Effects of HCV co-infection on apoptosis of CD4+ T-cells in HIV-positive patients

Christian KÖRNER*, Benjamin KRÄMER*, Daniela SCHULTE*, Martin COENEN*, Stefan MAUSS†, Gerd FÄTKENHEUER‡, Johannes OLDENBURG§, Jacob NATTERMANN*, Jürgen K. ROCKSTROH* and Ulrich SPENGLER*

*Department of Internal Medicine I, University of Bonn, Germany, Sigmund-Freud-Str. 25, Bonn 53127, Germany, †Center for HIV and Hepatogastroenterology, Düsseldorf 40237, Germany, ‡Department of Internal Medicine I, University of Cologne, Cologne 50937, Germany, and §Department of Experimental Hematology and Transfusion Medicine, University of Bonn, Bonn 53127, Germany

ABSTRACT

Apoptosis importantly contributes to loss of CD4+ T-cells in HIV infection, and modification of their apoptosis may explain why HIV/HCV (hepatitis C virus)-co-infected patients are more likely to die from liver-related causes, although the effects of HCV on HIV infection remain unclear. In the present study, we studied in a cross-sectional and serial analysis spontaneous ex vivo CD4+ T-cell apoptosis in HIV/HCV-co-infected and HIV-mono-infected patients before and after HAART (highly active antiretroviral therapy). Apoptosis of peripheral blood CD4+ T-cells was measured by both a PARP [poly(ADP-ribose) polymerase] and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) assay to detect cells with irreversible apoptosis. Although hepatitis C alone did not increase CD4+ T-cell apoptosis, HCV co-infection disproportionately increased elevated rates of apoptosis in CD4+ T-cells from untreated HIV-positive patients. Increased CD4+ T-cell apoptosis was closely correlated with HIV, but not HCV, viral loads. Under HAART, increased rates of CD4+ T-cell apoptosis rapidly decreased both in HIV-mono-infected and HIV/HCV-co-infected patients, without any significant difference in apoptosis rates between the two patient groups after 4 weeks of therapy. Nevertheless residual CD4+ T-cell apoptosis did not reach the normal levels seen in healthy controls and remained higher in HIV patients receiving protease inhibitors than in patients with other antiretroviral regimens. The results of the present study suggest that HCV co-infection sensitizes CD4+ T-cells towards apoptosis in untreated HIV-positive patients. However, this effect is rapidly lost under effective antiretroviral therapy.

INTRODUCTION

The hallmark of HIV infection is gradual loss of CD4+ T-cells, and apoptosis is now considered to contribute importantly to the declining numbers of CD4+ T-cells in HIV-infected patients. Apoptosis is a highly regulated process which is characterized by specific physiological and morphological cell changes resulting in the formation of membrane-enclosed apoptotic bodies which are taken up by surrounding cells without an inflammatory reaction. Induction of apoptosis is mediated via different pathways. The extrinsic pathway is triggered by binding of ligands from the TNF (tumour necrosis factor) family of death receptors (Fas, DR4/5) which initiates activation of proteolytic enzymes, the cascade of caspases. Alternatively, apoptosis is induced in the intrinsic pathway via signals resulting from altered mitochondrial integrity. Both pathways converge at the level of

Key words: apoptosis, CD4+ T-cell, hepatitis C virus, highly active antiretroviral therapy (HAART), HIV-1, protease inhibitor.

Abbreviations: APC, allophycocyanin; HAART, highly active antiretroviral therapy; HCV, hepatitis C virus; PARP, poly(ADP-ribose) polymerase; PBMC, peripheral blood mononuclear cell; PI, protease inhibitor; TNF, tumour necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling.

Correspondence: Dr Christian Körner (email christian.koerner@ukb.uni-bonn.de).
of CD4+ T-lymphocytes, resulting in CD4+ T-cell apoptosis [2–4].

Apoptosis is probably also a characteristic feature of HCV (hepatitis C virus) infection. Apoptosis of hepatocytes and peripheral blood lymphocytes has been reported in HCV-infected patients [5]; however, the underlying mechanisms have so far remained unclear. Moreover, both direct pro- and anti-apoptotic effects have been reported for single HCV proteins [6]. Thus the role of apoptosis is discussed controversially in the pathogenesis of chronic hepatitis C.

Owing to similar routes of transmission, 15–50% of HIV-infected patients are HCV co-infected, resulting in an estimated ten million patients worldwide who suffer from combined HIV and HCV infection. Of note, certain populations with special risk factors, such as HIV-positive haemophiliacs and HIV-positive intravenous drug users, have 70–90% prevalence of HIV/HCV co-infection among their individuals [7]. HIV/HCV co-infection has been identified to lead to increased liver-related mortality and is considered to accelerate the progressive course of HIV-infection towards severe immunodeficiency and AIDS, resulting in an increased overall mortality [8–13].

However, subsequent studies failed to confirm differences in survival between HIV-mono- and HIV/HCV-co-infected patients. For instance, analysis of the EuroSIDA cohort did not reveal any significant differences in decreased HIV loads and increased CD4 counts between HIV-positive and HCV-negative patients after the initiation of effective antiretroviral therapy [14,15]. HAART (highly active antiretroviral therapy) enables effective control over HIV replication and leads to recovery of CD4+ T-cell counts. Thus HAART can substantially increase the life expectancy of HIV-infected patients. Moreover, effective HAART results in reduced progression of HCV-associated liver disease and improved liver-related mortality in HIV/HCV-co-infected patients [16,17]. Finally, immune reconstitution under HAART is also associated with a marked reduction of CD4+ T-cell apoptosis [14,15].

However, concerning HIV/HCV-co-infected patients, it is still unclear to what extent HCV co-infection can modify rates of apoptosis in CD4+ T-cells and whether HAART can reduce apoptosis of CD4+ T-cells in these patients to the same degree as in HIV-mono-infected patients. Thus applying two different assays for apoptosis detection in cross-sectional as well as longitudinal studies of HIV/HCV-co-infected and HIV-mono-infected patients before and after the initiation of HAART, in the present study we directly compared CD4+ T-cell apoptosis between HIV- and HIV/HCV-infected patients.

MATERIALS AND METHODS

Patients and controls

A total of 94 patients and 12 healthy controls were enrolled in the present study comprising 47 HIV-mono-infected, 37 HIV/HCV-co-infected and 10 HCV-mono-infected patients. Of these, 25 HIV-mono-infected and 14 HIV/HCV-co-infected patients had never been treated with antiretroviral drugs or had been without any antiretroviral treatment for at least 1 year prior to the study. In total 22 patients with HIV mono-infection and 23 patients with HIV/HCV co-infection had been receiving effective HAART for at least 1 year, with HIV loads being consistently below the level of detection (less than 50 copies/ml). All HIV patients were infected with HIV-1. All patients with chronic HCV infection had not received any anti-HCV therapy prior to inclusion.

In addition to a cross-sectional analysis over all individuals, eight HIV-mono-infected and five HIV/HCV-co-infected patients could be studied sequentially during initiation of their first HAART (weeks 0, 4 and 12). Demographic and clinical details of all patients are summarized in Tables 1 and 2.

Written informed consent was obtained from all patients. The study had been approved by the local ethics committee of the University of Bonn, Germany.

Isolation and short-term culture of PBMCs (peripheral blood mononuclear cells)

PBMCs were isolated by density centrifugation [2400 rev./min for 20 min at room temperature (20–22°C) in a Beckman GH 3.8 swing-out rotor] from peripheral blood samples with Ficoll Hypaque density gradients (Biochrom). Isolated PBMCs were incubated for 20 h in 96-well plates at a concentration of 1 × 10^6 PBMCs/ml in RPMI 1640 medium (PAA) supplemented with 5% (v/v) autologous serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin.

Detection of apoptotic CD4+ T-cells

Rates of CD4+ T-cell apoptosis were measured by detecting cells via cleaved PARP and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) respectively. CD4+ T-cells in the early stages of apoptosis were detected with a specific antibody against caspase 3 activation, which induces irreversible steps of apoptosis such as cleavage of PARP [poly(ADP-ribose) polymerase] [1].

Several mechanisms of apoptosis induction have been identified in HIV-infected individuals. Examples are killing by bystander cells due to the secretion of death ligands TRAIL (TNF-related apoptosis-inducing ligand) and FasL, which are enhanced in HIV-infected patients. So called AICD (activation-induced cell death) is another mechanism of apoptosis induction in HIV infection contributing to the loss of activated CD4+ T-cells. Finally, HIV proteins themselves, such as HIV Tat or Env proteins, can directly interfere with host lymphocytes, resulting in CD4+ T-cell apoptosis [2–4].

Thus HAART can reduce apoptosis of CD4+ T-cells in these patients to the same degree as in HIV-mono-infected patients. Thus applying two different assays for apoptosis detection in cross-sectional as well as longitudinal studies of HIV/HCV-co-infected and HIV-mono-infected patients before and after the initiation of HAART, in the present study we directly compared CD4+ T-cell apoptosis between HIV- and HIV/HCV-infected patients.
Table 1  Patient characteristics  
Values are medians (interquartile range) for CD4+ and CD8+, all other values are means (range). MSM, men who have sex with men; n.a., not analysed.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Healthy controls</th>
<th>HIV</th>
<th>HIV/HCV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No HAART</td>
<td>HAART</td>
<td>No HAART</td>
</tr>
<tr>
<td>n</td>
<td>12</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>Female gender (n)</td>
<td></td>
<td></td>
<td>5 (20.0 %)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>36.7 (26–51)</td>
<td>40.5 (19–78)</td>
<td>42.5 (28–67)</td>
</tr>
<tr>
<td>Risk factors (n)</td>
<td></td>
<td></td>
<td>7 (70.0 %)</td>
</tr>
<tr>
<td>Intravenous drugs</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Blood transfusion</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Heterosexual</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Haemophilia</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MSM</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Endemic</td>
<td>–</td>
<td>–</td>
<td>3 (12.0 %)</td>
</tr>
<tr>
<td>Unknown</td>
<td>2 (20.0 %)</td>
<td>2 (8.0 %)</td>
<td>1 (4.5 %)</td>
</tr>
<tr>
<td>HIV status</td>
<td></td>
<td></td>
<td>39.9 (0.05–375.9)</td>
</tr>
<tr>
<td>HIV load (× 10^3 copies/ml)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CD4+ (cells/μl)</td>
<td>n.a.</td>
<td>n.a</td>
<td>371 (11–1235)</td>
</tr>
<tr>
<td>CD8+ (cells/μl)</td>
<td>n.a.</td>
<td>n.a</td>
<td>780 (324–1727)</td>
</tr>
<tr>
<td>HCV status</td>
<td></td>
<td></td>
<td>5.8 (0.02–25)</td>
</tr>
<tr>
<td>HCV load (× 10^6 copies/ml)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Genotype 1 (n)</td>
<td>10 (100 %)</td>
<td>10 (100 %)</td>
<td>–</td>
</tr>
<tr>
<td>Non-genotype 1 (n)</td>
<td>0 (0.0 %)</td>
<td>0 (0.0 %)</td>
<td>–</td>
</tr>
</tbody>
</table>

Table 2  Characteristics of HAART-treated patients  
Values are means (range). NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-NRTI.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HAART: all</th>
<th>HAART: PI-based</th>
<th>HAART: non PI-based</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>45</td>
<td>31</td>
<td>14</td>
</tr>
<tr>
<td>Female gender (n)</td>
<td>13 (28.9 %)</td>
<td>7 (23.3 %)</td>
<td>6 (42.8 %)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>42.3 (27–69)</td>
<td>41.7 (27–49)</td>
<td>43.7 (30–55)</td>
</tr>
<tr>
<td>HCV-co-infected (n)</td>
<td>23 (51.1 %)</td>
<td>17 (54.8 %)</td>
<td>6 (42.9 %)</td>
</tr>
<tr>
<td>PI (n)</td>
<td>9 (20 %)</td>
<td>9 (29.0 %)</td>
<td>–</td>
</tr>
<tr>
<td>Atazanavir</td>
<td>9 (20 %)</td>
<td>9 (29.0 %)</td>
<td>–</td>
</tr>
<tr>
<td>Lopinavir/Ritonavir</td>
<td>19 (42.2 %)</td>
<td>19 (61.3 %)</td>
<td>–</td>
</tr>
<tr>
<td>Saquinavir</td>
<td>2 (4.4 %)</td>
<td>2 (6.5 %)</td>
<td>–</td>
</tr>
<tr>
<td>Ritonavir+PI</td>
<td>5 (11.1 %)</td>
<td>5 (16.1 %)</td>
<td>–</td>
</tr>
<tr>
<td>Fosamprenavir</td>
<td>1 (2.2 %)</td>
<td>1 (3.2 %)</td>
<td>–</td>
</tr>
<tr>
<td>NNRTI (n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Efavirenz</td>
<td>9 (20 %)</td>
<td>–</td>
<td>9 (64.3 %)</td>
</tr>
<tr>
<td>NRTI (n)</td>
<td>22 (71.1 %)</td>
<td>24 (77.4 %)</td>
<td>8 (57.1 %)</td>
</tr>
<tr>
<td>Tenofovir</td>
<td>24 (53.3 %)</td>
<td>17 (54.8 %)</td>
<td>7 (50.0 %)</td>
</tr>
<tr>
<td>Emtricitabine</td>
<td>24 (53.3 %)</td>
<td>16 (51.6 %)</td>
<td>8 (57.1 %)</td>
</tr>
<tr>
<td>Lamivudin</td>
<td>12 (26.7 %)</td>
<td>7 (22.6 %)</td>
<td>5 (35.7 %)</td>
</tr>
<tr>
<td>Abacavir</td>
<td>5 (11.1 %)</td>
<td>0 (0.0 %)</td>
<td>5 (35.7 %)</td>
</tr>
<tr>
<td>Zidovudin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In brief, 1 × 10^6 PBMCs were stained with PerCP-conjugated anti-CD3 (BD Biosciences) and APC (allophycocyanin)-conjugated anti-CD4 (BD Biosciences) for 15 min at room temperature, washed with PBS and then fixed with paraformaldehyde (CellFIX; BD Biosciences) for 20 min at 4 °C. Fixed cells were washed with PBSF [PBS supplemented with 0.01 % (v/v) NaN3, 2.5 % (v/v) FCS (foetal calf serum; Biochrom)] and then permeabilized with digitonin (100 μg/ml in PBSF) for 30 min at 4 °C. After additional washing steps, apoptotic cells were detected by incubation with a FITC-conjugated antibody which was specific for cleaved PARP (Abcam) for 30 min at room temperature.

DNA fragmentation was measured with the APO-Direct-Kit (BD Biosciences) according to the manufacturer’s instructions. Briefly, 3 × 10^6 PBMCs were stained with APC-conjugated anti-CD4 antibody for 15 min at room temperature and then washed with PBS. Stained cells were fixed for 1 h with 1 % (w/v) paraformaldehyde in PBS on ice, rinsed twice with cold PBS and finally resuspended in 70 % (v/v) ethanol. Then, the cell suspension was stored for at least 24 h at –20 °C. After washing twice with washing buffer (from the APO-Direct-Kit) fragmented 3′-OH DNA ends were labelled by incorporating FITC-labelled dUTP by incubating the cells with a terminal deoxynucleotidyl transferase reaction mixture at 37 °C for 1 h. The reaction was stopped by washing twice with rinsing buffer (from the APO-Direct-Kit) and the cell suspension was incubated with RNase/PI (protease cleaved PARP [1]. A commercially available TUNEL assay was used to detect DNA fragmentation characteristic of cells in the late stages of apoptosis [18–21].
Flowcytometric analysis of CD4$^+$ T-cells

Representative dot plots from patients from each group with apoptosis determined by PARP (A, columns 2 and 3) and TUNEL assay (B). The dot plot of the isotype control is representative for all controls from each patient (column 1). Lymphocytes were gated by side and forward scatter. CD4$^+$ T-cells were defined as CD3$^+$/CD4$^+$-positive lymphocytes. Apoptotic cells are PARP-positive as determined in the lower-right quadrant. Late apoptotic cells show high levels of DNA fragmentation (TUNEL) as determined in the lower-right quadrant.

inhibitor) buffer (from the APO-Direct-Kit) for 30 min at room temperature prior to flowcytometric analysis.

Flowcytometric analysis was performed on a FACSCalibur flowcytometer (BD Biosciences) using CellQuest pro software (BD Biosciences) and Flowjo (Tristar).

Determination of HIV and HCV load

HCV genotypes were determined using the INNO-LiPA, HCV II kit (Innogenetics). HCV-RNA and HIV-RNA were measured by transcription-mediated amplification and branched DNA assays (TMA Versant, HCV 3.0 and HIV 3.0 Versant; Bayer Diagnostics).

Statistical analysis

Values are presented as median (interquartile range), unless otherwise stated. Statistical comparisons between groups were performed using the Mann–Whitney test using Prism 3.0 software (GraphPad). Correlations between apoptosis, CD4$^+$ T-cell counts and viral loads were analysed with Spearman’s rank correlation using Prism 3.0 software (GraphPad).

RESULTS

Both the PARP and the TUNEL assay consistently yielded identical patterns of CD4$^+$ T-cell apoptosis in the different patient groups (Figure 1), confirming that our assays were actually measuring spontaneous ex vivo apoptosis. Of note, we found marked differences in the levels of CD4$^+$ T-cell apoptosis between the different patient groups. Although the percentage of apoptotic CD4$^+$ T-cells was identical in patients with chronic hepatitis C and healthy controls (PARP, $P = 0.77$; TUNEL, $P = 0.32$), HIV-positive patients displayed significantly increased rates of apoptotic CD4$^+$ T-cells (PARP, $P = 0.0002$; TUNEL, $P < 0.0001$) (Figure 2). Importantly, the percentage of apoptotic CD4$^+$ T-cells was approx. 2-fold higher in HIV patients with HCV co-infection (PARP, $P = 0.0157$; TUNEL, $P = 0.0089$) than in patients with HIV mono-infection. Rates of CD4$^+$ T-cell apoptosis were positively correlated with HIV viral loads in untreated patients with HCV co-infection, as well as in untreated patients with HIV mono-infection (PARP: HIV, $r_s = 0.66$, $P = 0.0003$; HIV/HCV, $r_s = 0.82$, $P = 0.0002$; TUNEL: HIV, $r_s = 0.66$, $P = 0.0003$; HIV/HCV, $r_s = 0.84$, $P = 0.0001$).
Apoptosis in HIV/HCV co-infection

Figure 2  Impact of HCV co-infection on CD4\(^+\) T-cell apoptosis

(A and B) Percentage of CD4\(^+\) T-cells with apoptosis in different patient groups. (A) The results obtained with the PARP assay. (B) Rates of CD4\(^+\) T-cell apoptosis determined by DNA fragmentation (TUNEL assay). Box plots (median and interquartile range) show levels of apoptosis in healthy controls (n = 12), HCV-mono-infected patients (n = 10) and HIV/HCV-co-infected, patients (n = 14). Statistical comparisons between groups were performed with the Mann–Whitney test using Prism 3.0 software (GraphPad). (C and D) Associations between HIV load and CD4\(^+\) T-cell apoptosis. CD4\(^+\) T-cell apoptosis of HIV-mono-infected and HIV/HCV-co-infected patients is plotted against HIV loads. (C) CD4\(^+\) T-cell apoptosis determined by PARP assay is correlated with HIV loads in HIV-mono-infected and HIV/HCV-co-infected patients (HIV: \(r_s = 0.66, P = 0.0003\); HIV/HCV: \(r_s = 0.82, P = 0.0002\)). (D) Levels of CD4\(^+\) T-cell apoptosis determined by TUNEL assay is correlated with HIV loads (HIV: \(r_s = 0.66, P = 0.0003\); HIV/HCV: \(r_s = 0.79, P = 0.0003\)). Correlations between apoptosis and viral loads were analysed with Spearman’s rank correlation using Prism 3.0 software (GraphPad).

\(r_s = 0.79, P = 0.0003\). In contrast, neither in patients with HCV infection nor in patients with HIV/HCV co-infection was CD4\(^+\) T-cell apoptosis correlated with HCV loads (results not shown). In addition, we could not identify any correlations between CD4\(^+\) T-cell apoptosis and HCV genotypes, gender or age. However, the difference in CD4\(^+\) T-cell apoptosis between HIV-mono-infected and HIV/HCV-co-infected patients was disproportionately greater in patients with high HIV loads, indicating synergistic interactions between the two viruses.

Effect of HAART on CD4\(^+\) T-cell apoptosis

When we analysed untreated patients and patients on effective HAART in a cross-sectional comparison, both HIV-mono-infected and HIV/HCV-co-infected patients on HAART revealed significantly lower rates of CD4\(^+\) T-cell apoptosis than untreated patients (HIV: PARP, \(P = 0.032\) and TUNEL, \(P = 0.039\); HIV/HCV: PARP, \(P = 0.0005\) and TUNEL, \(P < 0.0001\)) (Figure 3). Furthermore, CD4\(^+\) T-cell apoptosis of HIV-mono-infected patients was not different from apoptosis in HIV/HCV-co-infected subjects under effective HAART. Nevertheless, rates of CD4\(^+\) T-cell apoptosis in treated HIV patients still remained above the level of healthy controls (HIV: PARP, \(P < 0.0001\) and TUNEL, \(P = 0.039\); HIV/HCV: PARP, \(P < 0.0001\) and TUNEL, \(P < 0.0001\)).

Stratification of all HAART-treated patients into PI- and non PI-based regimens revealed significant differences. Patients treated with PIs had higher rates of residual CD4\(^+\) T-cell apoptosis than patients treated...
Figure 3  Impact of effective HAART on CD4⁺ T-cell apoptosis
Percentage of CD4⁺ T-cells with apoptosis in HIV-mono-infected and HIV/HCV-co-infected patients with (n = 22, n = 23) and without (n = 25, n = 14) effective HAART respectively. (A and C) PARP assay. (B and D) TUNEL assay. In (C and D) patients were stratified with respect to the type of HAART, irrespective of HCV co-infection. Statistical comparisons between groups were performed with the Mann–Whitney test using Prism 3.0 software (GraphPad).

exclusively with NRTI [NRT (nucleoside reverse transcriptase) inhibitor] or NNRTI (non-NRTI)/NRT combinations (PARP,  P = 0.0033; TUNEL,  P = 0.0242) (Figure 3). This difference between PI- and non-PI-based HAART was independent of HCV co-infection.

Changes of CD4⁺ T-cell apoptosis during immune reconstitution
Longitudinal analysis of CD4⁺ T-cell apoptosis during immune reconstitution revealed decreasing levels of CD4⁺ T-cell apoptosis in both HIV-mono- and HIV/HCV-co-infected patients. The mean levels of CD4⁺ T-cell apoptosis was higher in HIV/HCV-co-infected patients than in HIV-mono-infected patients before HAART (Figures 4C and 4D), but decreased more vigorously than in HIV-mono-infected patients during the first four weeks of treatment (HIV: PARP, −1.22 % and TUNEL, −1.43 %; HIV/HCV: PARP, −4.96 % and TUNEL, −4.06 %), whereas increases in CD4⁺ T-cell counts (HIV, +124 cells/ml; HIV/HCV, +108 cells/ml) and inhibition of HIV replication (HIV, −2.47 log; HIV/HCV, −2.53 log) did not reveal significant differences between the two patient groups.

Measurements 12 weeks after initiation of HAART confirmed that levels of CD4⁺ T-cell apoptosis were identical in HIV/HCV-co-infected patients and HIV-mono-infected patients (Figures 4C and 4D). In addition, increases in CD4⁺ T-cell count (HIV, +135 cells/ml; HIV/HCV, +133 cells/ml), as well as a drop in HIV loads (HIV, −3.49 log; HIV/HCV, −3.46 log), were equivalent in HIV-mono- and HIV/HCV-co-infected patients 12 weeks after the start of HAART (Figures 4A and 4B).

DISCUSSION
Aptoptosis of CD4⁺ T-cells has been repeatedly implicated as a major process underlying the loss of CD4⁺ T-cells in HIV-positive patients. However, the role of
HCV co-infection for progression of HIV disease and particularly apoptosis of CD4+ T-cells has thus far remained controversial.

In the present study we investigated DNA fragmentation and cleavage of PARP in CD4+ T-cells which together reflect irreversible changes associated with induction of apoptosis. Our results confirm high rates of apoptosis in CD4+ T-cells of HIV-positive patients. Notably, apoptosis of CD4+ T-cells in HIV/HCV-co-infected patients markedly exceeded the levels observed in patients with HIV mono-infection, whereas HCV infection alone resulted in rates of apoptosis which were identical with healthy controls. Taken together, these findings indicate that, although HCV infection alone did not induce apoptosis in CD4+ T-cells, it sensitizes CD4+ T-cells towards the apoptotic effects of HIV infection.

These results corroborate a previous observation reporting that HCV/HIV-co-infected patients had particularly high rates of annexin V binding in CD4+ T-cells, a possible marker of early apoptosis [22]. However, unlike this previous study, the results of the present study were based on markers of irreversible apoptosis and also differentiated rates of apoptosis between HIV- mono- and HIV/HCV-co-infected patients, as well as treated and untreated patients respectively. Our results also seem to correspond to findings in HepG2 cells, where collaborative induction of apoptosis has been reported for the interaction of HCV and HIV envelope proteins [23]. However, in HepG2 cells either viral protein alone did not induce apoptosis, whereas in the present study apoptosis of CD4+ T-cells was already increased in HIV mono-infection. Thus further mechanisms must be involved, which sensitize CD4+ T-cells towards apoptosis induction in patients with untreated HIV/HCV co-infection.

Although it has been shown that HIV-driven immune activation is correlated with apoptosis of CD4+ T-cells in HIV mono-infection, Nunez et al. [22] did not find any association between T-cell activation and CD4+ T-cell apoptosis in HIV/HCV-co-infected patients. This does not rule out increased production of pro-inflammatory cytokines or altered expression of death receptors and their corresponding ligands. Indeed, up-regulated expression of the death receptor Fas, as well as TRAIL
Receptors I and II, has been independently described in HCV- and HIV-mono-infected patients and seems to correspond to increased serum levels of death receptor ligands FasL, TRAIL and TNF-α [24–30]. In particular, soluble TRAIL is increased in HIV infection closely related to the level of HIV replication. Moreover, HIV-Tat-stimulated monocytes have been demonstrated to kill CD4+ T-cells via TRAIL-dependent apoptosis [31,32]. In this context it is noteworthy that HCV core protein has been shown to sensitize Huh7 cells towards apoptosis mediated by TRAIL [33]. This finding has been attributed to enhanced apoptosis signalling via sequential induction of caspase 8, Bid cleavage, activation of the mitochondrial pathway and activation of effector caspase 3, as well as direct activation of caspase 9. Since cleavage of PARP and DNA fragmentation are apoptotic events downstream of caspase 3 activation, we could not differentiate which pathway of apoptosis activation was enhanced in CD4+ T-cells from HIV/HCV-co-infected patients.

Although the exact mechanisms underlying apoptosis sensitization remain unclear thus far, our results suggest a pivotal role for HIV replication. First, rates of apoptosis in HIV/HCV-co-infected patients were increased more over HIV-mono-infected patients the higher the level of HIV replication. Next, this effect was fully reversed when HIV replication was suppressed by effective HAART, as shown by our cross-sectional comparison and the individual serial longitudinal studies in single patients. Of note, the decline of apoptosis rates was more pronounced in HIV/HCV-co-infected patients at week 4 than in HIV-mono-infected patients, although HIV replication had been inhibited to the same degree in both groups. Although rates of apoptosis further declined in the subsequent weeks on HAART, residual apoptosis in CD4+ T-cells still remained above the level of healthy controls and HCV-mono-infected patients, but no longer revealed any significant differences between HIV- and HIV/HCV-co-infected patients. This may, on the one hand, reflect effects resulting from ongoing HIV replication below the level of detection. On the other hand, reflect effects resulting from the anti-retroviral agents must also be considered. In this context it was an intriguing finding that residual CD4+T-cell apoptosis was significantly higher in patients on PI-based HAART regimens than on non PI-regimens. Although PIs block cellular apoptosis at the level of mitochondrial pore proteins, paradoxical pro-apoptotic effects have been reported in vitro at high concentrations both in haematological cell lines and solid tumours [34,35]. Increased in vitro apoptosis from PIs was either associated with inhibited transcriptional activation of NF-κB (nuclear factor κB) together with inhibited expression of its targets Bcl-X1, survivin, c-Myc and cyclin D2 [34], or an increase in the cellular concentration of the antiproliferative and pro-apoptotic proteasome substrate CDK (cyclin-dependent kinase) inhibitor p21 [35]. This reasoning is consistent with an earlier report that HIV PIs have an impact on proteasome activity [36]. Furthermore, in vitro inhibition of cells with ritonavir, saquinavir or nelfinavir was associated with decreased expression of the anti-apoptotic protein McI-1 as well as blocking IL (interleukin)-6-mediated phosphorylation of ERK 1/2 (extracellular–signal-regulated kinase 1/2) and STAT3 (signal transducer and activator of transcription 3) [37].

Unfortunately, however, we do not have results to clarify whether resolution of hepatitis C by anti-HCV treatment can reduce CD4+ T-cell apoptosis to the same degree as antiretroviral therapy, since all our interferon-treated patients had been on antiretroviral therapy prior to anti-HCV treatment. Thus the relationship between anti-HCV treatment and CD4+ T-cell apoptosis still needs to be clarified in future projects.

Whatever the molecular and cellular mechanisms, the results of the present study clearly demonstrate that HIV/HCV-co-infected patients without effective HAART suffer increased apoptosis of CD4+ T-cells and, thus, are prone to progress more rapidly to severe immunodeficiency. Moreover, our results may help to explain why clinical data on HIV progression in patients with hepatitis C co-infection are controversial. In our comprehensive study a negative impact on disease progression was only noted in untreated patients, and rates of CD4+ T-cell apoptosis became identical between HIV and HIV/HCV-co-infected patients shortly after initiation of HAART. Although differences in outcome between HIV and HIV/HCV-co-infected patients have been reported [8,38,39], our findings would support several recent longitudinal observations of HIV/HCV-co-infected patients which suggest there are no relevant differences in immune recovery between HIV and HIV/HCV-co-infected patients on long-term HAART [14,40,41]. In line with these results, early initiation of antiretroviral therapy in HIV/HCV-co-infected patients is not only a good way to reduce liver-related mortality, but may also be an effective measure to prevent excessive loss of CD4+ T-cells in HIV/HCV co-infection [42].

**ACKNOWLEDGEMENTS**

We thank Dr J.-C. Wasmuth, Dr C. Schwarze-Zander and Dr Jetske Emmelkamp for taking care of the patients and for providing a crucial number of samples for the present study.

**FUNDING**

This work was supported by the German Federal Ministry of Education and Research (BMBF) within the network for resistance in hepatitis C [grant number 01KI0792]; and the H. W. and J. Hector Foundation [grant number M42 (to J. N.)].
REFERENCES


© The Authors Journal compilation © 2009 Biochemical Society


