence is associated with down-regulation of T cell immune responses in several ways,

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including apoptosis of activated T cells [14–17]. Indeed, chronic HCV infected patients display higher levels of peripheral T cell apoptosis than healthy controls [4]. Furthermore, in a HCV transgenic mouse model it was shown that peripheral CD4 and CD8 T cell apoptosis rates were higher compared to non-infected mice [18]. Moreover, increased FasL expression on hepatocytes suggested that the hepatic microenvironment promoted increased intrahepatic T cell apoptosis. This interaction between intrahepatic T cells and hepatocytes may also contribute to liver fibrosis, since hepatic stellate cells (HSCs) may become activated upon phagocytosis of apoptotic lymphocytes [19]. Activated HSCs are the main producers of extracellular matrix in liver fibrogenesis [20]. Therefore, the interplay between apoptotic intrahepatic T cells, HCV-infected hepatocytes and activated HSCs leads to liver fibrosis over time [21, 22]. Furthermore, previous studies on the breadth and proliferative capacity of T cells have shown a high degree of similarity between intrahepatic and peripheral T cells [23, 24]. These observations suggest that the peripheral T cell compartment mirrors its counterparts in the liver. Therefore, we hypothesized that peripheral T cell apoptosis could reflect liver fibrosis.

The present study was performed on freshly isolated PBMCs in order to further elaborate on our previous findings of increased peripheral T cell apoptosis in chronic HCV infected patients [4]. Here, we specifically aimed to evaluate whether level of peripheral T cell apoptosis is a reflection of the degree of liver fibrosis. To this end, patients with other liver disease [hepatitis B virus (HBV) infection or primary biliary cirrhosis (PBC)] resulting in liver fibrosis were included as disease controls.

Methods

Patients

Eighteen HCV monoinfected and 14 HIV–HCV coinfecting patients with known stages of liver fibrosis were included in this study. Controls comprised of 15 healthy controls and 10 HIV-monoinfected, 18 treated HBV infected and 14 PBC patients as disease controls. All patients were recruited from the Infectious Diseases Outpatient Clinic or from the Hepatology Outpatient Clinic of the University Medical Center Utrecht (UMCU). Data on T cell activation and exhaustion, including Fas-expression, of part of the subjects have recently been published elsewhere [25]. Patients with other clinical conditions possibly interfering with their immune system (e.g. liver disease from other causes, alcohol abuse, use of opioids, auto-immune disease, malignancy or any other severe systemic diseases) were excluded from the study. None of the patients received

treatment for HCV at the time of inclusion or within the preceding 12 months. All HIV-infected patients were on highly active antiretroviral therapy (ART) resulting in CD4⁺ T-cell-counts >200/mL and undetectable HIV-viral load (<50 copies/mL). Fibrosis stage was assessed by liver biopsy or transient elastography as previously described [25]. All HBV-infected patients were on antiviral nucleoside analog treatment, resulting in low HBV-DNA (<1,000 IU/mL).

Disease parameters

Levels of HCV-RNA and HIV viral load were measured with COBAS[®] AmpliPrep/COBAS[®] TaqMan polymerase chain reaction (PCR; lower limit of detection 15 IU/mL for HCV and 50 copies/mL for HIV). HBV-DNA loads were measured with COBAS AmpliPrep/TaqMan quantitative PCR (lower limit of detection 20 IU/mL). Blood from anonymous healthy controls was requested from the bloodbank Mini Donor Dienst of the UMCU and was tested negative for hepatitis B, hepatitis C and HIV. All patients and healthy controls were between 18 and 65 years old.

Informed consent was obtained in writing from all patients in accordance with the WMA Declaration of Helsinki and in accordance with the ICH guideline for Good Clinical Practice (6th revision, 2008). The Medical Ethics Committee for Research in Humans (METC) of the UMCU, The Netherlands, approved the protocol of this study.

Processing of blood for isolation of PBMCs and flow cytometric analysis

Whole blood was collected and processed as previously described [25]. Then, PBMCs were incubated with antibodies against CD3 (Horizon[™] V500, clone SP34, provided by BD Biosciences, San Diego, US), CD4 (APC-eF780, RPA-T4, eBioscience, San Diego, CA, USA; or PE-Cyanine7, RPA-T4, eBioscience), CD8 (Pacific Blue[™], RPA-T8, BioLegend, San Diego, CA, USA; or APC-eF780, RPA-T4, eBioscience) and Fas (APC, DX2, BD Biosciences), for 20 min at 4 °C. After washing, cells were incubated with fluorescent inhibitor of caspases for measuring of activity of extrinsic initiator caspase 8 or effector caspases 3 and 7 (FITC, ImmunoChemistry Technologies, Bloomington, MN, USA) at 37 °C (1 × 10⁶ cells/sample). For detection of surface phosphatidylserine (PS), cells were incubated with Annexin V (eF450, eBioscience) for 15 min at room temperature and then directly analyzed by flow cytometry. Per sample, 100,000 events were acquired. In all patients, laboratory procedures were performed within 16 h from venipuncture.

Table 1 Patient characteristics

	HCV <i>n</i> = 18	HIV–HCV <i>n</i> = 14	HIV <i>n</i> = 10	HBV <i>n</i> = 18	PBC <i>n</i> = 14	<i>p</i> Value ¹
General characteristics						
Age, median (IQR, years)	55 (51–60)	48 (40–54)	46 (42–58)	48 (40/56)	61 (54/69)	0.02
Gender (% male)	72	86	90	78	29	0.43
ALT, median (IQR, IU/L)	72 (48–144)	65 (54–134)	30 (19–41)	33 (23/38)	43 (28/56)	0.94
Liver fibrosis/cirrhosis						
F0–2, number (%)	9 (50)	8 (57)		8 (44)	7 (50)	0.69
F3–4, number (%)	9 (50)	6 (43)		10 (56)	7 (50)	
HCV characteristics						
HCV-RNA, med. (IQR, IU/mL)	3.5e5 (1.5e5–9.5e5)	4.5e5 (4.0e5–5.2e6)				0.05
Genotype (% genotype 1)	77	83				0.66

General and disease-specific characteristics of patient groups

ALT alanine transaminase, IQR interquartile range

¹ *p* Value for comparison of HCV with HIV–HCV

Fig. 1 Gating strategy and representative flow cytometry plots of apoptosis markers. Representative flow cytometry plots of T cell apoptosis in an HIV–HCV coinfecting patient. **a–c** Gating of T lymphocytes by lymphogate (**a**), CD3 positive T cells (**b**) and CD4 and CD8 positive T cells (**c**), **d** activated initiator caspase 8 in CD4 T cells, **e** activated effector caspases 3 and 7 in CD4 T cells, and **f** Annexin V positive CD4 T cells. Percentages of positive events are depicted in each plot

Statistical analysis

Medians were compared with Mann–Whitney test and, in case of multiple groups, preceded by Kruskal–Wallis. Two-way ANOVA was used for comparing medians with two independent variables. χ^2 test was used to test relationships between categorical variables. Dependence of variables was tested using Spearman's one-tailed correlation coefficient. Statistical analysis was performed with IBM SPSS Statistics version 19.0 (SPSS, Inc., IBM, USA) and GraphPad Prism 5 for Windows version 5.03 (GraphPad Software, Inc., USA).

Results

Patient characteristics

A total of 89 subjects, including 18 chronic HCV mono-infected patients, 10 HIV-1 mono-infected patients, 14 HIV/

HCV coinfecting patients, 18 HBV infected patients, 14 PBC patients and 15 healthy controls were included in this study. Patient characteristics are shown in Table 1 and most are comparable between the patient group. As expected, HCV-RNA loads were higher in coinfecting (1.9e6 IU/mL) compared to HCV mono-infected patients (3.5e5 IU/mL; $p = 0.07$). PBC patients (median 61 years) and HCV mono-infected patients (median 55 years) were older than other patients (group medians 46–48 years), but the degree of liver fibrosis was similar in all patient groups (Table 1).

Increased activation of the extrinsic apoptosis pathway in HCV mono-infected and HIV–HCV coinfecting patients

In order to determine levels of peripheral T cell apoptosis, we characterized surface expression of death receptor Fas [25], intracellular levels of activated initiator caspase 8 and

Fig. 2 T cell apoptosis affected in patient groups and healthy controls. Measurements of death receptor Fas (**a**), activated extrinsic initiator caspase 8 (**b**), activated effector caspases 3 and 7 (**c**), Annexin V-binding (**d**) in CD4 (left graph) and CD8 T cells (right graph) in healthy controls, HCV-monoinfected, HIV–HCV coinfecting and HIV monoinfected patients, as well as HBV- and PBC-patients as disease controls. KW Kruskal–Wallis p value

Fig. 3 Correlation between mutual apoptosis markers. Correlations and linear regression lines between Fas-expression and activated extrinsic initiator caspase 8 (a), caspase 8 and effector caspases 3 and 7 (b), caspases 3 and 7 and Annexin V-binding (c) CD4 T cells (left graph) and CD8 T cells (right graph) of HCV monoinfected (grey dots) and HIV–HCV coinfecting patients (black dots). R Spearman R , p p value

activated effector caspases 3 and 7 as well as Annexin V-binding to PS on peripheral CD4 and CD8 T cells (for gating see Fig. 1). In line with previous findings from our group [4], CD4 and CD8 T cell of HCV-infected patients depicted overall higher levels of markers of T cell apoptosis than healthy controls. This was evidenced by higher median percentages of Fas (CD4: 61.6 vs. 39.5 %, $p = 0.0012$ and CD8: 29.2 vs. 16.2 %, $p = 0.0971$), and activated caspases 3 and 7 (CD4: 1.0 vs. 0.63 %, $p = 0.07$ and CD8: 0.9 vs. 0.61 %, $p = 0.01$) compared to healthy controls. In contrast, caspase 8 (CD4: 5.5 vs. 4.9 %, $p = 0.16$ and CD8: 4.2 vs. 3.6 %, $p = 0.29$) and Annexin V-binding (CD4: 7.1 vs. 9.2 %, $p = 0.56$ and CD8: 7.5 vs. 10.3 %, $p = 0.58$) were similar to healthy controls (Fig. 2).

HIV–HCV coinfecting patients depicted a similar pattern in comparison to healthy controls, though activated caspase 8 was present at higher levels in coinfecting patients in comparison to HCV monoinfected patients (CD4: 8.8 vs. 5.5 %, $p = 0.04$; CD8: 9.2 vs. 4.2 %, $p = 0.02$) and healthy controls (CD4: 8.8 vs. 4.9 %, $p = 0.0018$; CD8: 9.2 vs. 3.6 %, $p = 0.0037$; Fig. 2). Correlations between Fas-expression and downstream caspase activation and Annexin V-binding further support the hypothesis that the extrinsic apoptosis pathway is upregulated in chronic HCV-infection, similar to infection with HIV (Fig. 3).

CD4 and CD8 T cells of treated HBV patients displayed higher levels of Fas-expression than healthy controls without downstream caspase-activation, while CD4 and

Fig. 4 Markers of T cell apoptosis in relation to liver fibrosis. Boxplots with median and min–max whiskers of percentages of Fas (a), caspase 8 (b), caspases 3 and 7 (c) and Annexin V-binding (d) in

HCV-monoinfected, HIV–HCV coinfecting, HBV-infected, and PBC patients with F0–2 (light grey) or F3–4 fibrosis (dark grey) liver fibrosis

CD8 T cells of PBC-patients depicted increased levels of Fas and activated caspases 3 and 7 in comparison with healthy controls (Fig. 2). This indicates that increased levels of peripheral T cell apoptosis is not specific for viral infection.

Peripheral T cell apoptosis does not reflect degree of liver fibrosis

To investigate whether peripheral T cell apoptosis reflects the degree of liver fibrosis, we compared the expression of apoptosis markers on T cells of HCV monoinfected patients and HIV coinfecting patients, as well as in disease control groups consisting of patients with treated HBV-infection or PBC with minimal or no fibrosis (F0–2) versus those with severe fibrosis or cirrhosis (F3–4). Although median percentages of Fas expression on CD4 T cells were higher in patients with F3–4 fibrosis in comparison to those with F0–2 fibrosis, this was only borderline significant ($p = 0.09$; Fig. 4). Neither Fas expression on CD8 T cells nor any other apoptosis marker showed a consistent pattern, indicating that peripheral T cell apoptosis does not reflect severity of liver fibrosis. Furthermore, no

correlations were found with level of HCV viremia, alanine transaminase (ALT) or CD4 T cell counts or HCV genotype (data not shown).

Discussion

In the present study, we demonstrate that markers of the extrinsic apoptosis pathway are upregulated in peripheral T cells of HCV monoinfected and HIV–HCV coinfecting patients. Interestingly, CD4 and CD8 T cells of HIV–HCV coinfecting patients depict higher levels of initiator caspase 8 compared to HCV monoinfected patients, but the downstream level of effector caspases 3 and 7 was similar (still higher than in healthy controls). Peripheral T cell apoptosis was not associated with degree of liver fibrosis or with other disease parameters.

Consistent with previous findings, our present data demonstrate that peripheral T cell apoptosis is increased in chronic HCV infection [4]. This may be the result of several mechanisms. For instance, HCV core protein enhanced Fas-mediated apoptosis in a T cell line [26], but may also inhibit apoptosis via enhanced Bcl-xL expression [27], or through upregulation of an inhibitor of caspase-activated

DNAse [28]. On the other hand, chronic antigen stimulation is also known to induce T cell death in order to maintain T cell homeostasis, in a process called activation-induced cell death, AICD [29, 30]. AICD may be exerted in a death receptor-dependent or -independent manner, as reviewed in by Brenner et al. [31]. However, in the present study, increased levels of peripheral T cell apoptosis were not only observed in HCV and HIV infection, but also in a non-viral liver disease like PBC. This may be the result of activation due to reactivity against mitochondrial autoantigen (PDC-E2) [32], or by abnormal cytokine regulation associated with PBC [33, 34].

Crispe et al. have proposed two main models to explain intrahepatic T cell apoptosis. First, lymphocyte activation and apoptosis may be induced in the circulation, after which these cells become trapped in the liver, making the liver a ‘graveyard’ [35]. In this model, T cell apoptosis could contribute to liver fibrosis by activation of HSCs through phagocytosis of apoptotic lymphocytes [19]. In an alternative model, the liver merely represents a ‘killing field’, with lymphocyte apoptosis being induced in the liver via clonal deletion or upon entering viral antigen presented by hepatocytes, as Iken et al. [18] proposed based on in vitro experiments [17]. In the ‘killing field’ model, apoptotic peripheral T cells may re-enter the circulation after apoptosis is induced, providing another theoretical basis for peripheral T cell apoptosis mirroring liver disease severity. However, we found no clear cut association between severity of liver fibrosis and cirrhosis and levels of peripheral T cell apoptosis in HCV-infected patients and HBV infected or PBC disease controls, indicating that T cell apoptosis in the periphery is not likely to play an important role in liver fibrosis. However, it should be noted that observations in peripheral T cells do not allow drawing definite conclusions about intrahepatic events.

Since all measurements were performed on freshly isolated PBMCs, to minimize influence of freezing and thawing of lymphocytes which is known to influence apoptosis-related proteins, our data is expected to closely reflect actual T cell apoptosis in the periphery [36]. However, apoptosis is by definition a dynamic process, which makes it difficult to draw definite conclusions from our data. Our findings do provide a basis for future studies to investigate whether T cell apoptosis may be involved in well-controlled coinfection with HIV. Although the clinical relevance of peripheral T cell apoptosis needs to be determined, it may be hypothesized that in HIV-infected patients this reflects ongoing activation and turnover of T cells which contributes to accelerated immune aging and occurrence of diverse late complications in patients with effective cART [37]. Interestingly, initiator caspase 8 activity was significantly higher in T cells of HIV–HCV coinfecting patients in comparison to HCV monoinfected

patients. This may be caused by increased AICD, as a consequence of higher levels of HCV viraemia in coinfecting patients. However, downstream caspase 3 and 7 activity was similar between HIV–HCV coinfecting and HCV monoinfected patients. Caspase 8 is not only a key player in activation of the extrinsic apoptosis pathway, but is also involved in T cell activation [38, 39]. Thus, increased levels of activated caspase 8 may provide one explanation for T cell activation in HIV–HCV coinfecting patients in comparison to HCV monoinfected patients [25].

In conclusion, our data confirm previous findings that HCV infection is associated with upregulation of the extrinsic apoptosis pathway. Furthermore, peripheral T cell apoptosis was not associated the stage of liver fibrosis, suggesting that T cell apoptosis in the peripheral level does not play a prominent role in liver fibrosis.

Conflict of interest The authors declare that they have no conflict of interest.

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