Experimental Neurology 219 (2009) 586–590

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Short Communication

Attenuated neurotoxicity of the transactivation-defective HIV-1 Tat protein in hippocampal cell cultures

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ARTICLE INFO

Article history:
Received 1 May 2009
Revised 10 June 2009
Accepted 6 July 2009
Available online 15 July 2009

Keywords:
Hiv-1 Tat
Neurotoxicity
Cell culture
Apoptosis
Cysteine-rich domain

ABSTRACT

This study reports that the cysteine 22→glycine 22 substitution in the HIV-1 Tat 1–86 B significantly attenuates its neurotoxicity. Consistent with previous studies, direct interactions of rat hippocampal cells with Tat 1–86 B were shown to cause dose-dependent and time-dependent neurotoxicity associated with activation of caspases from the mitochondrial apoptotic pathway. Despite the similar binding/uptake properties, Cys22 Tat 1–86 B failed to induce significant neurotoxicity and activation of caspases 9 and 3/7 in hippocampal primary cultures. Results of the study underscore the important role of cysteine-rich domain in mechanism of Tat-mediated neurotoxicity.

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In the era of highly-effective anti-retroviral therapy (HAART) neurological disorders linked to HIV-1 infection have become a major source of morbidity in AIDS (Navia et al., 1986; Koultsiliery et al., 2002; Manji and Miller, 2004). HIV-1 virus does not infect neurons. HIV-associated neurodegenerative pathology is widespread and is not proportional to viral load. Therefore, the neurotoxic potential of different HIV proteins attracts significant attention (Van de Bovenkamp et al., 2002; Jones and Power, 2006; Hult et al., 2008). Although mechanisms that incite neurodegeneration in AIDS are likely to be multifactorial, viral proteins appear to contribute to increased neuronal apoptosis in the HIV-infected brain (Roshal et al., 2001; Cossarizza, 2008).

The typical HIV genome has three major coding regions: core (gag); polymerase (pol); and envelope (env). The HIV genome also encodes 'accessory' or 'regulatory' proteins such as Nef, Tat, Vpr, Vif, Rev and Vpu that play a key role in the pathogenesis of HIV infection (Ozdener, 2005; Hult et al., 2008). The Tat protein is a key HIV-1 transactivator, which is essential for the control of HIV-1 gene transcription. The full-length open reading frame of HIV-1 Tat consists of the two exons of the viral *tat* gene and encodes a protein of approximately 101 amino acids (Hetzer et al., 2005). HIV-infected cells excrete Tat, and extracellular Tat can be then taken up by non-infected cells (Ensoli et al., 1993; Tardieu et al., 1992; Chang et al., 1997). Due to the intrinsic ability of Tat to interact with various cell components, this regulatory HIV protein can interfere with a variety of biochemical processes (Shojania and O'Neil, 2006). HIV-1 Tat is proven to be neurotoxic. Existing evidence for direct and indirect neurotoxicity of Tat rationalizes suggestions for an important role of this regulatory HIV protein in the pathogenesis of NeuroAIDS and stimulates the investigation of molecular mechanisms of Tat interactions with brain cells (King et al., 2006). Molecular determinants of Tat neurotoxicity are located within the 1–72 sequence encoded by the first *tat* exon. The second exon-encoded part of the full-length Tat sequence has been shown to be non-essential for the direct neurotoxicity (Nath et al., 1996). Amino acid residues 22–38 comprise highly conservative cysteine-rich domain of Tat. Experimental evidence, which indicates the important role of the cysteine-rich domain in the mechanisms of Tat toxicity, is accumulating rapidly (Misumi et al., 2004; Egelé et al., 2008; Mishra et al., 2008). The discovery of the lower cytotoxic potential of HIV-1 virus clade C, which is thought to be attributed to the mutation of cysteine 31 in the 1–101 version of Tat protein expressed in this HIV-1 subtype (Ranga et al., 2004; Mishra et al., 2008; Li et al., 2008), has fueled the interest to the role of individual cysteines located in the cysteine-rich domain in the ability of Tat to cause neurodegeneration. In this study, we investigate the effect of the mutation of the cysteine 22, which is essential for Zn^{+2}-chelating and trans-activation abilities of HIV-1 Tat (Saidae et al., 1990; Garber et al., 1998), with Gly on neurotoxic properties of the recombinant Tat 1–86 (clade B) in the primary culture of rat fetal hippocampal neurons.

Recombinant original Tat 1–86 and (Cys22→Gly22)-substituted Tat 1–86 (LAI/Bru strain of HIV-1 clade B, GenBank accession no. K02013) were purchased from Diatheva (Italy). Tat 1–101 clade C was purchased from Prospec (Israel).

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0014-4886/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.expneurol.2009.07.005

Primary hippocampal cell cultures were prepared from 18-day-old Sprague–Dawley rat fetuses as previously described (Aksenov et al., 2006). Cultures were used for experiments after 12 days in culture and were >85–90% neuronal as determined by anti-MAP-2/anti-GFAP/Hoechst fluorescent staining.

The treatment of hippocampal cell cultures was carried out by the addition of freshly-prepared stock solutions of the recombinant Tat polypeptides into the cell culture growth medium. Equal volume of the vehicle was added to control cell cultures. To determine dose–response neurotoxicity curves, groups of individually grown cell cultures were exposed to 10–150 nM concentrations of Tat or Cys22 Tat for 48 h. For the time course experiments, cell cultures were continuously incubated with 50 nM Tat or 50 nM Cys22 Tat for 2, 24, 48, and 96 h. To study binding/uptake, 50 nM Tat or 50 nM Cys22 Tat was added to the cultured cells and the incubation was carried out for different time periods from 1 min to 2 h. Cytotoxic effects induced during different time periods (1 min −2 h) of transient exposure of hippocampal cultures to Tat- or Cys22 Tat were studied as described in (Aksenova et al., 2009). For the comparison of the neurotoxic effects of Cys 22 Tat 1–86, Tat 1–86 (clade B), and Tat 1–101 (clade C), hippocampal cultures were exposed for 48 h to 100 nM dose of either of the recombinant Tat polypeptides.

Neuronal survival was determined using a Live/Dead viability/cytotoxicity kit from Molecular Probes (Eugene, OR) in rat fetal hippocampal cell cultures prepared in 96-well plates as described in (Aksenov et al., 2006, 2009). Fluorescence was measured using a Bio-Tek Synergy HT microplate reader (Bio-Tek Instruments, Inc., Winooski, VT).

Binding/uptake of Tat and Cys22 Tat was studied using anti-Tat immunocytochemistry in acetic alcohol-fixed cultures, immunoblotting of cell lysates, and the direct ELISA measurements of concentrations of Tat polypeptides in the cell culture growth medium (Aksenova et al., 2009). Rabbit polyclonal anti-Tat antibody (Diatheva, Italy) that recognizes both Tat and Cys 22 Tat immunoreactivities was used in these experiments.

Fluorochrome Inhibitor of Caspases (FLICA) Caspase 9 (red fluorescence) or Caspase 3/7 (green fluorescence) Apoptosis Detection Kit (Immunochemistry Technologies LLC, Bloomington, MN) were used to detect active caspases in cultures exposed to Tat and Cys22 Tat. Hoechst staining (blue fluorescence) was used to label cell nuclei. Microplate reader-based analysis and fluorescence microscopy imaging of specific fluorescence of SR-LEHD or FAM-DEVD complexes with active caspase 9 or 3/7 were carried out as described in (Aksenova et al., 2009).

Statistical comparisons were made using ANOVA and planned comparisons were used to determine specific treatment effects. Significant differences were set at $P<0.05$.

The dose–response curve of Tat 1–86 toxicity shown in Fig. 1A was consistent with our earlier reports (Aksenov et al., 2006, 2009). The analysis of dose–response curves demonstrated that Tat 1–86 was significantly ($P<0.05$) more toxic to hippocampal cell cultures than Cys22 Tat 1–86 following 48 h of treatment.

It could be argued that the substitution of cysteine 22 in Tat 1–86 simply delayed the development of Tat-mediated neurotoxicity. However, the comparison of the toxicity time courses in Tat – and Cys22 Tat-treated cell cultures (Fig. 1B) did not favor this suggestion since even the prolonged exposure to cysteine 22-substituted Tat variant failed to induce more than 10% decrease in cell viability. At 96-hour time point the Live/Dead ratio in Cys22 Tat-treated cultures was 90.7 ± 4.43% of control and this difference in cell viability did not reach statistical significance ($P>0.05$) due to high variation of individual numbers.

Binding to the cell membrane is the key step in the process of Tat neurotoxicity (Nath et al., 1999; Chandra et al., 2005; Aksenova et al., 2009). According to the literature, the cysteine 22 mutation does not affect intracellular distribution of the extracellular Tat protein following its internalization (Sadaie et al., 1990). Nevertheless, it

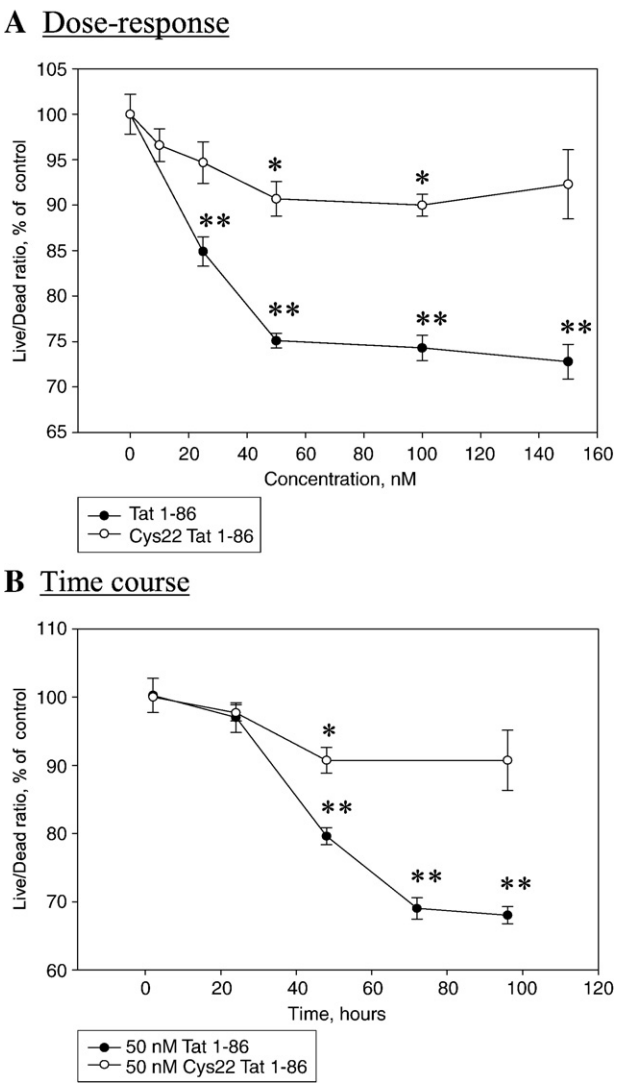

Fig. 1. Neuronal cell viability changes in hippocampal cell cultures induced by the original and cysteine 22-substituted Tat 1–86 clade B. (A) The dose–response of decreased neuronal cell viability in primary rat fetal hippocampal cell cultures exposed to Tat 1–86 or Cys 22 Tat 1–86. The graph shows the decrease in Live/Dead ratios produced by different doses of recombinant Tat 1–86 after 48 h of treatment. Data presented as mean values, n of sister cultures analyzed = 8–15 per each Tat 1–86 concentration. *—marks significant ($P<0.05$) differences in Live/Dead ratios between cultures treated with Cys22 Tat 1–86 and vehicle-treated controls. **—marks significant ($P<0.05$) differences in Live/Dead ratios between cultures treated with Tat 1–86 and cultures exposed to the same dose of Cys22 Tat 1–86. (B) The time course of the changes in neuronal cell viability in primary rat fetal hippocampal cell cultures exposed to Tat 1–86 or Cys 22 Tat 1–86. The graph represents relative (compared to non-treated controls) changes in Live/Dead ratios following the addition of 50 nM Tat 1–86 or 50 nM Cys 22 Tat 1–86. Individual measurements were carried out in 4–8 sister cultures (wells of the 96-well plate) per each time point and the experiment was repeated three times to ensure the reproducibility of the results. Data presented as mean values. *—marks incubation time points when significant ($P<0.05$) differences in Live/Dead ratios between cultures treated with Cys22 Tat 1–86 and vehicle-treated controls have been observed. **—marks incubation time points when significant ($P<0.05$) differences in Live/Dead ratios between cultures treated with Tat 1–86 and Cys22 Tat 1–86 have been observed.

was still possible that altered binding/uptake properties could be a reason for the attenuated neurotoxic ability of Cys22-substituted Tat 1–86. The presence of cell-bound/internalized Tat 1–86 or Cys22 Tat 1–86 in hippocampal neurons after 2-hour exposure to 50 nM dose to either one of the Tat variants was evident by immunofluorescent microscopy and Western blotting (Figs. 2A, B). The curves describing the specific absorption of Tat immunoreactivity by hippocampal cells in Tat 1–86- and Cys22 Tat 1–86-exposed cultures were not significantly different