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Chemokine mRNA levels in mononucleated cells of HIV-infected patients before and after initiation of PI- versus NNRTI-containing HAART

Jan-Christian Wasmuth[a,*], Hans-Dieter Nischalke[a], Alexander Jütte[b], Gerd Fätkenheuer[b], Bernd Salzberger[c], Tilman Sauerbruch[a], Ulrich Spengler[a], Jürgen K. Rockstroh[a], Franz L. Dumoulin[a]

[a] *Department of Medicine I, University of Bonn, Sigmund Freud Str. 25, 53105 Bonn, Germany*
[b] *Department of Internal Medicine I, University of Cologne, Cologne, Germany*
[c] *Department of Internal Medicine, University of Regensburg, Regensburg, Germany*

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Abstract

To compare CC chemokine mRNA levels from native peripheral blood mononucleated cells (PBMCs) before and 6 months after the initiation of two different regimens of highly active antiretroviral therapy (HAART), we treated group 1 ($n = 11$) with two nucleoside analogues and the protease inhibitor (PI) indinavir boosted by ritonavir (800/100 mg b.i.d.); group 2 ($n = 8$) was treated with the non-nucleoside reverse transcriptase inhibitor (NNRTI) efavirenz instead of PI. CC chemokine mRNA levels (regulated upon T cell activation expressed secreted [RANTES], macrophage inhibitory protein [MIP]-1α, MIP-1β, monocyte chemotactic protein [MCP]-1, MCP-2) were quantified from PBMCs before and 6 months after the initiation of HAART using a reverse transcription/real-time polymerase chain reaction (PCR) assay. The mRNA levels of MCP-1 and MCP-2 were significantly decreased in both groups ($P < 0.05$), while MIP-1α and MIP-1β were decreased significantly only in the PI-treated group, but not in the NNRTI group. A moderate decrease of RANTES was observed in both treatment groups. The data suggest that HAART regimens containing either NNRTI or PI are not equivalent with regard to modification of CC chemokine mRNA profiles.

Keywords: Chemokine mRNA levels; Mononucleated cells; Antiretrovirals; NNRTIs; PIs; HIV

1. Introduction

Protease inhibitor (PI)-based highly active antiretroviral therapy (HAART) regimens versus non-nucleoside reverse transcriptase inhibitor (NNRTI)-based HAART regimens are equally effective in suppressing viral replication and elevating CD4 cell counts (Staszewski et al., 1999). Whether these regimens differ in other aspects of immune reconstitution has not been studied in much detail. CC chemokine profiles are thought to be important markers of immune reconstitution following successful HAART (Cocchi et al., 1995). Here, we compared the changes of chemokine mRNA levels in 19 treatment-naïve HIV-1-positive patients who were prospectively studied after initiation with different HAART regimens. Eleven patients were treated with a PI-based regimen, eight with an NNRTI-based regimen. We measured CC beta-chemokine mRNA profiles of regulated upon T cell activation expressed secreted (RANTES), macrophage inhibitory protein (MIP)-1α, MIP-1β, monocyte chemotactic protein (MCP)-1 and MCP-2 from native peripheral blood mononucleated cells (PBMCs) to assess the immunological effects of HAART.

2. Patients and methods

2.1. Patients and treatment

A total of 19 consecutive patients presenting for the first time to the HIV-Outpatient Departments of the University Hospitals of Bonn and Cologne were studied prospectively. All patients were HIV-1-positive and had not received any antiretroviral treatment before. Initiation of HAART was based on international treatment guidelines (Carpenter et al., 1998). Patients were assigned to either treatment with a PI-based regimen containing indinavir (800 mg b.i.d.)

* Corresponding author. Tel.: +49-228-287-6558; fax: +49-228-287-5034.
E-mail address: j-c.wasmuth@uni-bonn.de (J.-C. Wasmuth).

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Table 1
Patient characteristics at baseline

	Group 1 (PI) ($n = 11$)	Group 2 (NNRTI) ($n = 8$)	Control group ($n = 10$)
Age			
Median (range)	46.0 (35–63)	35.5 (29–49)	29.0 (24–37)
Sex			
Male/female	9/2	7/1	6/4
CDC stage			
A	4	2	n.a.
B	4	4	
C	3	2	
Viral loads			
log copies/ml (mean ± S.D.)	5.88 ± 6.17	5.30 ± 5.02	n.a.
CD4 cell counts			
Cells/μl (mean ± S.D.)	235 ± 185	180 ± 178	n.a.
Nucleosides			
D4T + 3TC	9	6	n.a.
AZT + 3TC	2	2	

All patients were antiretroviral naïve; 11 patients were treated with indinavir/ritonavir and two nucleoside analogues (group 1), 8 patients were treated with efavirenz and two nucleoside analogues (group 2). n.a.: not applicable.

boosted by baby-dose ritonavir (100 mg b.i.d.) and two nucleosides (group 1; $n = 11$) or an NNRTI-based regimen containing efavirenz (group 2; $n = 8$). Nucleoside analogues were 3TC in combination with either AZT or D4T at the treating physician's choice. There were no significant differences in the baseline characteristics between the two groups (Table 1). All patients were screened for HBV, HCV, CMV, EBV infections or syphilis, which could be excluded in all of the patients. All study procedures were done in accordance with the current revision of the Helsinki Declaration of 1975.

All patients were followed for 24 weeks and continued on their initial regimen throughout the observation period. Blood samples for study procedures were drawn at baseline and after 24 weeks; routine laboratory testing and clinical evaluation were done at shorter intervals. Ten HIV-negative, healthy, matched controls were included and measured at baseline as a normal reference group.

2.2. RNA extraction, reverse transcription and real-time polymerase chain reaction (PCR)

Blood samples were processed within 1 h after drawing. Total RNA was isolated from native peripheral blood using Trizol LS reagent (Life Technologies, Eggenstein, Germany) and 1 μg was reverse transcribed for 45 min at 42 °C and for 30 min at 55 °C using 0.5 μg random primers (Promega, Heidelberg, Germany) and 200 U of Superscript Plus RT (Life Technologies). Real-time PCR was carried out as previously described (Altfeld et al., 2000; Dumoulin et al., 2000). PCR was set up using 9 μl PCR master mix supplemented with 3.5 mM $MgCl_2$, 0.5 μM custom-synthesized primers (Life Technologies) and 1 μl of either external standards or a cDNA equivalent to 20 ng total RNA to give a final reaction volume of 10 μl. The settings for the thermal profile were as follows: initial denaturation (30 s/95 °C), followed by 35 amplification cycles: 3 s/65 °C (β-actin 60 °C); 15 s/72 °C (β-actin: 30 s); 0 s/95 °C and a melting curve analysis (increasing temperature from 60 to 95 °C). Acquisition temperature for the fluorescence signal was set to 85 °C (β-actin, RANTES, MIP-1α, MIP-1β) or 84 °C (MCP-1 and MCP-2), respectively. As an additional control of specificity, PCR products were size-fractionated on 2.5% agarose gels and visualized under ultraviolet light. All PCRs were run in duplicate.

2.3. Calculation of steady-state mRNA levels and statistics

Raw data for chemokine mRNAs from duplicate measurements were averaged and are reported as ratios relative to mRNA of the housekeeping gene β-actin, thus allowing a correction for possible variations in the efficiency of RNA extraction and reverse transcription. Comparison of 6 months' chemokine mRNA levels, HIV viral loads and peripheral blood CD4 cell counts to baseline values as well as differences between treatment groups was performed using the non-parametric Wilcoxon's rank sum test for paired and unpaired data, respectively. *P*-values (two sided) <0.05 were considered to indicate statistical significant results. Statistical analysis was performed with the SPSS software (Version 6.1.3; SPSS Inc.).

3. Results

3.1. Viral loads and CD4 cell counts

After initiation of HAART HIV viral loads were suppressed below the limit of detection in 68% of the patients (73% in group 1, 63% in group 2). There was no

statistically significant difference between the PI-treated (group 1) and the NNRTI-treated groups (group 2), although patients receiving a PI-based regimen tended to have a more pronounced reduction in viral loads (group 1: from 5.88 ± 6.17 to 1.56 ± 1.80 log copies/ml, $P = 0.005$; group 2: from 5.30 ± 5.02 to 3.31 ± 3.75 log copies/ml, $P = 0.012$; mean $\pm$ S.D.). Follow-up of the patients beyond the 24-week observation period required a change in treatment for two patients in group 1 and one patient in group 2, respectively, because viral replication was not fully suppressed. Subsequently to the reduction in viral loads, an increase in CD4 cell counts was observed in both groups (group 1: $235 \pm 185/\mu l$ versus $402 \pm 249/\mu l$, $P = 0.003$; group 2: $180 \pm 178\mu l$ versus $350 \pm 217/\mu l$, $P = 0.017$; mean $\pm$ S.D.). There was no statistical significant difference between the two treatment arms. We observed four

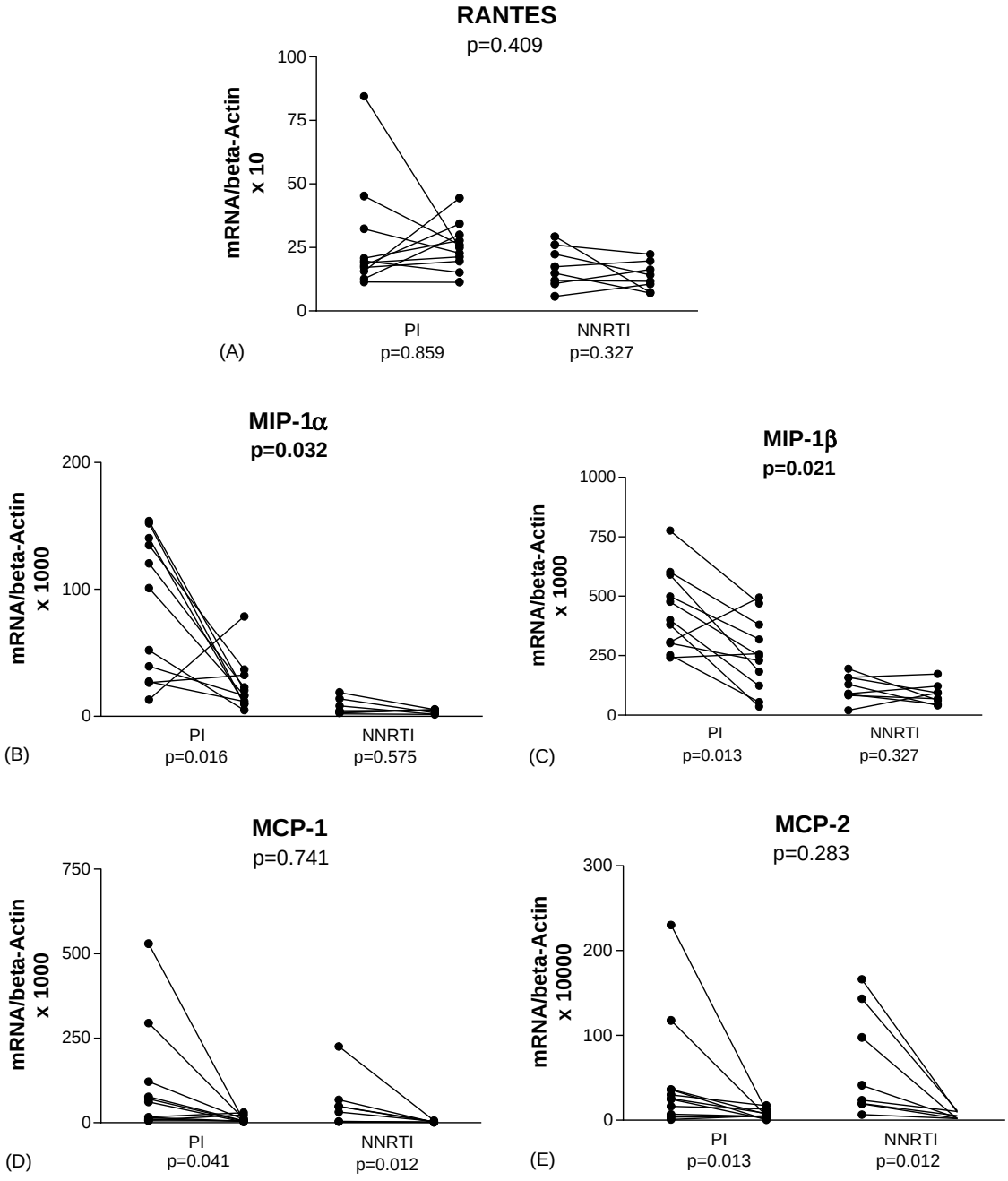

Fig. 1. Changes of chemokine mRNA levels at week 24 compared to baseline. Values are given as chemokine/β-actin mRNA ratios. For better readability values were multiplied 10× for RANTES, 1000× for MIP-1α, MIP-1β, and MCP-1, and 10,000× for MCP-2, respectively. Dots at the left indicate baseline values, dots at the right values at week 24. Significances in comparison to baseline are given below the groups, significances comparing the two treatment groups are given in the upper middle of the diagrams. There were 11 patients in the PI group and 8 patients in the NNRTI group. There were no statistical significant differences between the two groups with respect to RANTES (A), MCP-1 (D), and MCP-2 (E) mRNA levels, while there was a statistical significant difference in the change of MIP-1α (B) and MIP-1β (C) levels.

adverse events (group 1: elevation of transaminases, nausea; group 2: rash, efavirenz-associated neurotoxicity), which, however, did not require change of treatment in any patient.

3.2. Native peripheral blood chemokine mRNA levels

After initiation of HAART, mRNA levels of all investigated chemokines, except for RANTES, decreased significantly (mean ± S.D.; RANTES: 2.288 ± 1.740 at baseline to 2.036±0.962 at week 24, $P = 0.717$; MIP-1α: 0.081±0.057 to 0.031 ± 0.020, $P = 0.014$; MIP-1β: 0.424 ± 0.175 to 0.276±0.151, $P = 0.009$; MCP-1: 0.086±0.133 to 0.007± 0.008, $P = 0.002$; MCP-2: 0.006±0.006 to 0.001±0.0004, $P < 0.001$).

Analyzing the results for the two treatment groups, there were no statistical significant differences with respect to RANTES, MCP-1, and MCP-2 mRNA levels, while there was a statistical significant difference in the change of MIP-1α and MIP-1β levels between the two groups (Fig. 1). MIP-1α and MIP-1β showed a significant decrease compared to baseline for group 1 ($P < 0.05$), but not for group 2, where only a moderate decrease was observed ($P > 0.3$). There was an unexpected difference in baseline values of MIP-1α and MIP-1β mRNA levels between groups 1 and 2. After adjustment for lower baseline values in group 2, the difference between the two treatment arms remained statistically significant.

MCP-1 and MCP-2 levels fell by more than 85% after 24 weeks of treatment ($P < 0.05$) with no difference between both groups. RANTES mRNA levels decreased in both arms after 24 weeks of treatment, although no statistical significance compared to baseline was reached.

There was a significant correlation between younger age and lower levels of MIP-1β ($r = 0.490$; $P = 0.033$). However, there was no significant correlation between other chemokine mRNA levels and CD4 counts, CD8 counts, viral loads, age, sex, or stage of HIV disease, respectively.

4. Discussion

Chemokines are thought to play an important role in the HIV-1-specific immune response (Cocchi et al., 1995; Nicastri et al., 2001). Several groups demonstrated a change in chemokine profiles after initiation of HAART, and these alterations were interpreted as evidence for immune restoration (Aleman et al., 1999; Bisset et al., 1997; Carter et al., 2000; Clerici et al., 2000). However, the biological consequences of the observed changes in beta-chemokines are still unknown.

During the last 2 years NNRTI have emerged as an alternative to PI-based regimens due to their good efficacy and tolerability (Bartlett et al., 2001). This report is to our knowledge one of the first to directly compare the impact of a PI-based regimen versus an NNRTI-based combination therapy on chemokine expression patterns in treatment-naïve patients. Reduction in viral loads and rise of CD4 cells were adequate and within the expected range in either treatment group.

Chemokine mRNA levels of RANTES, MIP-1α, MIP-1β, MCP-1, and MCP-2 were found to be decreased 24 weeks after initiation of HAART. We found a significant difference in the decrease of MIP-1α and MIP-1β mRNA levels between the NNRTI- and the PI-treated groups after 24 weeks, whereas there was no difference between the two groups with respect to MCP-1, MCP-2, or RANTES. These findings are in line with a study reported by Burton et al., who compared the changes of chemokine levels in antiretroviral-naïve patients treated with NNRTI- or PI-containing HAART (Burton et al., 2002). Similarly to our results they found a difference in changes of MIP-1α and MIP-1β levels between patients treated with NNRTI and PI. In their study, levels of both chemokines decreased in the NNRTI group, but increased in the PI-treated group. The patterns observed for RANTES, which was not changed, and for MCP-1, which decreased, were also similar to ours. These findings indicate that antiretroviral regimens containing NNRTI instead of PI might not be equivalent with regards to modification of chemokine profiles.

It is possible that higher levels of MIP-1α and MIP-1β in NNRTI-treated than in PI-treated patients in both studies are associated with residual viral replication (Burton et al., 2002). Although this might be beneficial through generation of HIV-1-specific immune responses and although intermittent viral replication at less than 200 copies/ml seems not be clinically significant (Havlir et al., 2001), there are no clinical data which compare the long-term outcome of PI-treated versus NNRTI-treated patients and which would allow to draw conclusions on the clinical consequences of the observed findings.

Data published by other groups showed an inconsistent pattern of changes in chemokine profiles: For instance, Aleman et al. found decreases in RANTES and MCP-1, while MIP-1α and MIP-1β were unchanged (Aleman et al., 1999); in contrast, Carter et al. showed increased MIP-1α production by cultured whole blood samples, but no effect of HAART on MIP-1β and RANTES levels (Carter et al., 2000). Giovannetti et al. demonstrated decreased RANTES and MIP-1α levels, while MIP-1β levels were not consistently changed (Giovannetti et al., 1999). Finally, Bisset et al. treated patients with late-stage HIV infection with the PI indinavir and showed an increase of RANTES, MIP-1α and MIP-1β, while MCP-1 decreased (Bisset et al., 1997). Only the decrease of MCP-1 is consistent in all studies. It probably indicates effective suppression of viral replication, as production of MCP-1 has been shown to be correlated to cellular infection and replication with HIV (Mengozzi et al., 1999). However, the variability of the results for the other chemokines might well reflect methodological difficulties and/or heterogeneity of study populations and treatments. For example, Bisset et al. studied patients with advanced HIV disease, pretreated with nucleoside analogues (Bisset

et al., 1997), whereas we and Burton et al. (2002) included only antiretroviral-naïve patients with mainly asymptomatic to moderate HIV disease.

In our study, there was an unexpected difference in baseline mRNA levels of MIP-1α and MIP-1β between both treatment arms. There was no statistical difference in any other parameter (i.e. CD4 cells, viral loads, gender, age) at baseline which could explain this finding. In addition, strict internal quality control was implemented to minimize technical problems (i.e. healthy controls, positive controls, duplicate runs). Although not statistically significantly different, the younger age in group 2 might be a reason for lower baseline values, as a younger age was correlated to lower levels of MIP-1β. However, as the difference between week 24 and baseline was the primary end point of our study, an unknown underlying systematic error should have been neutralized.

Other known factors possibly influencing the chemokine profile can also be excluded, since concomitant diseases such as viral hepatitis or other infectious diseases were excluded. Nevertheless, there are some limitations to our study: Thus, the size of the groups is small, and larger groups might be needed to confirm significant differences between the two treatment arms. Longer follow-up periods might be needed as well. On the other hand, most processes of immune restoration take place within the first 24 weeks, and no such effects are seen thereafter (Bart et al., 2000). We measured chemokine mRNA expression only at two time points, and we cannot exclude fluctuation of mRNA levels. However, there have been reports that showed no marked variability of chemokine expression over time (McKenzie et al., 1996). In addition, Burton et al. (2002) measured chemokine levels at weeks 4, 8, and 16, which makes it unlikely that our comparable results should be just an effect of change over time. It has to be clearly stated, that we have measured chemokine mRNA levels and not chemokine protein levels, and we cannot exclude that chemokine protein secretion is not directly related to mRNA expression. The applied real-time PCR uses β-actin as the housekeeping gene. The use of housekeeping genes has been debated throughout the last years, as their levels depend on factors such as cell activation and metabolic state and may vary considerably (Bustin, 2002). Although strict internal quality control was implemented and many other factors known to influence chemokine and housekeeping gene levels can be excluded as mentioned above, the use of β-actin might be influencing the results to a significant extent. Finally, we cannot infer from our data on the chemokine profiles of pretreated patients, for whom no reliable data on chemokine profiles during salvage therapy have been published.

Taken together, our data give a first hint, which has to be confirmed by further and larger prospective studies, that antiretroviral regimens containing NNRTIs instead of PIs may not be equivalent in regard of modification of chemokine profiles during immune reconstitution in antiretroviral-naïve patients. Thus, CD4 cell counts and HIV viral loads probably reflect only partial aspects of immune reconstitution and need to be supplemented by additional immune reconstitution markers.

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