Brief Definitive Report

Opposing Regulatory Effects of Thioredoxin and Eosinophil Cytotoxicity-enhancing Factor on the Development of Human Immunodeficiency Virus 1

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Summary

Exogenous recombinant human thioredoxin (rTRX, ≥500 nM), a dithiol reductase enzyme, inhibited the expression of human immunodeficiency virus (HIV) 1_{BaL} in human macrophages (Mø) by 71% (range, 26–100%), as evaluated by p24 antigen production and the integration of provirus at 14 d after infection. The stoichiometric reducing agent *N*-acetylcysteine (NAC) also inhibited HIV production, but to a lesser degree, and only at 30,000-fold higher concentrations. Exogenous rTRX is cleaved by Mø to generate the inflammatory cytokine, eosinophil cytotoxicity-enhancing factor (ECEF). In contrast to rTRX, rECEF (concentrations from 50 pM to 2 μM) enhanced the production of HIV by 67% (range, 33–92%). Thus, whereas TRX is a potent inhibitor of the expression of HIV in human Mø, cleavage of TRX to ECEF creates a mediator with the opposite effect. TRX also inhibited the expression of integrated provirus in the chronically infected OM 10.1 cell line, showing that it can act at a step subsequent to viral infection and integration.

As an intracellular dithiol reductase enzyme, thioredoxin (TRX) (13–14 kD) is thought to play a role in a number of oxidation reduction-dependent biochemical pathways (1). Human monocytes, lymphocytes, and other cell types also secrete TRX or related molecules (2–5) or display these species on the cell surface (4, 6). The extracellular TRX and related species exhibit cytokine-like activities, including the induction of IL-2 receptors on T-lymphocyte cell lines (7) and the enhancement of eosinophil cytotoxic function (4, 8, 9). With respect to eosinophil function, the most potent stimulus is a TRX species truncated at the COOH terminus (eosinophil cytotoxicity-enhancing factor [ECEF], 10 kD), that is devoid of dithiol reductase enzymatic activity (4, 8, 9).

In many cell types, the expression of mature HIV from integrated provirus is controlled by cellular oxidation-reduction status and/or cytokine stimulus. Oxidants or substances favoring the production of oxidants, including inflammatory cytokines, favor nuclear factor (NF)-κB activity and expression of HIV (10–12). Substances that suppress cellular levels of oxidants, including cysteine, *N*-acetylcysteine (NAC), glutathione, and ascorbic acid, suppress NF-κB activity and HIV expression (13, 14). It should be noted that intracellular levels and plasma concentrations of glutathione decrease in patients infected with HIV, and this decrease in glutathione is correlated with disease progression in AIDS (15, 16). For this reason, therapy with NAC (a glutathione precursor) has been suggested for AIDS patients (17, 18).

The relationship of HIV expression to cellular oxidation-reduction status and cytokine stimulus prompted us to investigate the effects of exogenous recombinant TRX/ECEF polypeptides on the growth of HIV_{BaL} in the human macrophages (Mø).

Materials and Methods

Recombinant TRX/ECEF Species. Recombinant TRX/ECEF species were generated in *Escherichia coli* as described (9). The two species chosen for evaluation were: (*a*) the full-length 104 amino acid TRX translation product with dithiol reductase activity but only weak cytokine activity (9); and (*b*) a form truncated after amino acid 80 with strong cytokine activity but no dithiol reductase activity (9). Endotoxin content of these preparations was <50 pg/ml at concentrations used for experiments, as assayed by the Limulus Amebocyte Lysate System (BioWhittaker Inc., Walkersville, MD).

To obtain metabolically radiolabeled rTRX (0.4×10^6 cpm/mg), TB1 cells transformed with p-MALc vector expressing rECEF/TRX-104 were grown to 2×10^8 cells/ml, stimulated with 0.3 mM isopropylthiogalactoside (Sigma Chemical Co., St. Louis, MO), and cultured with [^{35}S]methionine/cysteine (250 uCi/ml of Tran-^{35}S-Label; ICN Radiochemicals, Irvine CA). The fusion protein was purified by amylose resin-maltose binding pro-

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tein affinity chromatography, cleaved with factor Xa, and dialyzed in RPMI 1640, as previously done (9).

Culture of Cells and HIV Infection. As described (19), human Mø were derived by the culture of 10^7 peripheral blood mononuclear cells in IMDM (Hyclone Laboratories, Logan, UT) plus 10% pooled HIV-negative human male serum (Lampire Biological Laboratories, Pipersville, PA) in 25-cm^2 tissue culture flasks (Costar Corp., Cambridge, MA). After 3–5 d, the nonadherent cells were removed.

The Mø (99% pure as determined by nonspecific esterase staining) were infected, after a total culture period of 7 d, for 4 h with 0.1–1 infectious units of HIV_{BaL} (National Institutes of Health AIDS Research and Reference Reagent Program) per cell. After infection, the Mø were washed twice with HBSS (BioWhittaker, Inc.) and cultured in fresh medium in the presence or absence of rTRX/ECEF species. Medium was removed on days 2, 7, and 14 for the determination of HIV p24 antigen. At these time points, cells were either harvested for analysis of integrated provirus DNA or fed with fresh medium.

The OM 10.1 cell line, a derivative of the HL-60 myelomonocytic leukemia cell line with a single integrated copy of $HIV\text{-}1_{LAV}$ provirus (20), was generously provided by Dr. Salvatore Butera (Centers for Disease Control, Atlanta, GA). The cells were maintained in IMDM with 10% fetal bovine serum (Hyclone Laboratories). 4 d before the experimental treatments, the cells were treated with 10 μg/ml of 3'-azido-2'',3'-dideoxythymidine (AZT; Sigma Chemical Co.) to inhibit background expression of HIV. 1 d before experimental treatment, the cells were washed and resuspended in AZT-free medium. The next day, the cells were seeded at 10^6 in polypropylene culture tubes in 1 ml of medium alone, medium plus 100 nM PMA (Sigma Chemical Co.), or medium plus 100 U/ml TNF (Cetus Corp., Emeryville, CA), with or without 500 nM rTRX. The culture supernatants were harvested at 48 h for the determination of reverse transcriptase (RT) activity.

Parameters of HIV Infection. The production of mature HIV was determined by quantitative ELISA for the p24 core antigen in culture supernatants (p24 Core kit; DuPont-NEN Research Products, Boston, MA). Control experiments showed that the TRX/ECEF reagents did not interfere with the p24 antigen assays. For determination of RT activity, virus was precipitated from supernatants with 30% polyethylene glycol 8000 (Sigma Chemical Co.) overnight at 4°C. RT activity was assayed by a nonradioactive, colorimetric assay of the precipitate (RT-Assay No. 1468-120; Boehringer Mannheim, Indianapolis, IN).

For the determination of integrated proviral DNA, total DNA was extracted from Mø by the guanidinium thiocyanate/phenol/chloroform method (21), using Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH). 1 μg of isolated DNA was amplified by PCR, using the primer pairs SK100 and SK104 (22, 23). These primers bracket a highly conserved 290-bp region of HIV-1 *gag*. Quantitation of HIV-1 DNA was performed using the K4 deletion mutant, for which the primer pair brackets a 177-bp region, as a competitive internal standard (24). K4 was provided generously by Dr. David T. Scadden, New England Deaconess Hospital (Boston, MA). Control experiments showed that the internal standard and the wild-type gene were amplified at similar rates and that no amplified species could be generated from uninfected Mø.

Determination of rTRX Cleavage by Mø. Radiolabeled rTRX was added to washed, confluent-uninfected or 7-d HIV-infected Mø in 24-well tissue culture plates (Costar Corp.), at a concentration of 60 μg/ml (~5 μM). After incubation for 1 min or more, as described, the reagent was removed and the cells were rinsed once with ice-cold PBS. The Mø were then lysed by the addition of 33 μl/well of SDS-PAGE sample buffer, and lysates of three equivalent cultures (total volume, 100 μl) were analyzed by SDS-PAGE in a 15% reducing gel and autoradiography.

Results and Discussion

The Mø plays a central role in the pathogenesis of HIV infections. Transmission of HIV-1 infection from one individual to another appears to be primarily due to monocytotropic strains that do not induce formation of syncytia (25). The chronically infected Mø also serves as a constant reservoir for the virus (26). For these reasons, we selected the macrophage and the monocytotropic strain $HIV\text{-}1_{BaL}$ to investigate the effects of rTRX and rECEF.

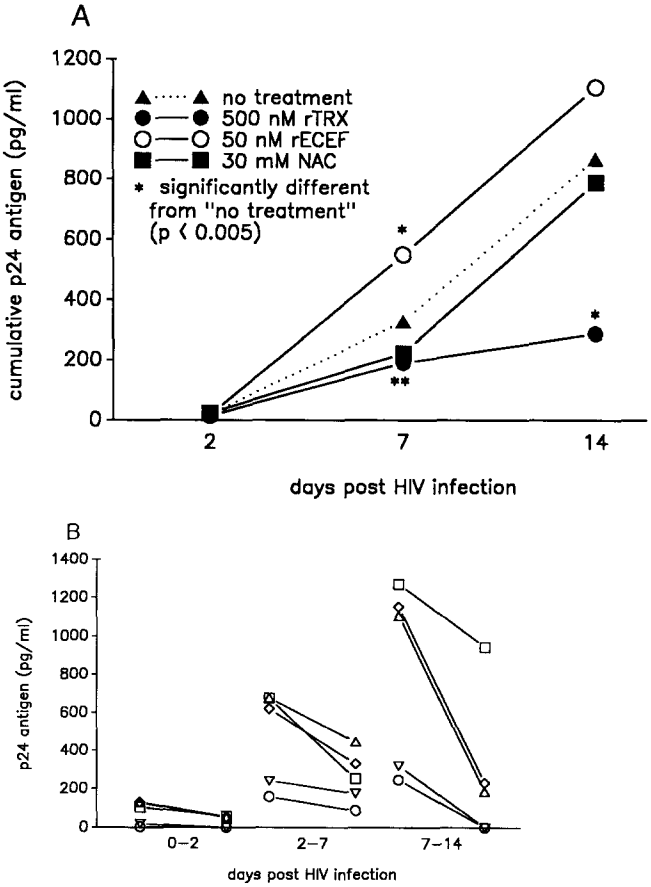

Figure 1. Regulation of HIV production in infected Mø by TRX/ECEF. Human Mø derived by 5–10-d culture of isolated blood monocytes were exposed to 0.1–1 infectious units per cell of $HIV\text{-}1_{BaL}$ for 4 h. After washing, the infected Mø were cultured with the indicated reagents. Medium was harvested on days 2, 7, and 14 for analysis of p24 antigen content. On days 2 and 7, the replacement medium contained fresh TRX/ECEF or NAC. (*A*) Representative experiment showing cumulative p24 antigen production under the different experimental conditions. Data are pooled from four experiments, with statistical analysis by the Wilcoxon signed-rank test. (*B*) Summary of five experiments (each symbol represents a different Mø donor) showing the effect of rTRX on HIV production within each time segment. The left data point in each pair shows p24 antigen produced by untreated cultures; the right data point shows production in cultures treated continuously with 500 nM rTRX.

Mø were exposed to HIV-1_{BaL} strain for 4 h washed in medium, and cultured with test substances as indicated. At optimal concentrations (≥0.5 μM), rTRX suppressed the expression of HIV by up to 100% (n = 5), as evaluated by p24 antigen content of the cultures. NAC (30 mM) had a modest suppressive effect, that was apparent at day 7, but not after longer incubations. In contrast, rECEF induced a 33–92% increase in virus expression (n = 4), that was most clear at day 7 (Fig. 1 *A*). The suppressive effect of rTRX on p24 antigen production was most prominent in the 7–14-d period after infection (Fig. 1 *B*). No cytopathic effects were observed under any of the conditions of treatment, using phase contrast microscopy.

The effects of the rTRX/ECEF species on HIV growth were characterized further by quantitative PCR analysis for the HIV-1 *gag* gene in cellular DNA extracts. Quantities of HIV-1 DNA generally correlated with p24 antigen production. In the example shown (Fig. 2), the amount of detectable HIV proviral DNA in rTRX-treated Mø was about fivefold lower than in rECEF-treated Mø. In other experiments, in which rTRX-treated Mø had no detectable p24 antigen, no HIV DNA was detectable at day 14 (control experiments showed that the threshold of detection in this system was ~10^4 HIV DNA copies). These results show that rTRX inhibited either the spread of HIV to uninfected cells or superinfection.

The suppressive effects of NAC and rTRX on HIV expression were lost at concentrations below 15 mM and 500 nM, respectively (Fig. 3). If both substances act by creating intracellular reducing conditions, the ~30,000-fold difference in dose requirements probably reflects the mechanistic differences between a stoichiometric reactant (NAC) and a catalyst (rTRX).

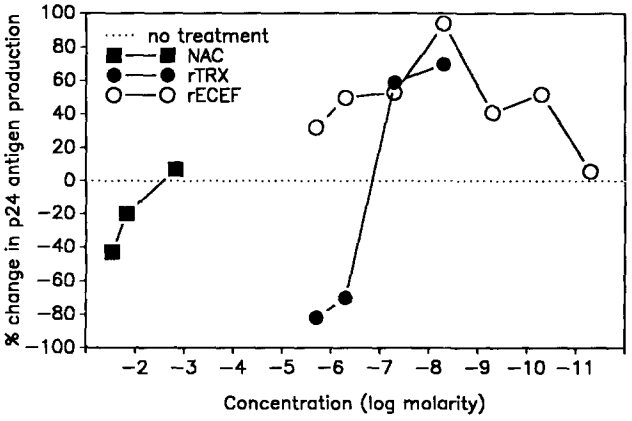

Figure 3. Dose dependence of regulatory effects. rTRX, rECEF, and NAC were studied for their effects on p24 antigen production at the indicated concentrations. Data are pooled from three experiments.

The enhancing effect of rECEF on HIV expression was detectable at concentrations as low as 50 pM, a similar potency to that observed in the assay of eosinophil cytotoxic function (9). In addition, lower concentrations of rTRX (5 and 50 nM) also enhanced HIV expression, in a manner similar to rECEF-80 (Fig. 3).

Under the conditions of the experiments described here, it is possible that the suppressive effect of rTRX on HIV expression was eroded by ongoing metabolic cleavage of rTRX to the truncated ECEF species. To test for cleavage, ^{35}S-radiolabeled rTRX was prepared and added to uninfected and HIV-1–infected Mø. Within 1 min, essentially all cell-associated rTRX was detected as a smaller 10-kD species, though recombinant maltose-binding protein was unaltered (Fig. 4). Analysis also showed that in the culture supernatant there was a great excess of uncleaved rTRX (data not shown). Furthermore, the suppressive effect on HIV expression could be

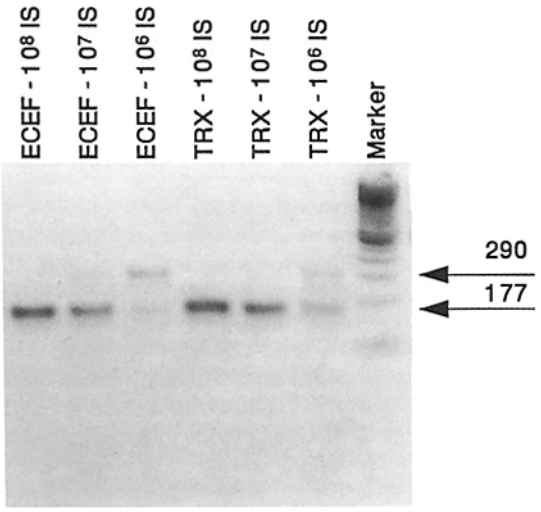

Figure 2. Effects of TRX/ECEF species on quantities of integrated HIV provirus. Under conditions similar to day 14 in Fig. 1 *A*, the levels of integrated DNA were analyzed by a competitive PCR and agarose gel (2%) electrophoresis. The species indicated by the 290-bp arrow represent amplified fragments of the *gag* gene from integrated HIV-1_{BaL} provirus. The species indicated by the 177-bp arrow represent amplified fragments of the titrated internal standard (IS). (*Top*) The number of IS copies added to the PCR reaction.

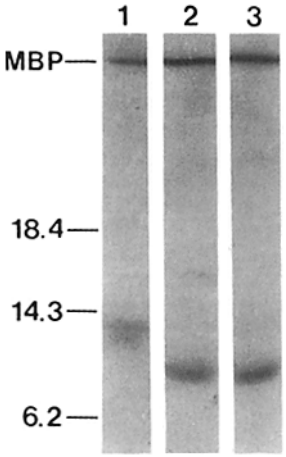

Figure 4. Cleavage of exogenous rTRX by uninfected and HIV-infected Mø. Human Mø derived by 7-d culture of isolated blood monocytes were exposed to the ^{35}S-radiolabeled rTRX reagent, also containing radiolabeled maltose-binding protein (MBP, the fusion protein partner). After 1 min, the Mø were rinsed briefly in ice-cold medium and lysed immediately with SDS-PAGE sample buffer. The lysate was analyzed by SDS-PAGE (15% gel) and autoradiography. (Lane *1*) Composition of the radiolabeled rTRX reagent. (Lanes *2* and *3*) Lysates of uninfected and HIV-infected Mø. Note that the MBP present in the Mø lysate was not altered. The adherent rTRX was converted to a 10-kD species.

maintained only by supplementing the medium with exogenous rTRX on a regular basis. It is thus likely that the low-dose enhancing effect of rTRX on HIV expression resulted from metabolic processing of rTRX to ECEF, under conditions when there was not a great excess of rTRX in the supernatant (i.e., concentrations below 500 nM).

To determine whether rTRX acts directly on virus particles or on the virus–Mø interaction, 500 nM rTRX was added directly to aliquots of HIV stock for 4 h. The rTRX-treated HIV stock was then added directly to the Mø. After a 4-h period of infection, Mø were washed and cultured in fresh medium. As measured at 7 and 14 d, there was no difference in p24 antigen production between rTRX-treated or untreated cultures, indicating that rTRX did not act on the virus directly or on the initial virus–cell interactions.

We wanted to determine whether rTRX could inhibit HIV expression at later stages of infection. When the addition of rTRX was delayed for 72 h after the infection of Mø, there was 22–83% inhibition of p24 antigen accumulated at 14 d, indicating that rTRX can act at a stage subsequent to the initial infection and integration.

To determine the effects of rTRX in a model of viral latency, we used the OM10.1 cell line. The OM10.1 cell contains a single integrated HIV provirus that is expressed at very low levels but induced by as much as 1,000-fold by various stimuli (20). We found that 500 nM rTRX suppressed both PMA- and TNF-induced HIV expression by 61 and 99%, respectively, as evaluated by RT activity at 48 h after induction. Since spread of the virus to uninfected cells or superinfection was not a significant factor during this brief period, the data show that rTRX acts by inhibiting a step in the life cycle subsequent to integration of the provirus. Since this experiment involved a different cell type and HIV-1 strain (HIV-1_{LAV}), the results suggest a broad spectrum of cell type and viral strain for the suppressive effect of rTRX.

Our findings demonstrate that the cleavage of TRX to ECEF is a potential controlling event in the life cycle of HIV, and indicate that rTRX deserves evaluation as a potential antiviral therapeutic agent.

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