Cell Death and Differentiation (2004) 11, 782–784
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www.nature.com/cdd

Letter to the Editor

HIV-Tat down-regulates telomerase activity in the nucleus of human CD4 + T cells

Cell Death and Differentiation (2004) **11,** 782–784. doi:10.1038/sj.cdd.4401346
Published online 12 March 2004

Dear Editor,

HIV-1 Tat is essential for viral gene expression and replication. Soluble Tat is released by acutely infected T cells,[1] either *in vitro* or *in vivo*. Extracellular Tat activates or suppresses cell growth, presumably through regulation of genes controlling cell proliferation or death.[2] Actually, the factor, immobilized on polystyrene plates or free at picomolar concentrations, promotes the growth of activated endothelial[1] or CD4 + T cells.[3] Micromolar concentrations of extracellular Tat are instead capable of inhibiting antigen-driven T-cell proliferation.[3]

Telomerase complex (i.e. hTR RNA template and human telomerase reverse transcriptase (hTERT) catalytic subunit) adds repetitive TTAGGG sequences to the 3′ ends of chromosomes, thus compensating for the progressive telomeric loss that occurs at each cell division. While hTR is present in many different cell types, including telomerase-negative cells, hTERT is generally expressed in cells endowed with telomerase activity. However, hTERT can be detected in telomerase-negative lymphocytes. In this case, telomerase activity can be induced by mitogenic stimuli through activation and transfer of hTERT into the nucleus.[4] This would ensure an adequate telomere size following multiple proliferative events of a given cell clone, as required for effective and long-lasting immune functions.

It is reasonable to hypothesize that impairment of the replicative potential of T lymphocytes could be involved in peripheral CD4 + T-cell decline during HIV infection. Actually, increased CD4 + T-cell senescence[5] and reduced telomerase activity have been described in hematopoietic progenitors of HIV-1-infected patients.[6] Preliminary studies performed in our laboratory in 1999, showed that Tat downregulates telomerase function of activated lymphocytes *in vitro* (unpublished data). These results were further supported by the observation that telomerase activity of PHA-stimulated lymphocytes is also inhibited by HIV infection.[7]

The present study was dedicated to investigate the effect of recombinant soluble Tat on telomerase activity and hTERT expression in the nucleus and cytoplasm of non-adherent mononuclear cells, and of purified CD4 + T cells obtained from healthy donors.

Non-adherent mononuclear cells (NA-MNC), separated from plastic-adherent MNC, were obtained by gradient centrifugation on Ficoll-Hypaque of peripheral blood. Furthermore, CD4 + T cells (95% pure at FACS analysis) were isolated from NA-MNC by magnetic beads coated with monoclonal antibodies (mAbs) against CD4 antigen (Dynabeads M-450, Dynal, Oslo, Norway). On day 0, cultured lymphocytes were activated with PHA (2 μg/ml, Sigma Chemical, St Louis, MO, USA). Purified recombinant Tat protein from the IIIB isolate (expressed in *Escherichia coli* and purified to homogeneity by heparin-affinity chromatography and HPLC, as described previously[1]) was added to the cultures on day 0. All experiments were repeated with at least four different lymphocyte preparations. Telomerase activity was determined according to the telomeric repeat amplification protocol (TRAP) adopted by Liu *et al.*[4] in lymphocytes, using an identical number of control or treated viable cells. Band intensity was quantified by bidimensional densitometry (Bio-Rad, Richmond, CA, USA). The sum of signal intensity of each ladder band was calculated and expressed in arbitrary units. The assay was performed separately on nuclear and cytoplasmic extracts, obtained as described by Andrews and Faller.[8] In all, 30 μg of extracts were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad), probed with anti hTERT polyclonal antibody (Calbiochem, Darmstat, Germany) at a 1 : 200 dilution, and detected using the ECL Plus Western detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). To test the quality of cell extracts, anti-H1 histone mAb (Upstate, Lake Placid, NY, USA) was used for Western Blot analysis.

NA-MNC were stimulated with PHA, exposed to Tat (1000 ng/ml) and analyzed for telomerase activity 24 h later. The results, illustrated in panel ‘a’ of Figure 1, show that: (a) in nonstimulated NA-MNC (group 1), telomerase activity was low in the nuclear compartment and absent in the cytoplasm; (b) in PHA-stimulated cells (group 2), enzyme activity was high in the nucleus, and almost undetectable in the cytoplasm; (c) in PHA-stimulated cells exposed to Tat (group 3), telomerase activity in the nuclear compartment was much lower than that found in stimulated cells not treated with Tat. In contrast, in the cytoplasmic compartment of PHA-treated cells, telomerase activity was significantly higher in cells exposed to Tat than in untreated controls. Similar results were obtained in NA-MNC stimulated with anti-CD3/anti-CD28 mAbs (data not shown).

Purified CD4 + T lymphocytes were activated with PHA and exposed to graded concentrations (from 10 pg/ml to 1000 ng/ml) of soluble Tat at the time of mitogenic stimulus. Concentrations of Tat, ranging from 100 to 1000 ng/ml, reduced telomerase activity in the nuclear compartment

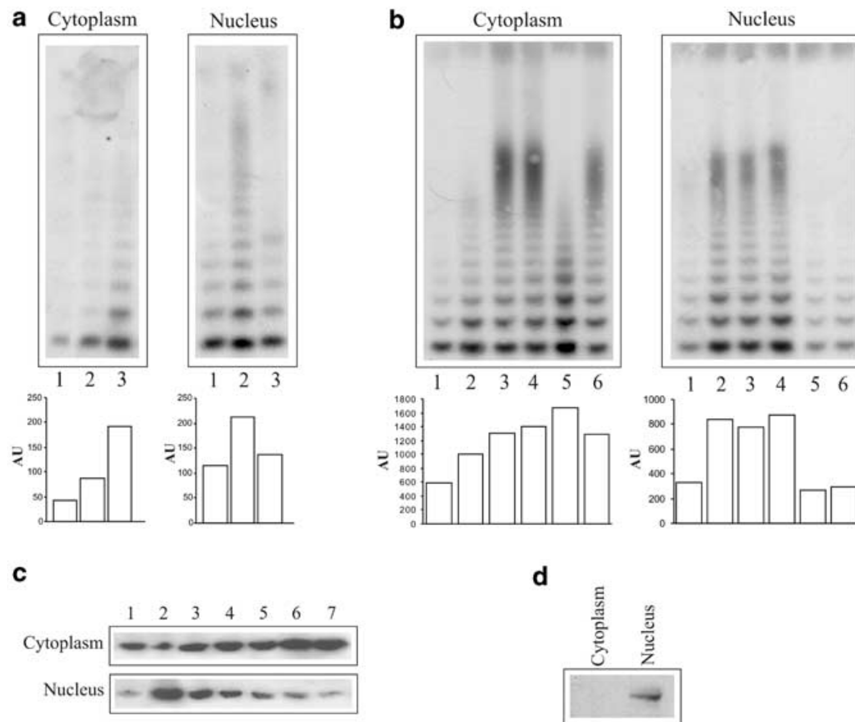

Figure 1 (**a**) Effect of soluble recombinant Tat protein added to NA-MNC at the time of mitogenic stimulation induced by PHA (2 μg/ml) on the telomerase activity (TRAP assay) of cytoplasmic and nuclear extracts, tested after 24 h culture. Lane 1: untreated control; lane 2: PHA alone; lane 3: PHA + Tat 1000 ng/ml. Columns indicate the extent of telomerase activity expressed as 'arbitrary units' (AU, see Methods). (**b**) Effect of Tat protein on telomerase activity in cytoplasmic and nuclear extracts of purified CD4 + T lymphocytes activated by PHA (2 μg/ml). Effect of Tat protein added to purified CD4 + T cells at the time of mitogenic stimulation, on the telomerase activity of cytoplasmic and nuclear extracts corresponding to 5×10^4 cells, tested after 24 h of culture. Lane 1: untreated control; lane 2: PHA alone; lane 3: PHA + Tat 10 pg/ml; lane 4: PHA + Tat 1 ng/ml; lane 5: PHA + Tat 100 ng/ml; lane 6: PHA + TAT 1000 ng/ml. Columns indicate the extent of telomerase activity expressed as 'arbitrary units' (AU, see Methods). (**c**) Effect of Tat protein on hTERT expression, tested after 24 h of culture. Lane 1: untreated control; lane 2: PHA alone; lane 3: PHA + Tat 10 pg/ml; lane 4: PHA + Tat 100 pg/ml; lane 5: PHA + Tat 1 ng/ml; lane 6: PHA + Tat 100 ng/ml; lane 7: PHA + Tat 1000 ng/ml. (**d**) Western Blot analysis of H1 histone expression tested on cytoplasmic and nuclear extracts of purified CD4 + T lymphocytes

(panel 'b' of Figure 1), but not in the cytoplasm. Again, similar results were obtained when target cells were stimulated with anti-CD3/CD28 mAbs and telomerase activity tested after 24–48 h (data not shown). Positive controls (i.e. extracts obtained from Jurkat T-cell line) and negative controls (i.e. Rnase-treated extracts) were used in each experiment (data not shown). Moreover, a linear correlation was found between graded numbers of activated lymphocytes and the corresponding telomerase activity expressed in terms of density values (arbitrary units, data not shown). It is noteworthy that Tat treatment did not affect the slight increase of CD4 + T-cell proliferation and induction of CD25 following 24 h exposure to PHA (data not shown).

Several studies have shown that activation of telomerase in CD4 + T cells does not require increase of hTERT mRNA or protein, but rather phosphorylation and transport of hTERT to the nuclear compartment.[4] Therefore, we investigated whether Tat-induced downregulation of telomerase activity in lymphocyte nuclei could be associated to a differential distribution of hTERT protein in cellular compartments. Western Blot analysis of nuclear and cytoplasmic extracts (panel 'c' of Figure 1) showed that in stimulated CD4 + T cells not exposed to Tat, the expression of hTERT protein was detectable in the nucleus, but not in the cytoplasm. Treatment of stimulated CD4 + T cells with Tat was associated with increased levels of hTERT in the cytoplasm and reduced amounts of the protein in the nucleus in a concentration-dependent manner. The quality of nuclear and cytoplasmic preparations utilized in this study has been tested in order to rule out the contamination of cytoplasmic extract with nuclear material. Therefore, experiments have been performed using an mAb able to recognize histone H1. The results illustrated in Figure 1d show that nuclear, but not cytoplasmic, extract from purified CD4 + cells was positive for the presence of H1 histone, as evidenced by Western Blot analysis.

The results of the present investigation pointed out that relatively high concentrations of soluble HIV-Tat protein are able to downregulate telomerase activity in the nuclear compartment of activated lymphocytes. Tat-mediated inhibition of telomerase activity in CD4 + T lymphocytes was detectable in the range of nano- to micromolar concentrations (i.e. 100–1000 ng/ml) that can be considered to be of clinical significance. Actually, these Tat concentrations may reach locally high levels in the lymphoid organs of infected individuals.[9]

In line with the findings of Liu *et al.*,[4] who demonstrated that phosphorylation and nuclear translocation of hTERT is required to raise telomerase activity in activated lymphocytes,

we found that Tat is able to reduce the amount of hTERT component in the nucleus, that is, in the cell compartment where this subunit is required for telomere elongation. Consistent with this observation are the results of telomerase activity in the two different compartments. In fact, telomerase activity is inhibited in the nucleus of Tat-treated lymphocytes, where reduced concentrations of hTERT have been demonstrated by Western blot analysis. In contrast in the cytoplasm where the transactivating factor induced a substantial increase of hTERT levels, treatment with Tat was not followed by reduction, but rather by increase of telomerase activity.

It is known that ageing is accompanied by increased susceptibility to infections due to T-cell senescence revealed by shortened telomeres, and inability to upregulate telomerase activity.[10] Moreover, accelerated replicative senescence of the immune system, usually accompanied by apoptotic death of stimulated lymphocytes, was described by Bestilny *et al.*,[11] who found shorter telomeres in the T cells of HIV-infected patients. These authors did not detect downregulation of telomerase activity in T lymphocytes of seropositive subjects. However, no determination of telomerase activity was carried out selectively in the cell nucleus of patient's lymphocytes.

The mechanism by which Tat interferes with telomerase activity has not been fully elucidated. The present results seem to favor the hypothesis that the effect of Tat could be directed to the machinery involved in the nuclear translocation of hTERT. The possibility that Tat would affect telomerase activity by preventing lymphocyte activation and proliferation seems to be excluded. In fact, thymidine incorporation and CD25 induction were not downregulated by Tat in PHA-treated CD4+ T cells (data not shown).

In conclusion, Tat-induced telomerase impairment in the nuclei of lymphocytes could be of clinical relevance and would contribute to understand, at least in part, the mechanism underlying the increase of CD4+ T-cell senescence that has been suggested to occur in patients with advanced AIDS.

Acknowledgements

We thank Mr Graziano Bonelli for technical assistance; Ms Angela Lippa and Ms Annamaria Carinci for editorial assistance. This work was supported by grants from the National AIDS Project of the Istituto Superiore di Sanità, Rome, Italy, to EB and BE, and by grants from the Associazione Italiana per la Ricerca sul Cancro (AIRC) to BE.

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