Cellular HIV-1 DNA load predicts HIV-RNA rebound and the outcome of highly active antiretroviral therapy

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Objective: To assess whether cellular HIV-1 DNA prior to highly active antiretroviral therapy (HAART) initiation predicts its outcome.

Design and methods: Patients included all 51 hemophiliacs of the Greek component of the Multicenter Hemophilia Cohort Study who had initiated HAART and for whom cryopreserved lymphocyte samples before HAART initiation were available. Cellular HIV-1 DNA quantification was performed by a molecular beacon-based real-time PCR assay in multiple samples per patient with a median (interquartile range) follow-up of 76 (45–102) weeks.

Results: The median (range) baseline HIV-1 DNA load was 297 (< 10 to 3468) copies per 1×10^6 peripheral blood mononuclear cells. Baseline HIV-1 DNA load did not predict initial virological response (VR). None of the patients with initial VR and baseline HIV-1 DNA load at or below the median experienced a subsequent virological rebound, while the cumulative probability of virological rebound by week 104 was 55% among those with HIV-1 DNA load greater than the median ($P < 0.008$). Cellular HIV-1 DNA load was the only parameter associated with sustained virological response as shown by univariate or multivariate analyses [adjusted odds ratio (95% confidence interval) 0.197 (0.048–0.801) per 1 $\log_{10}$ increase in DNA copies, $P = 0.023$].

Conclusion: Low cellular HIV-1 DNA load is a marker of sustained virological response in patients with initial VR and it can reliably predict the long-term success of HAART.

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Keywords: cellular HIV-1 DNA load, HIV-1 RNA rebound, antiretroviral therapy

Introduction

The widespread use of highly active antiretroviral therapy (HAART) after 1996 had a profound effect on the prognosis of HIV infection [1–4]. HAART is usually effective in suppressing plasma HIV-1 RNA levels and restoring CD4 cells to levels where opportunistic infections are rare. Since durable HIV-1 suppression is a primary goal of therapy, many authors have described various predictors of short- and long-term success of HAART. Apart from treatment potency, pharmacokinetics and adherence [5–8], predictors of successful HAART include naive treatment history, no history of AIDS, age, no pre-existing resistant HIV

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strains, higher baseline CD4 counts, and lower baseline HIV-1 RNA levels [9–17].

Plasma viral load and CD4 cell counts have been extensively studied. However, only low baseline CD4 cell count ($< 200 \times 10^6$/l) or relatively high baseline HIV-1 RNA values ($> 10^5$ copies/ml) are consistently associated with lower success rates of HAART [14,15,18]. HIV-1 RNA nadir, or measurements made 1–16 weeks after HAART initiation seem to be more predictive than baseline HIV-1 RNA values [19–21]. Importantly, baseline CD4 cell count, baseline HIV-1 RNA or the patterns of HIV-1 RNA response cannot predict subsequent virologic rebound among patients who initially respond to HAART [14,22].

In a recent study, we showed that a marker of HIV-1 DNA concentration in peripheral blood mononuclear cells (PBMC), representing cellular HIV-1 DNA forms that have undergone the second template switch (HIV-1 STS DNA), was associated with HIV-1 disease progression in the absence of therapy, independently of HIV RNA and CD4 cell count, in a cohort of HIV-1-infected hemophiliac patients with known times of seroconversion [23]. In this study, we evaluated cellular HIV-1 DNA load as a predictor of short- and long-term success of HAART in the HIV-1-positive patients of the same cohort who were treated with triple combination therapy.

Methods

Patients

All hemophilia patients who initiated HAART within the HIV-1-infected Greek patients enrolled in the Multicenter Hemophilia Cohort Study, with at least one cryopreserved PBMC sample available before HAART, were eligible for this study [24]. Although an institutional review board (IRB) was not obtained before study initiation, such an exemption complied with the policy of the local IRB that is in accordance with the principles of the Decleration of Helsinki. The 158 HIV-1-infected Greek hemophilic individuals have known seroconversion dates and have been prospectively followed up for more than 19 years since seroconversion. Individual HAART medications were categorized as protease inhibitors (PI) and nucleoside and non-nucleoside reverse transcriptase inhibitors (NRTI and NNRTI, respectively). Of the 158 participants, 130 (83.3%) had at least one cryopreserved PBMC sample available for measurement of cellular HIV-1 DNA load [23]. Fifty-six patients initiated HAART. Three of them were excluded from the current analysis due to lack of available PBMC sample within 1 year prior to HAART initiation (baseline value) and two were excluded because their viral load was below 50 copies/ml at HAART initiation. In the longitudinal studies evaluating cellular HIV-1 DNA load kinetics after initiation of HAART, 47/51 (92.2%) of patients with at least two HIV-1 DNA load measurements (one before and one after initiation of HAART) were included.

The following definitions of virological response to HAART were used: (i) initial virological response (VR) was defined as plasma HIV-1 RNA lower than 50 copies/ml in two consecutive measurements; (ii) virological rebound was defined as relapse of HIV-1 RNA above 500 copies/ml in two consecutive measurements in patients with initial VR; (iii) sustained VR was defined as viral load lower than 50 copies/ml in two consecutive measurements after HAART initiation retained throughout the follow-up. Occasional HIV-1 RNA blips between 50–500 copies/ml followed by fewer than 50 copies/ml occurred; these patients were classified as sustained virological responders; (iv) virological failure was defined as failure to reach lower than 50 copies/ml in two consecutive measurements or relapse above 500 copies/ml in two consecutive measurements in patients who previously reached lower than 50 copies/ml. The short-term VR was evaluated by initial VR while the sustained VR, virologic rebound and virologic failure represent parameters of the long-term VR.

Laboratory methods

Clinical and laboratory data including PBMC were collected approximately every 6 months [23–25]. Quantification of PBMC-associated HIV-1 STS DNA, previously defined as 'cellular HIV-1 DNA load' or 'cellular viral load', was determined by a molecular beacon-based real-time PCR assay as described previously [23]. A molecular beacon real-time PCR assay was also used for the measurement of T-cell-receptor rearrangement excision DNA circles (TREC) as described previously [25].

Statistical analysis

Individual HIV-1 DNA load measurements were $\log_{10}$-transformed before statistical evaluation. Patients' baseline characteristics were compared by Spearman correlation coefficients, Mann–Whitney U test or Fisher's exact test when appropriate. For patients initiating a successful second-line HAART regimen (change of at least two drugs) after the failure of their first HAART regimen, follow-up time was right-censored at the time of second HAART regimen initiation. Predictors of time to initial VR and of time from initial VR to virological rebound were assessed by Cox proportional hazards models. Kaplan–Meier survival curves were constructed to estimate cumulative rates of response whereas log–rank tests were used to compare response rates among subgroups. The independent prognostic value of baseline HIV-1 DNA load for the probability

of sustained response was evaluated by logistic regression models. Repeated measurements of HIV-1 DNA levels during patients follow-up were analysed by random effects models, allowing for subject-specific baseline values and left censoring in HIV-1 DNA values (i.e., < 10 copies/1×10^6 PBMC). These models provided estimates of the rates of cellular HIV-1 DNA load changes after HAART initiation.

Results

Baseline characteristics

The baseline characteristics of the 51 hemophilic HIV-1-infected individuals who initiated HAART are shown in Table 1. Patients had long-standing HIV-1 infection with a median time (range) from seroconversion to HAART of 15.0 (11.8–18.6) years. Seven of them had developed AIDS before HAART, while three were naive to previous antiretroviral treatment. The median (range) of baseline cellular HIV-1 DNA load values was 297 ($<$ 10 to 3468) copies per 1×10^6 PBMC. Baseline cellular HIV-1 DNA was not significantly correlated with time from seroconversion to HAART (Spearman's r, -0.003, $P = 0.981$), concurrent CD4 cell count (r, 0.090; $P = 0.528$), TREC ($r = 0.157$; $P = 0.270$), or HIV-1 RNA levels (r, 0.191; $P = 0.179$); it was marginally associated with age at seroconversion (r, 0.254; $P = 0.072$) and age at HAART initiation (r, 0.265; $P = 0.060$). The first HAART regimen included two NRTI plus one PI, two NRTI plus one NNRTI, and one NRTI plus one PI plus one NNRTI in 42 (82.4%), eight (15.7%), and one (2.0%) patients, respectively. Of the 51 subjects seven initiated a successful second-line HAART regimen.

Assessment of virologic response

Among the 51 study participants, initial VR was observed in 19 (37.3%) patients, of whom seven (36.8%) experienced a subsequent virologic rebound within a median (range) 34 (21–111) weeks after the time when HIV-1 RNA reached < 50 copies/ml. Overall, 39 of the 51 (76.5%) subjects experienced virologic failure while the remaining 12 subjects had sustained VR during a median (range) follow-up time of 2.9 (1.6–4.8) years after initial VR.

Predictors of virological response

Results from Cox proportional hazards models showed that time to initial VR was not predicted by any variable examined, such as age at seroconversion, age at HAART initiation, time from seroconversion to HAART, plasma HIV-1 RNA, CD4 cell counts, AIDS diagnosis, use of saquinavir hard gel capsules, or TREC levels. Median [interquantile range (IQR)] baseline cellular HIV-1 DNA load was 280 (101–627) and 507 (69–1338) copies/1×10^6 PBMC in patients with non-response and with initial VR, respectively. The cumulative probability of response by duration of treatment was not associated with cellular HIV-1 DNA levels (Fig. 1a).

Cellular HIV-1 DNA load was the only parameter that was significantly associated with viral rebound among the initially responding patients (Table 2). The median (IQR) baseline HIV-1 DNA load level was 1338 (1083–1374) and 91 (39–459) copies/1×10^6 PBMC in treated patients with virologic rebound and sustained VR, respectively ($P = 0.002$). None of the initial responders with baseline HIV-1 DNA load at or below the median value (297 copies/1×10^6 PBMC) experienced a subsequent virologic rebound, while among those with HIV-1 DNA load greater than 297 copies/1×10^6 PBMC, the cumulative probability of virological rebound by week 104 was 54.6% ($P = 0.008$) (Fig. 1b).

The distribution of demographic, immunological, virological and treatment parameters by long-term VR is shown in Table 2. Cellular HIV-1 DNA was the only

Table 1. Baseline characteristics of the 51 haemophilia patients who initiated highly active antiretroviral therapy (HAART).

Median age at seroconversion [years (range)] (range)	18.2 (2.2–40.0)
HAART use begun	
Median age [years (range)]	33.4 (16.3–54.1)
Median calendar year	1997
Median time from seroconversion to HAART [years (range)]	15.0 (11.8, 18.6)
Median plasma HIV-1 RNA [copies/ml (range)]	8097 (242–5 458 000)
Median CD4 cell count [$\times 10^6$/l (range)]	169 (2–556)
Median TREC [copies/1×10^6 PBMC (range)]	3303 ($<$ 10 to 53 709)
Median HIV-1 STS DNA [copies/1×10^6 PBMC (range)]	297 ($<$ 10 to 3468)
AIDS [n (%)]	7 (13.7)
Treatment naive [n (%)]	3 (5.9)
HAART based on [n (%)]	
Protease inhibitor (not saquinavir hard gel)	20 (39.2)
Saquinavir hard gel	22 (43.1)
Non-nucleoside reverse transcriptase inhibitor	9 (17.7)

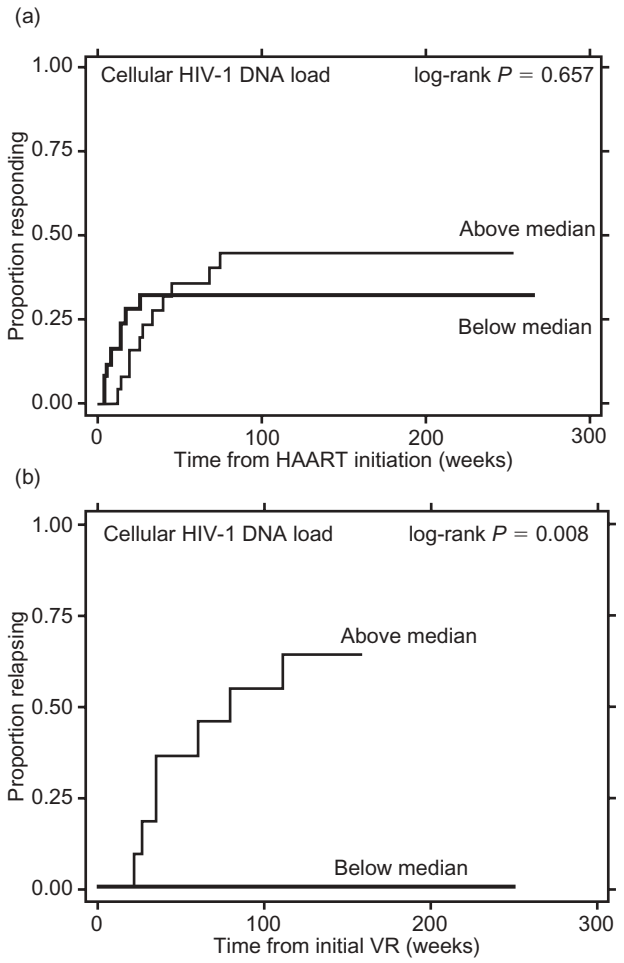

Fig. 1. Kaplan–Meier plots by median cellular HIV-1 DNA load (297 copies/1 × 10^6 PBMC) in patients with initial VR (a) or virological rebound (b).

immunological or virological parameter associated with sustained VR. We further evaluated this association with multivariate analysis using logistic regression models. Cellular HIV-1 DNA remained a significant predictor of sustained VR even after adjustment for the other virological, immunological and treatment factors in multivariate analysis with the adjusted odds ratio [95% confidence interval (CI)] of having a sustained VR per 1 $\log_{10}$ increase in baseline levels of HIV-1 DNA being 0.197 (0.048–0.801) ($P = 0.023$). That is, subjects with higher baseline HIV-1 DNA levels by 1 $\log_{10}$ copies/1 × 10^6 PBMC had reduced (by about 80%) odds of sustained VR. Sensitivity analysis excluding the three treatment-naive subjects yielded practically identical results to those of the main analysis (data not shown).

Longitudinal trends in cellular HIV-1 DNA levels

The HIV-1 DNA longitudinal trends after initiation of HAART were studied. Forty-seven out of the 51 patients (92.2%) were included based on the availability of the baseline and at least one sample after starting HAART. The median (IQR) number of samples available per patient was three, ranging from two to eight. The median (IQR) follow up was 76 (45–102) weeks, ranging from 9 to 156 weeks.

There was a tendency for decreasing cellular HIV-1 DNA load over time in subjects taking HAART. The overall mean (95% CI) slope as estimated by a random effect model was −0.160 (−0.309, −0.011) $\log_{10}$ copies/10^6 PBMCs/year ($P = 0.036$), which is equivalent to a 30.8% (2.4-50.9) relative decrease per year.

We further evaluated cellular HIV-1 DNA trends in

Table 2. Baseline characteristics of the 51 patients with virologic rebound, sustained virologic response and virological failure.

	Virological rebound (n = 7)	Sustained virological response (n = 12)	Virological failure (n = 39)[a]
Median age at seroconversion [years (IQR)]	16.9 (13.8–29.1)	19.8 (15.2–26.4)	18.0 (10.9–24.8)
HAART use begun			
Median age [years (IQR)]	32.6 (28.2–43.3)	34.9 (30.5–41.5)	32.6 (25.5–39.5)
Median calendar year	1997	1998	1997
Median time from seroconversion to HAART [years (IQR)]	14.7 (12.0–15.7)	15.3 (13.6–16.3)	14.7 (14.2–15.7)
Median plasma HIV-1 RNA [copies/ml (IQR)]	8600 (8097–24700)	7349 (2450–20904)	8210 (5000–18070)
Median HIV-1 STS DNA [copies/1 × 10^6 PBMC (IQR)]	1338 (1083–1374)[b]	91 (39–459)[b,c]	441 (109–1163)[c]
Median CD4 cell count [× 10^6/l (IQR)]	190 (62–350)	240 (132–385)	143 (80–267)
Median TREC [copies/1 × 10^6 PBMC (IQR)]	7320 (2286–22981)	4489 (1852–10919)	2769 (577–7121)
AIDS [n (%)]	1 (14.3)	1 (8.3)	6 (15.4)
Treatment naive [n (%)]	0 (0)	3 (25.0)[d]	0 (0.0)[d]
HAART based on [n (%)]			
Protease inhibitor	3 (42.9)	4 (33.3)	16 (41.0)
Saquinavir hard gel	2 (28.6)	6 (50.0)	16 (41.0)
Non-nucleoside reverse transcriptase inhibitor	2 (28.6)	2 (16.7)	7 (18.0)

[a]Virological failure: subjects without initial virological response (n = 32) plus subjects with virological rebound (n = 7). [b]Comparing subjects with virological rebound to subjects with sustained virological response ($P = 0.002$). [c]Comparing subjects with sustained virological response to subjects with virological failure ($P = 0.069$). [d]Comparing subjects with sustained virological response to subjects with virological failure ($P = 0.011$). IQR, Interquartile range.

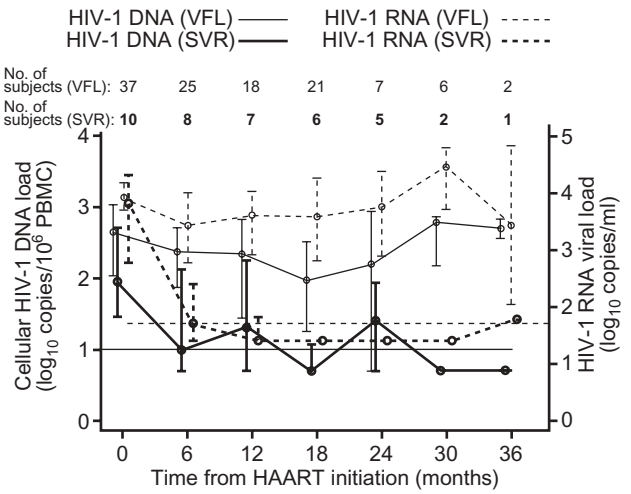

Fig. 2. Median (IQR) levels of cellular HIV-1 DNA load (solid lines) and HIV-1 RNA viral load (dashed lines) according to time since HAART initiation in patients with virological failure (VFL) or sustained virological response (SVR). Horizontal lines represent lower detection limits for cellular HIV-1 DNA (solid line) and HIV-1 RNA viral load (dashed line).

subjects with sustained VR and in failures. Fig. 2 shows the cross-sectional medians (IQR) of cellular HIV-1 DNA by time since HAART initiation and virologic response. For comparison, corresponding medians (IQR) of HIV-1 RNA are also shown. Subjects with sustained VR had on average lower baseline HIV-1 DNA levels and steeper declines over time compared to failures. The average (95% CI) slope in subjects with sustained VR and in failures was -0.49 (-0.83 to -0.16) and -0.06 (-0.23 to 0.10), respectively, corresponding to a 67.9% (29.9–85.3%) decrease per year in subjects with sustained VR and to a 13.2% decrease per year (40.7% decrease to 27.0% increase) in failures ($P = 0.025$).

Discussion

In this study, by using a real-time PCR assay and a molecular beacon-based detection system, we accurately quantified PBMC-associated HIV-1 DNA forms that have undergone the second template switch (HIV-1 STS DNA). This pool of unintegrated and integrated, linear, double-stranded HIV-1 DNA structures was used as a marker of cellular HIV-1 DNA load [23]. Various PCR-based methodologies have been used to describe cell-associated unintegrated or integrated HIV-1 DNA forms [26–33]. There is agreement that unintegrated full-length or near full-length HIV-DNA is the dominant HIV DNA in both resting and activated CD4 lymphocytes and macrophages infected by HIV-1. Some of the unintegrated HIV DNA is in the form of 1- and 2-long terminal repeat circles. Only a fraction of this unintegrated or integrated HIV-1 DNA is replication competent [34–36]. A distinctive characteristic of the assay used in this study is the simultaneous assessment of the PBMC number, which is the source of amplified HIV-1 DNA [23]. Based on this assay, we had two major findings in the pre-HAART era. First, the progression to AIDS and death was independently associated with cellular HIV-1 DNA load. And second, the HIV-1 DNA load of each individual was relatively steady in longitudinal samples up to 16 years from seroconversion.

We herein extended our observations to the post-HAART period. The HIV-1 DNA load declined in agreement with previous studies [37,38]. Furthermore, the rate of decline was clearly associated with the long-term success of HAART (sustained VR), indicating that this marker has some value in monitoring HIV-1 infection after initiation of HAART, especially when HIV-1 RNA is below the threshold level of detection [39,40]. Despite this decline of infected cells, it has been proven that many years after initiation of HAART replication-competent viruses can be isolated from blood cells, including resting CD4 lymphocytes, macrophages, and natural killer cells [41–44]. Moreover, plasma HIV-1 RNA rebounds within 2–4 weeks to the pre-treatment levels after treatment interruption, suggesting the existence of stable viral reservoirs not sensitive to antiretroviral treatment [45–47].

According to our analysis, the pre-HAART levels of cellular HIV-1 DNA predict the long-term success of HAART and they especially predict the patients who will rebound after the initial viral response. It is of interest to note that the CD4 cell counts and HIV-1 RNA levels were not statistically significant predictors in this study. In large clinical studies it has been shown that until the time of rebound the HIV-1 RNA response profiles and pre-treatment CD4 cell count or HIV-1 RNA values do not differ between the persons who experience rebound and those who do not [10,13,14,37].

Prediction of HIV-1 RNA rebound after the initially successful HAART is a finding with clinical utility. In this context, cellular HIV-1 DNA load may also serve as an indicator when to initiate HAART in the HIV-1-infected patient with CD4 cell count $> 200 \times 10^6$/l or HIV-1 RNA $< 10^5$ copies/ml where uncertainty exists. Our data suggest that assessment of cell-associated HIV-1 DNA as an early marker of long-term success of HAART should be evaluated in large clinical studies.

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