Augmentation of Cytotoxic Effect of Tumor Necrosis Factor on Human Immunodeficiency Virus-infected Cells by Staurosporine, a Potent Protein Kinase C Inhibitor

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ABSTRACT

We have examined the effect of the protein kinase C (PKC) inhibitor, staurosporine, on tumor necrosis factor (TNF)-induced cytotoxic action and augmentation of human immunodeficiency virus (HIV) expression on the chronically HIV-infected T-cell line, MOLT-4/HIV (HTLV-IIB strain). Staurosporine enhanced the decrease in the number of viable cells caused by TNF treatment for 3 days (1 ng/ml of TNF, 43% decrease; 1 ng/ml of TNF + 20 nm staurosporine, 94%), whereas the cytotoxic action on that cell line induced by 10 ng/ml of 12-O-tetradecanoylphorbol-13-acetate (TPA), which was known to be an activator of PKC, was partially inhibited by staurosporine. In addition, staurosporine augmented the TNF cytotoxic activity against other cell lines including HIV-uninfected U937 cells (100 ng/ml of TNF, 53% decrease in the number of viable cells; 100 ng/ml of TNF + 5 nm staurosporine, 86%). However, staurosporine did not change the sensitivity of cells to TNF; thus, those insensitive to TNF were not changed to TNF sensitive by staurosporine. Furthermore, staurosporine did not affect the augmentative effect of TNF on HIV expression evaluated by levels of p24 antigen. Moreover, HIV long terminal repeat (LTR)-directed chloramphenicol acetyltransferase assay showed that staurosporine strongly inhibited the TPA-induced activation of HIV LTR, while that caused by TNF was little affected (10 ng/ml of TPA, 98.4% conversion; 10 ng/ml of TPA + 40 nm staurosporine, 22.2%, 1 ng/ml of TNF, 98.5%; 10 ng/ml of TNF + 40 nm staurosporine, 93.9%). These results suggest that TPA and TNF facilitate HIV replication by different pathways and that staurosporine augments TNF cytotoxicity by possible suppression of PKC activity in both HIV-infected and uninfected cells.

INTRODUCTION

We have previously reported that TPA facilitates HIV replication in chronically MOLT-4/HIV cells (1). Interestingly, this TPA effect was also associated with selective cytoidal effect on MOLT-4/HIV cells even at the concentrations at which uninfected MOLT-4 cells were not killed (1). Nabel and Baltimore (2) found that TPA augmented HIV replication through the induction of a DNA-binding protein, which bound to the viral enhancer, indistinguishable from NF-kB, one of the transcription factors. Since TPA is well recognized as a potent activator of PKC (3), it is generally believed that activation of PKC is involved in the induction of NF-kB by TPA. Further studies have demonstrated that PKC inhibitors can block the TPA-augmented HIV expression (4, 5).

Recently, it was shown that cytokines including LT and TNF also augmented HIV replication (6–10). Furthermore, we have demonstrated that LT and TNF not only enhance HIV expression but, like TPA, also selectively kill HIV-infected cells (6, 10). Little is known about the mechanism of TNF cytotoxic action on HIV-infected cells. However, it is well established that the activation of the HIV enhancer by the NF-kB-like molecule is involved in the TNF-induced augmentation of HIV expression as well as in that by TPA (8, 9).

In this study we have examined whether the cytotoxic action of TNF on HIV-infected cells and the stimulatory effect of TNF on HIV expression are associated with PKC activation by using one of the most potent PKC inhibitors, staurosporine (5, 11).

MATERIALS AND METHODS

Chemicals. Human recombinant TNF, provided by Dr. D. Mizuno (Biotechnology Research Center, Teikyo University), was purified from Escherichia coli carrying the TNF-Sam gene as reported previously (12). The specific activity of the TNF used in this study, determined by using 1,929 cells, was 2.8 × 10^6 units/mg. TPA (Sigma Chemical Co., St. Louis, MO) and staurosporine (Kyorwa Medex Co., Tokyo, Japan) were prepared as stock solutions in dimethyl sulfoxide (TPA, 1 mg/ml; staurosporine, 1 mm). In all experiments, staurosporine was added 10 min before the addition of TNF or TPA.

Cell Lines. MOLT-4 (13), MOLT-4/HIV (MOLT-4 chronically infected with HIV), CCRF-CEM (14), CCRF-CEM/HIV, Jurkat (15), Jurkat/HIV, H9 (16), H9/HIV, MT-4 (17), MT-4/HIV, U937 (18), and U937/HIV cells were used in this study. All cell lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Each chronically HIV-infected cell line was established in our laboratory by infection with the HTLV-IIB strain of HIV (19), and the percentage of HIV antigen-positive cells tested by the indirect immunofluorescence method (19) exceed 80% in all lines.

Cytocidal/Cytostatic Assay. Cells (3 × 10^5 cells/ml) were cultured in 2 ml of medium with or without various concentrations of chemicals in 12-well plates (Corning). The viable cell number was calculated at indicated times by the trypan blue dye exclusion method. In one specific experiment (Fig. 3), we evaluated the cell growth by the MTT assay as previously reported (20). In brief, 1 × 10^5 cells were cultured in a 96-well plate with 100 μl of medium for 3 days in the presence of various concentrations of chemicals. Ten μl of MTT (5 mg/ml) was added to each well and incubated for 2 h at 37°C. Acid isopropanol (100 μl) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. Plates were read with Easy Reader (EAR400FW; SLT-Lab Instruments, Salzburg, Austria), using a test wavelength of 550 nm and a reference wavelength of 690 nm.

Assay for HIV p24 Antigen. HIV p24 antigen, in a cell-free culture medium of MOLT-4/HIV cells, was determined by an enzyme immunoassay kit (Abbott Laboratories) according to the manufacturer's instructions.

CAT Assay. HIV LTR-directed CAT assay was performed as described previously (21). In brief, 5 μg of pH3LTRCAT, which contains −451 to +185 of LTR of HIVm fused to the bacterial CAT gene (22), was transfected to 2 × 10^6 of MOLT-4/HIV cells by the DEAE-dextran method. Twenty-four h after transfection, cells were further incubated for 16 h in the presence of various substances. At the end of incubation, cells were lysed by 100 μl of 0.5% NP-40 in 0.25 M Tris-Cl (pH 8.0) buffer. Cell lysates were prepared by 3 cycles of freezing (−80°C) and
PROTEIN KINASE INHIBITOR-AUGMENTED TNF CYTOTOXICITY

RESULTS

Effect of Staurosporine on TPA- or TNF-induced Cytotoxic Action. We examined the effect of staurosporine on cytotoxicity of TPA or TNF in chronically HIV-infected cells. As shown in Fig. 1, 10 ng/ml of TPA reduced the number of viable MOLT-4/HIV cells to about 45% of untreated cells on day 3 after treatment (Fig. 1, left). Staurosporine was able to partially restore this reduction of the viable cell number by TPA in a dose-dependent manner. In contrast, staurosporine strongly augmented the cytotoxic action of TNF rather than inhibit it in this cell line (Fig. 1 right). This augmentative effect of staurosporine on TNF-induced cytotoxicity was observed at various concentrations of TNF (0.1, 1, and 10 ng/ml) in response to doses of staurosporine (Fig. 2). The enhancement of TNF cytotoxic action did not seem to be caused by direct toxicity of the PKC inhibitor, because the cell growth was not inhibited by treatment with staurosporine alone at the concentrations used in the experiment (Fig. 2).

To assess whether the augmentation of TNF-induced cytotoxic/cytostatic effect would be limited to only MOLT-4/HIV cells, we next examined the effect of staurosporine on TNF-insensitive MOLT-4 cells and TNF-sensitive U937 cells (Fig. 3). It is clear from this figure that the cytotoxic action of TNF on U937 cells was enhanced by staurosporine in a dose-dependent manner. However, MOLT-4 cells remained resistant to TNF even though they were treated with 20 nm staurosporine. This phenomena was further confirmed with several other cell lines. Various cell lines listed in Table 1 were cotreated with 100 ng/ml of TNF and 5 nm staurosporine for 3 days. Under these experimental conditions, 5 nm staurosporine alone did not affect the cell growth in all cell lines used (Table 1, Column 3). Among these cell lines, U937, Jurkat, Jurkat/HIV, CCRF-

Fig. 1. Comparison of the effects of staurosporine on TNF- or TPA-induced cytotoxic/cytostatic activity on MOLT-4/HIV. MOLT-4/HIV cells (3 x 10^5 cells/ml) were cultured in the presence of 10 ng/ml of TPA (Left) or TNF (Right) in combination with various concentrations of staurosporine (Ø, 0; △, 2.5; ∆, 5.0; ■, 10.0; ▲, 20.0 nm). On each day the number of viable cells was counted by the trypan blue dye exclusion method. The number of viable cells in reagent-free control culture is also shown (Ø). The data represent the average (Points) and range (Bars) of duplicate determinations.

Fig. 2. Augmentation of TNF cytotoxic effect by staurosporine in MOLT-4/HIV cells. MOLT-4/HIV (3 x 10^5 cells/ml) were cultured in the presence of various concentrations of TNF (Ø, 0, Ø, 0.1, 1; T, 10 ng/ml) in combination with various concentrations of staurosporine. On day 3 the number of viable cells was counted and the relative viable cell number was compared to MOLT-4/HIV, which was cultured in medium alone. The viable cell number of control MOLT-4/HIV cells was 2.2 x 10^6 cells/ml. The data represent the Average (Points) and range (Bars) of duplicate determinations.

Fig. 3. Effect of staurosporine on TNF-induced growth inhibition in TNF-sensitive U937 cells and TNF-insensitive MOLT-4 cells. MOLT-4 (Ø) and U937 (Ø) cells were cultured in the presence or absence of 10 ng/ml of TNF together with various concentrations of staurosporine. The growth of cells was evaluated by MTT assay as described in "Materials and Methods." The relative growth was determined by the ratio of A_{50} value which was calculated as follows:

Relative growth = \frac{A_{50} of well in the presence of TNF}{A_{50} of well in the absence of TNF}

Data are means (Points) and ranges (Bars) of two independent experiments, each of which was done in quadruplicate and whose SD was always <10% of the means.

The CEM/HIV, U937/HIV, H9, and CCRF-CEM cells were sensitive to the cytotoxic activity of TNF to varying degrees (Table 1, Column 2). Considerable augmentation of TNF cytotoxicity by staurosporine was observed in all of these cell lines except for Jurkat/HIV cells (Table 1, Columns 4 and 5). In contrast, the cell lines which were not susceptible to the TNF activity were not affected by treatment with staurosporine (MT-4, MT-4/HIV, H9/HIV, and MOLT-4). Up to 20 nm staurosporine failed to cause cell death in TNF-insensitive cell lines (data not shown).

Effect of Staurosporine on TNE-induced Augmentation of HIV Expression. Next, in order to evaluate the effect of staurosporine on TNF-induced augmentation of HIV expression, we determined the amount of p24, an HIV gag gene product, in cell-free culture supernatants. The level of p24 released from MOLT-4/HIV cells treated with 1 ng/ml of TNF or TPA was nearly 4-fold higher in comparison to untreated cells (Fig. 4).
Table 1  Effect of staurosporine on TNF-induced cytotoxic effect in various cell lines

Cells (3 × 10^6 cells/ml) were cultured in medium with or without 100 ng/ml of TNF in the presence or absence of 5 nM staurosporine. On day 3 after culture the viable cell number was determined by the trypan blue dye exclusion method. The data represent means of duplicate determinations whose ranges were always <10% of the average.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>No. of viable cells 10^6 cells/ml (relative no.)</th>
<th>Augmentation index a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium</td>
<td>TNF</td>
</tr>
<tr>
<td>U937</td>
<td>177 (1.00)</td>
<td>84 (0.47)</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>230 (1.00)</td>
<td>190 (0.83)</td>
</tr>
<tr>
<td>U937/HIV</td>
<td>159 (1.00)</td>
<td>105 (0.66)</td>
</tr>
<tr>
<td>Jurkat</td>
<td>191 (1.00)</td>
<td>119 (0.55)</td>
</tr>
<tr>
<td>CCRF-CEM/HIV</td>
<td>281 (1.00)</td>
<td>173 (0.62)</td>
</tr>
<tr>
<td>H9</td>
<td>178 (1.00)</td>
<td>123 (0.69)</td>
</tr>
<tr>
<td>Jurkat/HIV</td>
<td>211 (1.00)</td>
<td>116 (0.55)</td>
</tr>
<tr>
<td>MOLT-4</td>
<td>200 (1.00)</td>
<td>200 (1.00)</td>
</tr>
<tr>
<td>H9/HIV</td>
<td>122 (1.00)</td>
<td>113 (0.93)</td>
</tr>
<tr>
<td>MT-4/HIV</td>
<td>99 (1.00)</td>
<td>93 (0.92)</td>
</tr>
<tr>
<td>MT-4</td>
<td>147 (1.00)</td>
<td>135 (0.92)</td>
</tr>
</tbody>
</table>

a) SS, staurosporine.

b) Augmentation index (AI) was calculated as follows:

\[ AI = \frac{\text{viable cell number}_{\text{control}}}{\text{viable cell number}_{\text{SS}+\text{TNF}}} \times \frac{\text{viable cell number}_{\text{SS}+\text{TNF}}}{\text{viable cell number}_{\text{control}}} \]

Numbers in parentheses, relative cell numbers to the untreated control.

Fig. 4. Effect of staurosporine on TPA- or TNF-induced augmentation of HIV expression in MOLT-4/HIV cells. MOLT-4/HIV cells (3 × 10^6 cells/ml) were cultured in the presence of 1 ng/ml of TPA (C), TNF (B), or medium alone (A) with or without various concentrations of staurosporine for 24 h. At the end of culture, culture supernatants were harvested and passed through 0.22-μm filters, and then assayed for p24. Data are means (Points) and ranges (Bars) of duplicate determinations. The amount of p24 in the control supernatant (cultured with medium alone) was 54 ng/ml.

The increase in p24 caused by TPA was inhibited by staurosporine in a dose-dependent manner which was consistent with our previous report (5), whereas that induced by TNF was not inhibited by treatment with staurosporine (Fig. 4). The level of p24 released from MOLT-4/HIV cells treated with staurosporine alone was only little decreased as the dose of staurosporine was increased. Further evidence that staurosporine affected the regulation of HIV expression by TPA but not by TNF was obtained with an experiment using an HIV LTR-directed CAT assay (Fig. 5). It is clear from this figure that staurosporine can inhibit TPA-caused enhancement of HIV LTR activation in a dose-dependent manner as expected (4), while the augmentation induced by TNF was affected only slightly by the same treatment at higher concentrations of staurosporine.

DISCUSSION

We have previously reported that TPA (1), LT (6), and TNF (10) enhance HIV replication and preferentially kill cells chronically infected with HIV as compared to noninfected cells. Since TPA is a substance which is rarely encountered under natural conditions in vivo, the action of physiological substances such as TNF in the regulation of the immune system is of more interest to examine. Although the role of TNF in the regulation of the immune system remains unclear, our finding partially suggests that the pathways to induce the NF-κB-like molecule are different from those to induce TPA.

Fig. 5. Effect of staurosporine on TPA- or TNF-induced augmentation of HIV LTR activation. Transfected MOLT-4/HIV cells with p3H-LTRCAT as described in Materials and Methods were incubated with medium alone (A: Lanes 1–4), TPA (B: Lanes 1–4, 1 ng/ml; Lanes 5–8, 10 ng/ml), or TNF (C: Lanes 1–4, 1 ng/ml; Lanes 5–8, 10 ng/ml) in the presence of 0 (Lanes 1 and 5), 10 nM (Lanes 2 and 6), 20 nM (Lanes 3 and 7), or 40 nM (Lanes 4 and 8) of staurosporine for 16 h. Percentages of conversion of acetylated chloramphenicol (AcCM) are listed.
by TPA and TNF would be different, based on their observation that TPA and TNF synergistically augment HIV replication.

In the present study we used a potent PKC inhibitor, staurosporine, to investigate the role of PKC on the TNF-induced augmentation of HIV replication and cytotoxic activity. The data in this communication clearly demonstrate that TNF might induce a NF-kB-like molecule in the PKC-independent pathway. Our data also show that the cytotoxic activity of TNF is separate from its activity to augment HIV replication because suppression of PKC activity modulated the cytotoxic action of TNF but did not affect the expression of HIV. The mechanism of the augmentation of the cytotoxic activity of TNF by staurosporine is not clear. However, we showed that it was not due to the increased binding of TNF to cells, since treatment of cells with PKC inhibitors did not affect the binding of TNF to the cells (data not shown). To explain the mechanism we tentatively suggest that there is a cellular protective molecule against the cytotoxic action of TNF. If the protective molecule was induced or synthesized in a PKC-dependent manner, inhibition of PKC activity could lead to inactivation of the protective molecule, resulting in the augmentation of the activity of TNF. Indeed, it has been reported recently that phosphorylation of three M, 28,000 proteins in bovine aortic endothelial cells may be responsible for the resistance of these cells to TNF (27).

However, the precise roles of the PKC system in HIV-infected cells are unknown and further studies are essential to clarify this question.

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REFERENCES


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