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CCTTT-repeat polymorphism of the inducible nitric oxide synthase is not associated with HIV pathogenesis

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SUMMARY

Nitric oxide (NO) produced by the inducible form of nitric oxide synthase (iNOS) has bactericidal and virocidal effects. Although NO synthesis and iNOS expression in macrophages affect several aspects of human immunodeficiency virus (HIV) type-1 pathogenesis, their role in HIV disease remains largely unknown. In humans, the expression of iNOS is influenced by a functional CCTTT-repeat polymorphism in the promoter region of the gene. We investigated the association of this polymorphism with HIV pathogenesis in naive HIV-infected patients before the initiation of antiretroviral therapy. The allele frequencies of the *iNOS* CCTTT-repeat polymorphism were assessed by PCR in 857 patients from the Swiss HIV Cohort Study, including rapid progressors and long-term nonprogressors, and in 240 healthy volunteers. In HIV-infected patients, the initial viral load and the decline in total CD4 cells was calculated to estimate disease progression. Allele frequencies of the *iNOS* CCTTT-repeat polymorphism were similar between the HIV-infected and noninfected blood donors. In treatment-naive HIV-positive patients, there was no association of the *iNOS* polymorphism with viral load or with the course of CD4 cells. Regulation of *iNOS* expression by the functional CCTTT-polymorphism does not modify HIV pathogenesis.

Keywords HIV inducible nitric oxide synthase polymorphism pathogenesis

INTRODUCTION

Nitric oxide (NO) is a labile intercellular messenger molecule and an effector molecule in macrophage cytotoxicity [1,2]. NO is produced by a family of enzymes, the NO synthases (NOS). Monocytes contain the constitutively expressed endothelial NOS and the inducible NOS (iNOS) [3]. Expression of iNOS is increased by a variety of stimuli, including microorganisms, pro-inflammatory cytokines and interferon-α and -γ [4]. Up-regulation of iNOS leads to increased NO production, which has bactericidal and virocidal effects *in vitro* and *in vivo* [5]. Compared to littermate controls, mice deficient in *iNOS* or pretreated with NOS inhibitors exhibited higher mortality, greater viral replication, and delayed clearance of virus when challenged with different viruses [6,7].

Because NO mediates other viral infections, it is a tempting potential target for therapeutic intervention in treatment of HIV disease. However, NO's role in HIV infection remains largely unknown. While most *in vitro* studies concluded that HIV [8] or HIV proteins [9,10] increase iNOS [11] and subsequently NO production, the ultimate effects of increased NO production on HIV replication are controversial. In some studies, NO increased HIV replication in monocyte-derived macrophages (MDM) [12] and caused apoptosis of cells near the HIV-infected MDM [13], potentially contributing to disease progression. In other studies, NO inactivated HIV protease [14], reverse transcriptase [15], and increased MIP-1α in MDM [16], suggesting a protective role for NO in HIV infection.

In vivo studies support the up-regulation of NO by HIV. Compared to noninfected controls, NO production substantially increased in HIV-infected peripheral blood monocytes [17] and sera from patients with advanced HIV infection. Serum levels of

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NO metabolites correlated with viral load and with activation of mononuclear phagocytes [18,19]. Furthermore, iNOS expression is associated with the development of the AIDS dementia complex [17,20–22] and with HIV-associated cardiomyopathy [23,24].

The gene for human *iNOS* has a functional CCTTT polymorphism within its promoter region that alters the transcriptional activity of the gene. Thirteen different alleles have been detected in Caucasians. Alleles with eight or nine CCTTT-repeats have lower levels of transcriptional activity and are less inducible by Il-1β than alleles with 12–15 repeats [25]. Thus, alleles with lower repeat numbers likely produce less iNOS and subsequently less NO than alleles with higher repeat numbers.

To investigate the role of NO in HIV pathogenesis, we examined the association of specific CCTTT alleles with HIV infection, progression rate and HIV RNA over time.

PATIENTS AND METHODS

Study participants

Written consent was obtained from all participants according to good clinical practice. We recruited 857 HIV-1-infected patients from the Genetic Core of the Swiss HIV Cohort Study (SHCS). Patients had to be at least 18 years of age. The decline in numbers of CD4 cells was calculated in untreated patients with at least two CD4 measurements separated by more than 1 year. Viral load was determined in untreated patients as the mean of $\log_{10}$ of HIV-1 RNA copies/ml with at least three independent measurements of HIV RNA levels (Roche Amplicor HIV-1 Ultrasensitive Monitor assay, version 1·5) over time before the start of treatment. Two CD4 cell count measurements separated by one year reflect quite precisely CD4 decline. In contrast HIV RNA is more inclined to variability and therefore, we claimed three independent measurements of HIV RNA to calculate the mean of $\log_{10}$ of HIV-RNA. The control group consisted of HIV-negative blood donors ($n = 240$).

iNOS *Genotype*

Blood samples supplemented with EDTA were extracted with the QIAmp DNA Mini Kit (QIAGEN AG, Basel, Switzerland). To amplify the region of the *iNOS* CCTTT repeat polymorphism, 50 ng DNA was added with primer U5553 ACCCCTGGAAGC CTACAACTGCAT and the fluorescently (Joe) labelled primer L5735 GCCACTGCACCCTAGCCTGTCTCA (Microsynth, Balgach, Switzerland) with the Amplitaq Gold System (Applied Biosytems, Foster City, CA, USA) in a total volume of 12·5 μl. PCR products were then analysed on an ABI310 genetic analyser with GeneScan 500 ROX as internal standard. Allele assignment was done with the ABI Genotyper software (all from Applied Biosystems).

Statistical analysis

Data were analysed with the statistical software packages R and SAS 8·1. Genotype frequencies between groups were compared with the chi–square test. Associations between CD4 decline or viral load and *iNOS* repeat number were analysed by linear regression. Differences between the presence and absence of a *iNOS* CCTTT 14 repeat allele were analysed using a *t*-test assuming equal variance. All *P*-values are two-sided, and *P*-values below 0·05 were considered statistically significant.

RESULTS

Allelic distribution of functional iNOS *CCTTT repeat polymorphism*

To investigate the role of the functional CCTTT repeat polymorphism in *iNOS* on HIV pathogenesis, we analysed the *iNOS* genotype of 857 HIV-positive patients and of 240 noninfected blood donors (Table 1). We detected 13 different CCTTT-alleles and an allele frequency similar to Caucasians reported in other studies [25,26]. No significant difference in *iNOS* allele frequencies was observed between the HIV-infected patients and noninfected blood donors.

HIV RNA and iNOS *CCTTT repeat polymorphism*

We next analysed the association of *iNOS* alleles with HIV RNA levels in HIV-positive patients ($n = 183$). Although there was a wide range in the means of HIV RNA levels in the study group, linear regression revealed no association of the *iNOS* CCTTT repeat polymorphism with the mean HIV RNA in a dominant (i.e. with the longer of the two alleles for each patient, $P = 0{\cdot}54$, data not shown) or in a codominant model (i.e. the sum of the two alleles, $P = 0{\cdot}87$, Fig. 1a). The power of the study to detect a difference of 0·2 $\log_{10}$ HIV-1 RNA copies in the dominant and codominant model was 82% and 99%, respectively, suggesting that this repeat polymorphism has no effect on HIV replication *in vivo*.

CD4 decline and iNOS *CCTTT repeat polymorphism*

To assess the influence of the *iNOS* polymorphism on HIV disease progression, we analysed the association of the polymorphism with the decline in CD4 cells over time (CD4 decline) in 246 naive patients (Fig. 1b). Since the study included rapid progressors and long-term nonprogressors, a broad variation in CD4 decline over time was observed (–300 to + 100 cells/year). Nevertheless, there was no association between the *iNOS* CCTTT repeat number and the CD4 decline in a dominant ($P = 0{\cdot}10$) or a codominant model ($P = 0{\cdot}44$). The power of the study to detect a difference of 10 CD4 cells/μl in the dominant and codominant model was 82% and 99%, respectively. These results suggest that this polymorphism does not influence disease progression rate as defined by the CD4 decline in HIV-infected patients.

Table 1. iNOS Allele frequencies in the study

iNOS alleles (repeats)	HIV Infected ($n = 857$)	Blood donors ($n = 240$)
6	0·01	
8	0·08	0·004
9	0·041	0·037
10	0·128	0·123
11	0·185	0·19
12	0·325	0·335
13	0·172	0·165
14	0·087	0·071
15	0·039	0·056
16	0·011	0·013
17	0·002	0·006
18	0·001	
19	0·001	

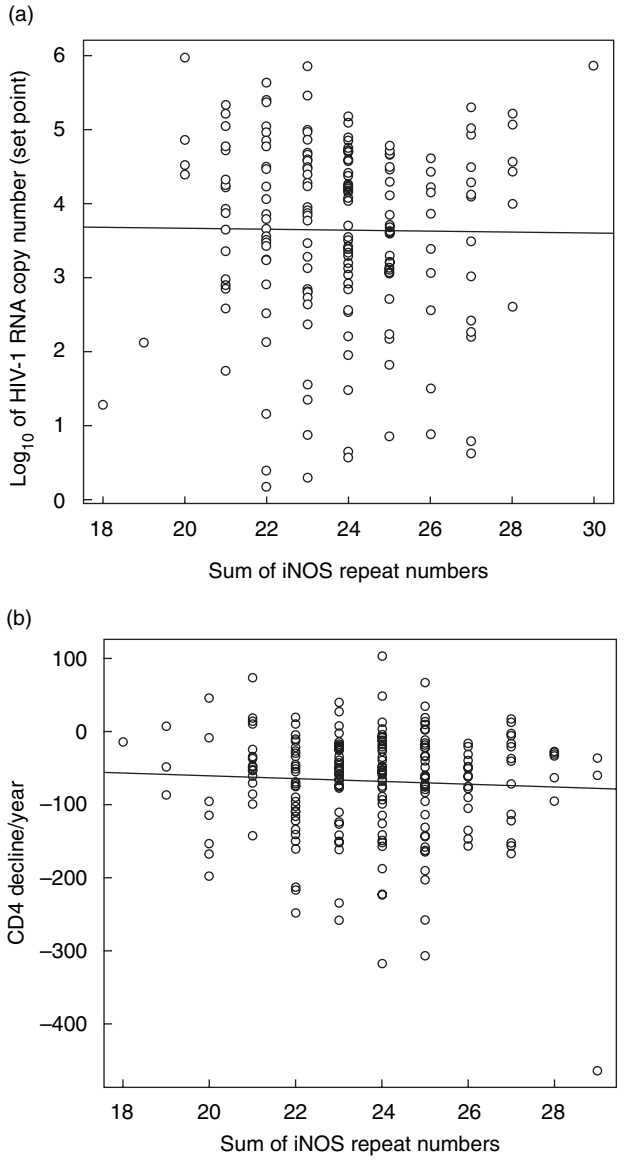

Fig. 1. (a) Log_{10} of HIV-1 RNA copy numbers in naive patients, according to the *iNOS* CCTTT genotype in a codominant model (sum of the repeat numbers). (b) Decline of CD4 numbers per year in naive patients, according to the *iNOS* CCTTT genotype in a codominant model (sum of the repeat numbers).

Since the *iNOS* 14 CCTTT repeat allele was previously associated with a variety of disease states, we analysed this allele for an association with HIV disease progression. 34 out of 246 patients included in the CD4 decline analysis had at least one *iNOS* 14 CCTTT repeat allele. However, no significant association with CD4 decline ($P = 0{\cdot}64$) was detected. Similarly, there was no significant difference in viral load ($P = 0{\cdot}13$) between the patients having at least one *iNOS* 14 CCTTT repeat allele ($n = 30$) and the rest ($n = 153$).

DISCUSSION

HIV progression rate is influenced by distinct host genetic factors, such as CCR5Δ32 and Rantes (reviewed in [27]). However, these factors explain progression in only a minority of patients. The effect of NO on HIV pathogenesis is controversial but *iNOS*-dependent NO production is assumed to be a pathogenic factor in malaria [28], dementia [29], and diabetic retinopathy [25]. This perception is based on an association of a CCTTT repeat polymorphism with the outcome in these diseases. Therefore, we hypothesized that the CCTTT repeat polymorphism in the *iNOS* gene may impact transmission or progression of HIV disease.

We detected all known CCTTT repeat alleles of the *iNOS* gene among the HIV-positive patients but the distribution of the alleles was similar to that of a control group of HIV-negative volunteers. This implies that HIV transmission is independent of this *iNOS* polymorphism. When we investigated the effect of the CCTTT repeat polymorphism on disease progression, we also detected no association with either the mean HIV RNA levels or the CD4 decline before treatment assuming a codominant or a dominant model. Thus, the CCTTT repeat polymorphism has also no impact on disease progression and no prognostic significance in HIV pathogenesis

Our findings may be explained in a couple of different ways. First, the lack of an association of this polymorphism with HIV disease progression may be due to variability in NO levels produced by the different *iNOS* alleles that are too small to be reflected in distinct HIV progression rates. Alternatively, NO may not influence HIV pathogenesis in man. However, this is less likely considering the manifold functions of NO in the immune system. It is also possible that another region of the *iNOS* gene is more critical for NO production and thus for HIV pathogenesis. Several other single nucleotide polymorphisms have recently been identified in the promoter and the coding region of the iNOS gene which revealed an association between certain iNOS haplotypes and the outcome of hepatitis C infection [30]. We focused on a polymorphism within the iNOS promoter which showed functional consequences in *in vitro* assays. Definition of transcriptional activity of the iNOS gene due to different iNOS polymorphisms and haplotypes will be critical for dissecting the genetic impact of iNOS on disease in the future.

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APPENDIX

The members of the Swiss HIV Cohort Study are:

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