Transmembrane protein polymorphisms and resistance to T-20 (Enfuvirtide, Fuzeon®) in HIV-1 infected therapy-naïve seroconverters and AIDS patients under HAART-T-20 therapy

Vladimir A. Morozov · Alexei V. Morozov · Dirck Schürmann · Heiko Jessen · Claudia Kücherer

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Abstract The human immunodeficiency virus type 1 fusion inhibitor T-20 (Enfuvirtide, Fuzeon®) has recently been introduced into clinical practice. T-20 in combination with HAART efficiently inhibits HIV-1 replication, however T-20 resistance has been reported and the number of confirmed resistant-associated mutations is growing.

In this study we aimed to analyze HIV-1 gp41 transmembrane protein (TM) variability and primary resistance to T-20 in plasma viruses from 10 HIV-1 subtype B infected homosexuals. Nine out of ten were documented seroconverters. Nine individuals (including one long time infected therapy naïve individual) were part of four linked virus infection chains. We also examined TM polymorphism in two AIDS patients under HAART and T-20 therapy. Obtained TM amplicons were examined for minor variants by clonal analysis.

Sequences polymorphism of the N-terminal regions of the fusion domain (FD) and the heptad repeat 2 (HR2) domain were demonstrated in examined seroconverters. Analysis of the heptad repeat 1 (HR1) domain revealed T-20 resistance in cloned sequences from 3/10 individuals. In two individuals these mutations were present as minor viral quasispecies. Transmission of the resistant virus to the sexual partner was traced in virus infection chain.

Baseline TM amplicons (population sequence) and clones from two patients under HAART did not contain T-20 resistance associated mutations. After onset of T-20 therapy only resistant viruses were identified in plasma from the patients. As shown by clonal analysis of plasma from one patient, treatment interruption results in viruses reverting to a T-20-sensitive genotype.

Keywords HIV-1 · Seroconverter · Transmission chains · env transmembrane protein · T-20 · Natural resistance

Introduction

Polymorphism of HIV-1 envelope (env) is the most prominent as compared to other viral genes [1] and in combination with variation in glycosylation pattern [2] it allows HIV-1 to escape the immune pressure representing a serious obstacle in efficient anti-viral therapy and vaccine design.

The components of the envelope glycoprotein complex are synthesized as a glycosylated precursor (gp160	extsuperscript{env}), which is cleaved in host cells by furin (subtilysin proteinase) to give rise to gp120 surface (SU) and gp41
transmembrane (TM) [1, 3–5]. These glycoproteins are present as a trimeric complex on the virion surface.

HIV-1 TM protein is a key mediator of virus-to-cell fusion. The TM has a complex structure and three of its functional domains are directly involved in fusion. At the N-terminus of the TM there is a nearly 20 amino acid (aa) long fusion domain (FD) that is indispensable for the interference with the plasma membrane at the initial step of fusion. Downstream to the FD there are two helical repeats—heptad repeat 1 (HR1 or N-terminal repeat) and heptad repeat 2 (HR2 or C-terminal repeat). Both repeats (nearly 50 aa long) contain hydrophobic stretches that are interacting during virus-to-cell fusion with each other in an anti-parallel manner forming a six-helical-bundle structure [6]. Integrity of both elements is crucial in formation of the functional fusogenic complex and inhibition of the bundle structure formation prevents target cells from virus infection. In the extra cellular virus particle the HR1 and the HR2 domains are largely “shielded” by gp120 and during virus-to-cell fusion the HR1 domain is exposed only for a short window.

Both—the HR1 and the HR2 domains are attractive targets for the anti-HIV-1 therapy. T-20 (Enfuvirtide, Fuzeon) is the first approved drug that targets HR1 [5, 7]. T-20—a 36-amino acid long peptide—is a replica of the C-terminal region of the HR2 (HIV-1LAI) [8–9]. T-20 acts by zipping into the grooves on the HR1 trimer in place of HR2, thus, preventing fusion intermediate from progressing into six-helix structure [9–11]. It is an advantage, that T-20 acts extra cellular. However, T-20 access to the HR1 domain is likely of a short time; thus, a permanent high concentration of drug is required for an efficient inhibition of fusion [11]. T-20 therapeutic effect may vary as different HIV-1 isolates have variable susceptibility to T-20 [12].

The LLGIV (position 33–38) motif was shown to be crucial for T-20 attachment to HR1 [13]. Resistance-associated mutations were initially discovered in vitro at position 36–38 (numbering from A—the first amino acid in TM, acc. number AF324493) of the HR1 domain [14]. The “resistance-associated region” now is spanning the positions 32–45 with a gradual increase of double mutations [10, 15–17]. Some of the frequent mutations are given below Q32/H/R; G36/D/S; I37/V; V38A/M; Q39/R; Q40H; N42D; N43D; V38A + N42T; N42T + N43S; N42T + N43K. The impact of known mutations on susceptibility to T-20 treatment differs nearly by 100 times [18].

The mechanism of virus escape during T-20 therapy is not always clear, but it is likely associated with conformational changes in α-helix (which reduce the efficacy of T-20 attachment to HR1). Recently, the first substitution that might have an impact on T-20 susceptibility was described in HR2 (S138A) [19, 20].

Limited data are available concerning TM variation and natural resistance to T-20 therapy in naïve individuals and seroconverters [21–23]. Nothing is known about horizontal transmission of T-20-resistant virus if present together with a sensitive virus strains. All that might have crucial importance regarding therapy outcome.

To address the question of the FD-HR1 variability and investigate natural resistance to T-20, we examined plasma viruses from 10 clade B infected therapy naïve homosexuals from Berlin. Nine of the individuals were from four virus infection chains. We also examined FD-HR1 diversity and resistance to T-20 in two patients under HAART and T-20 therapy.

Materials and methods

Patients

Study patients are given in Table 1. All patients were German men who have sex with men (MSM) and were infected with HIV-1 subtype B (pol and env gp120 C2V5). Nine of ten individuals were engaged in four virus infection chains as reported by the patients and confirmed by phylogenetic analysis of pol and env sequences (data not shown). Source patient 02–029 was not treatment-naïve and was infected for more than 2 years (Table 1, chain 3). Most of the individuals were documented seroconverters with a known date of infection and the blood samples were collected no longer than 1 month after seroconversion. Plasma specimens from two AIDS patients (K. and B.) under HAART were analyzed before and during T-20 therapy and from patient K. after T-20 therapy interruption also.

Isolation of plasma viruses and RNA

Plasma specimens (0.3–0.5 ml) were centrifuged at 3000 g for 5 min and supernatants were used for virus isolation. Viruses were isolated by high-speed centrifugation (1,10,000 g for 1 h 10 min, at 4°C) through 20% sucrose cushion. RNA was isolated from virus containing pellets using TRISOL reagent (BRL, USA) or TRI reagent (Sigma, USA) according to the protocol of manufacturers. RNA was immediately used in RT-PCR reaction.

RT-PCR

RT-PCR was performed using Titan-one tube RT-PCR kit or C. therm. Polymerase One-Step RT-PCR kit (both kits from Roche Diagnostics, Germany) according to the protocols of the manufacturer. Six primers were used for amplification (Table 2). Primers 7487f and 8459r were used in RT-reaction and 7528f and 8035r pair in RT-PCR. Two
additional primers (7581f and 8014r) were used as supplementary in case of poor amplification with the 7528f and 8035r pair. Absence of DNA contamination in analyzed specimens was controlled by omitting the RT step. Reverse transcription was carried out at 50°C for 1 h followed by PCR using the following conditions: initial denaturation at 95°C for 4 min; followed by 30 cycles of 94°C—40 s; 52°C–54°C (depending on primers)—30 s; 72°C—1 min; final extension of 5 min at 72°C. About 2 μl of the RT-PCR reaction were subjected to 40–42 cycles of PCR with corresponding nested primers (conditions are as above).

Cloning and sequence analysis

Amplicons were purified from 2% agarose gels according to the protocol of the supplier (JetQuick Berlin, Germany) and cloned in pTOPO-TA (Invitrogen Life Technologies, USA). Analysis for the insert was carried out by EcoR1 restriction. Amplicons and clones were sequenced in both directions using the BigDye Terminator sequencing kit (Perkin-Elmer, Biosystems, Germany) and Applied Biosystem model 3100 automatic DNA sequencer. Alignments of sequences were done using Lasergene (Version 6, DNASTAR Inc. USA) program.

GenBank accession numbers

pNL 4–3 (AF324493), HXB2 (NC001802). HIV-1 env sequences from seroconverters 01–0209 and 01–0336 were deposited to GenBank under accession numbers EF192132 and EF192133 respectively.

Results

Diversity in FD-HR1 domains in viruses from therapy-naive seroconverters

The FD is the first stretch in TM that is involved in virus-to-cell fusion. T-20 is not directly interfering with the FD
and mutations associated to T-20 resistance were not reported so far. It is known, that FLGFL motif (amino acids 8–12; acc. AF324493) in FD is crucial for the cell membrane attachment. Thus, mutations in FLGFL motif could create a low or even non-fusogenic virus variants.

We examined amplicons and cloned sequences from 10 individuals including nine therapy-naive seroconverters. Comparative analysis of clones (Fig. 1 A) demonstrated significant polymorphism including frequent deletions and insertions at the N-terminal part of the FD (amino acids 1–7). Substitutions at position 2 (V) and deletion at position 3 (G) were revealed in a majority of sequences. A tyrosine (T) insertion between position 3 and 4 was detected in two individuals from a linked infection in chain 3. Finally, L7M/V/T substitutions were revealed in majority of the amplicons.

The impact of revealed rearrangements in the FD on fusogenic properties of the virus was not examined. However, stability of the FD sequence in virus infection chains argues in favor of low (if any) contribution of these mutations to the virus-to-cell fusion. In addition, in virus infection chains we revealed frequent M25I/L substitutions close to the C-terminus of the FD. It is noteworthy to mention, that the FLGFL motif was intact in all examined clones, indicating equal efficacy of the viral interaction with the plasma membrane at the very first step of fusion.

The HR1 domain is located immediately downstream to the FD. Analysis of the HR1 domain polymorphism is of a principal importance since it is a target sequence for T-20. Direct sequencing of 10 amplicons results in recovery of resistance-associated mutation (N43S) only in 01–004 (as shown by nearly equal double picks at the same position on chromatogram). Thus, matching reported 10% frequency of natural resistance to T-20 among subtype B infected patients [22]. Clonal analysis of the amplicons confirmed this mutation in two out of five examined clones, giving quasi-equal amount of resistant to non-resistant virus variants in 01–004. Clonal analysis of remaining nine amplicons demonstrated T-20 resistant viruses in two more specimens (as a minor population) from virus infection chain 2, thus, bringing the proportion of viruses with resistant genotypes in examined group to 3 out of 10. Significant diversity of sequences was revealed in 02–642 (source patient of virus infection chain 2). Five variations of TM were revealed in seven clonal sequences and between those two clones contained resistance-associated mutations. In clone # 1 it was one mutation (N43S) and in clone # 6 two mutations (I38V and N43S) were revealed. The same resistance-associated mutations were shown in viruses from 02–663—a sexual partner of 02–642 and resistances to T-20 were revealed also in 2/7 clones (clones # 2 and # 4 respectively). Thus, in the virus infection chain 2 transmissions of viruses (as a minor population) with retained resistant genotype took place. It should be mentioned that 3/5 sequence variants of TM revealed in 02–642 (source) were identical to 3/5 sequence variants in 02–663 (recipient). Interestingly, no resistance-associated mutations were revealed in four cloned TM sequences from another recipient (02–679) in virus infection chain 2. Cloned sequences revealed in 02–679 were identical to clone # 4 (non-resistant variant) from the 02–642 (source).

No resistance-associated mutations were shown in infection chains 1, 3 and 4 and no T-20 resistant viruses were demonstrated in plasma from the AIDS patient under HAART (02–029)—source patient of the chain 3. It is noteworthy to mention, that two mutations Q41R and I37T revealed in clones from patients 01–206 (cl. 2) and 01–336 (cl. 2) respectively, are not associated with resistance to T-20. Interestingly, the LLQCTVW “hydrophobic stretch” (amino acids from 54 to 60 in pNL4-3) was intact in all examined viral sequences.

Sequence diversity in HR2 domain

It was shown that minor changes in the HR2 domain might influence the T-20 therapy outcome [19]. Thus, we extended sequence analysis of viruses from 4 seroconverters to the HR2 domain.

FD-HR2 amplicons from two seroconverters (01–206 and 01–209) from virus infection chain 1 and specimens from two other seroconverters (02–663 and 01–336) were analyzed (Fig. 1 B).

Clonal analysis (n = 15) revealed frequent substitutions in proximity to the N-terminal region (amino acids 129–137). This variable stretch corresponds to the N-terminal region of T-20. Analysis of clones from seroconverters revealed from 2–5 amino acid mismatches and up to 4 mismatches when compared to corresponding regions in HXB2 and pNL4-3. In fact, clones from one patient demonstrated up to three amino acids mismatches in between. Interestingly, two motifs—NYT (amino acids126–128)—putative glycosylation site and ELDKVA stretch (amino acids 151–156)—target for the 2F5 neutralizing antibodies were conserved in all examined specimens. Two substitutions S138A and N145L previously shown to influence HR2 to HR1 binding [19] were not detected in examined clones.

Diversity of the N-terminal region of the HR2 domain has been reported for subtype B [19] and subtype C, however, other fragments (amino acids 138–162) were more variable in subtype C compared to subtype B [24].

T-20 associated resistances in patients under HAART and T-20 therapy

We examined FD-HR1 polymorphism in plasma viruses from two patients (K. and B.) under HAART. Plasma
samples were taken before and during T-20 therapy and were analyzed by cloning for minor virus variants. Plasma from patient K. was examined for about 2 months and from patient B. — 4 months after therapy initiation respectively. In addition, we examined plasma specimen from patient K. nearly 5 months after his T-20 treatment was interrupted.

Compared to the FD polymorphism in viruses from seroconverters in FD viral sequences from patients K. and B it was not significant. In addition, no T-20 resistance-associated mutations in the HR1 domain were marked in examined 10 clones (from each patient) from baseline plasma samples. However, during T-20 therapy in all examined clones (15 clones from K. and 13 clones from B.) we revealed T-20-associated resistances (Fig. 2). The following resistance-associated mutations were revealed in plasma viruses from patient K. (Fig. 2 A): single mutation V38A (11 clones), double mutation NN43-44TK/TS (4 clones). Interestingly, both types of mutations were not detected together, but only in separate clones. Mutations revealed in cloned FD-HR1 fragments from patient B. (Fig. 2 B) were different, but double mutations at amino acid position 43–44 were identical to that in viruses from patient K. Only double mutations were detected in viruses from patient B.: V38M + L45M (4 clones). NN43-44TK
Thus, during T-20 therapy population of plasma viruses from both patients contained a set of resistance-associated mutations. These mutations were diverse and were either single or double. Interestingly, no other resistant mutation(s) were present in clones with mutated NN43–44. Among the other possibilities, this observation might indicate that combination of some mutations in the HR1 domain dramatically impair the efficacy of virus-to-cell fusion. Based on published data [9] we estimate that resistance-associated mutations revealed in patients K. and B. reduce susceptibility to T-20 from 50 to 100 fold.

Patient K. was under HAART when T-20 therapy was interrupted because of failure and 5 months later his plasma was examined for resistant viruses. Sequence analysis of the amplicon (not shown) and clones (n = 13) demonstrated that all plasma viruses were T-20 sensitivity (Fig. 2A). More of that, alignment of sequences revealed identities between five clones (same as clone # 1N) before therapy and six clones (same as clone # 2S) after therapy interruption, thus, indicating sequences reversion to the wild type. Since intermediate plasma specimens were not available, the time elapsed until resistant virus emerged or until virus reverted to the wild type upon arrest of T-20 treatment could not be estimated precisely. Recent analysis of TM amplicons from two patients who discontinued T-20 revealed reversion of the virus to the wild type within 3 months [25].

Discussion

Clonal analysis is a relatively fast and powerful approach for the estimation of HIV-1 polymorphism in a given individual. In particular, it is useful in retrieving pre-existing minor population of drug resistant variants. In fact, it allows detection of the minor population of viruses that correspond to less than 25–30% of total virus bulk, thus, undetectable by direct sequence analysis.

Data on resistance to T-20 during therapy are accumulating fast. However, our knowledge about viruses with natural resistance to the drug, genetic stability of resistant viruses and efficacy of infection are still limited.

By clonal analysis we examined TM polymorphism in a group of documented seroconverters (homosexuals) infected with HIV-1 subtype B. The group was of a special interest because it was composed of individuals from virus infection chains (documented donors and acceptors of the virus).
Clonal analysis of the FD-HR1 region in plasma viruses demonstrated significant polymorphism in N-terminal stretch of the FD. In 3/10 individuals we revealed mutations in the HR1 domain associated with resistance to T-20. In 2/3 cases viruses with resistant genotype were present as a minor population, thus, were not detected by initial genotype assay. Viruses with resistant genotype could be transmitted as it was shown in one virus infection chain and in fact, that argues in favor of relative stability and efficient replication of resistant viruses in vivo. Since our clonal analysis was limited, persistence of additional resistant variants cannot be excluded.

Successful virus-to-cell fusion is dependent on binding efficiency of hydrophobic grooves between N- and C-terminal helices thus, reciprocal match of both repeats is essential. Thus, polymorphism in the HR2 domain (besides S138A and N145L) might to some extend reflect the efficacy of HR1-T20 interaction. In this regard, mutual polymorphism in both HR1 and HR2 might explain variable susceptibility of non-resistant viruses to T-20.

Fifteen clones from four patients were examined for the HR2 domain polymorphism. Comparative sequence analysis revealed that amino acids 129–137 were the most diverse, indicating significant natural variability of N-terminal region of the HR2 domain. Two revealed substitutions—L130V and E137N did not effect virus replication in vitro and resistance to T-20, as shown by recombinant phenotypic assay (using pNL4-3 as a backbone with TM insert from 01–336) (VM, personal communication). The impacts of other substitutions revealed in the HR2 domain on virus-to-cell fusion remains to be determine.

In conclusion, using cloning approach we demonstrated notable polymorphism of the FD and HR2 N-terminal regions in plasma viruses from therapy naive seroconverters. Clonal analysis of the FD-HR1 region allowed us to detect T-20 resistant viruses in 3/10 (in 2 as a minor population) seroconverters and we traced transmission of resistant virus in virus infection chain. Thus, in case of horizontal transmission of resistant virus, one might expect rapid creation of a stable persistent reservoir of T-20 resistant virus in resting CD4+ T lymphocytes and, as a result, a fast creation of a stable persistent reservoir of T-20 resistant viruses. Follow up studies of individuals from infection chains might shed light on viral fitness and stability of resistant genotype in vivo. Further investigations on natural and acquired T-20 resistance are required to predict therapy outcome.

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References