Tumor Necrosis Factor-Dependent Production of Human Immunodeficiency Virus 1 in Chronically Infected HL-60 Cells

By Kiyoshi Kitano, Coralia I. Rivas, Gayle Cocita Baldwin, Juan Carlos Vera, and David W. Golde

Tumor necrosis factor (TNF) may play a central role in proviral activation and release from latency in cells infected with the human immunodeficiency virus (HIV). We studied viral production and its relation to TNF in a HL-60 cell line (J22-FL) infected with a monocytotropic strain of HIV-1 Jr.FL. Viral production was stimulated to similar levels by TNF, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), and 1,25-dihydroxyvitamin D3 (1,25(OH)2D3). Production of the virus was not suppressed by 3-azido-3-deoxythymidine (AZT), indicating that viral production was not caused by superinfection. Low concentrations of TNF (0.1 ng/mL) induced viral production with a short lag period of 8 hours, and this proviral activation was specifically suppressed by anti-TNF antibodies. However, induction of virus production by 1,25(OH)2D3 showed an extended lag period of 2 to 3 days. The effect of 1,25(OH)2D3 on virus production was also blocked by anti-TNF antibodies, which suggests the direct participation of TNF in this process. TNF accumulated in the culture supernatant of cells stimulated with 1,25(OH)2D3 with a kinetics consistent with its involvement in the action of 1,25(OH)2D3 on viral production. The J22-FL cell line produced low levels of virus when cultured in the absence of an external stimulus; however, this basal viral production was suppressed greater than 80% in the presence of anti-TNF antibodies. Corresponding low levels of TNF were detected in the culture supernatants. Viral production decreased slowly with increasing passage of the cells, and no virus was detected in the supernatants of cells maintained in culture for several months. Concomitantly, TNF was no longer detected in the supernatant of these cells, which suggests that endogenous autocrine production of TNF drives viral production in the unstimulated cells. However, viral production was stimulated in these cells by low concentrations (0.1 ng/mL) of added TNF. These results argue for a central role for TNF in HIV proviral activation in chronically infected myeloid cells.

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Submitted December 21, 1992; accepted June 30, 1993.

Supported by Grants No. R37 CA30388, RO1 HL42107, and NCI-P30-CA08748 from the National Institutes of Health, by Memorial Sloan-Kettering Cancer Center Institutional funds, and by a Grant-in-Aid for Scientific Research on Priority Areas by The Ministry of Education, Science and Culture of Japan.

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MATERIALS AND METHODS

Cells. The establishment of a HL-60 cell line (J22-FL) infected with a monocytotropic strain of HIV-1 Jr.FL and containing a...
single, clonally integrated HIV virus has been previously described.\textsuperscript{24} The JRFL primary isolate was reported in 1987.\textsuperscript{37} We used frozen stocks from this isolate expanded briefly in phytohemagglutinin (PHA)-treated normal T lymphocytes to infect the HL-60 cells and isolate the J\textsubscript{Zz} clone. The cells were cultured in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% glutamine, and antibiotics. The medium was changed every 3 days. Unless otherwise indicated, cells were suspended at a concentration of 1 x 10\textsuperscript{5} cells/mL. Cell viability was assessed by exclusion of trypan blue.

Agent, growth factors, and antibodies. Human recombinant TNF-\textalpha was obtained from R&D Systems (Minneapolis, MN). 25(\OH)\textsubscript{2}D\textsubscript{3}, TPA, and 3'-azido-3'-deoxythymidine (AZT) were all purchased from Sigma Chemical Co (St Louis, MO). A polyclonal anti-human TNF-\textalpha neutralizing antibody (raised in goats, affinity purified IgG fraction) was obtained from R&D Systems. This antibody, at a concentration of 1 \mu g/mL, inhibits 100% of the biologic activity of 0.25 ng/mL of TNF-\textalpha. A second anti-TNF-\textalpha antibody obtained from Boehringer-Mannheim Corporation (Indianapolis, IN) was also used in some of the experiments with similar pharmacologic activity of 0.25 ng/mL of TNF-\textalpha. A control sample incubated with medium alone is also shown (0). The antibody used in this experiment was a polyclonal antibody (IgG fraction) obtained from R&D Systems, and similar results were observed. To test for the specificity of TNF-\textalpha action, cells were incubated in the presence of both maximally stimulatory concentrations of TNF-\textalpha and blocking TNF-\textalpha antibodies. As expected, TNF-\textalpha-induced viral production was specifically and totally suppressed by the anti-TNF antibodies (Fig 2A). At least two other different TNF-\textalpha antibodies were used in these experiments with similar

\textbf{Virus measurement.} To measure virus production, 1 x 10\textsuperscript{5} J\textsubscript{Zz}-HL-60 cells were cultured under the conditions described (Figs 1-7). Cell culture supernatants were assayed for the presence of HIV p24 core antigen using a commercially available p24 capture enzyme-linked immunosorbent assay (ELISA) kit (Coulter HIV-1 p24 antigen kit; Coulter Corp., Hialeah, FL).

\textbf{TNF-\textalpha measurement.} TNF-\textalpha was measured in cell culture supernatants using a commercially available ELISA kit (R&D Systems).

\textbf{RESULTS}

When J\textsubscript{Zz}-HL-60 cells were cultured in the presence of 1 ng/mL of TNF-\textalpha for varied periods of time, a major increase in viral production was observed as compared with control cells (Fig 1A). As a positive control for viral production, J\textsubscript{Zz}-HL-60 cells were stimulated with TPA. Viral production induced by TNF-\textalpha was quantitatively equivalent to that induced by a maximally stimulatory concentration (1 \mu mol/L) of TPA (Fig 1A). The kinetics of accumulation of virus in the cell culture supernatants was also similar for the two agents, with maximal accumulation observed 24 hours after initiating the culture (Fig 1A). These studies indicated that J\textsubscript{Zz}-HL-60 cells were highly sensitive to the action of TNF-\textalpha, with a substantial increase in viral production observed at concentrations of TNF-\textalpha as low as 30 pg/mL (Fig 1B). Maximal viral production was observed at 100 pg/mL, with a 50% effective dose (ED\textsubscript{50}) of about 60 pg/mL. Further studies indicated that the kinetics of viral production was similar for all concentrations of TNF-\textalpha tested, from 0.1 to 30.0 ng/mL (data not shown). Similar results were observed with TPA at concentrations ranging from 0.3 to 100.0 \mu mol/L (data not shown). To test for the specificity of TNF-\textalpha action, cells were incubated in the presence of both maximally stimulatory concentrations of TNF-\textalpha and blocking TNF-\textalpha antibodies. As expected, TNF-\textalpha-induced viral production was specifically and totally suppressed by the anti-TNF antibodies (Fig 2A). At least two other different TNF-\textalpha antibodies were used in these experiments with similar

\begin{figure}[h]
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\caption{(A) Kinetics of HIV-1 production by J\textsubscript{Zz}-HL-60 cells treated with TPA or TNF-\textalpha. J\textsubscript{Zz}-HL-60 cells were cultured in the presence of 1 \mu mol/L TPA (C), 0.1 ng/mL of recombinant human TNF-\textalpha (\#), or medium alone (\*). At the indicated times, culture supernatants were harvested and stored at \textdegree \text{-70} before being tested for the presence of the HIV-1 p24 core antigen by using a capture ELISA kit (see Materials and Methods section). (B) Dose response of HIV-1 production to TNF. J\textsubscript{Zz}-HL-60 cells were cultured for 24 hours in the presence of the indicated concentrations of TNF-\textalpha, and culture supernatants were harvested and analyzed for the presence of the HIV-1 p24 core antigen. Data are presented as nanograms of p24 core antigen per milliliter of supernatant and correspond to the mean of triplicate experiments.}
\end{figure}

\begin{figure}[h]
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\caption{(A) Effect of anti-TNF-\textalpha antibodies on HIV-1 production in response to TNF-\textalpha. J\textsubscript{Zz}-HL-60 cells were cultured in triplicate for 24 hours in medium containing 0.1 ng/mL of TNF-\textalpha in the presence of the indicated concentrations of anti-TNF-\textalpha (\#) antibody. A control sample incubated with medium alone is also shown (0). The antibody used in this experiment was a polyclonal antibody (IgG fraction) obtained from R&D Systems, and similar results were obtained using monoclonal TNF-\textalpha antibodies obtained from Boehringer-Mannheim (see Materials and Methods section). (B) Effect of different antibodies on the determination of the HIV-1 p24 core antigen by ELISA. Antibodies (1 \mu g/mL) were added to cell culture supernatants containing known amounts of commercially available (Coulter) HIV-1 p24 core antigen before measuring the presence of the p24 antigen. The following antibodies were tested: anti-TNF-\textalpha (R&D Systems) (C), anti-IL-5 (R&D Systems) (\textcircled{3}). A control sample (\#) contained medium alone. Results are expressed as millioptical density units/min per milliliter of supernatant and are presented as the mean of duplicate experiments.}
\end{figure}
results (data not shown). A potential problem with these experiments was the possibility that the TNF-α antibodies present in the samples could interfere with the capture ELISA system used for the quantitation of the HIV p24 core antigen. A control experiment was performed in which different antibodies were added to a culture supernatant containing known amounts of exogenously added HIV p24 core antigen. As can be seen in Fig 2B, the presence of the antibodies did not affect the quantitative determination of the HIV p24 core antigen, which confirmed the specificity of the inhibitory activity of the anti-TNF-α antibodies on viral production.

AZT was used to test for superinfection in cells cultured in the presence of TNF-α. As shown in Fig 3A, AZT did not affect viral production induced by TNF-α, ruling out the possibility of further production of virus through the active superinfection of cells during the experiment. This was an important control, because the J22-HL-60 cells replicated actively over the course of the experiment, with a doubling time of about 18 hours (Fig 3B). No effect of TNF-α on cell viability (data not shown) or cell proliferation (Fig 3B) was observed in these experiments relative to control cells cultured in the presence of medium alone. At the concentrations used AZT did not affect cell proliferation, as assessed by thymidine uptake experiments (data not shown).

1,25(OH)2D3 is a well-known differentiating agent for myeloid cells,38 and shows effects similar to TNF-α on the differentiation of HL-60 cells.34-41 No viral production was observed during the first 2 days of culture (Fig 4A) when J22-HL-60 cells were incubated in the presence of 1,25(OH)2D3. Viral accumulation was only evident after 3 days of incubation and reached final levels somewhat lower than those observed in cells stimulated with TNF-α. The delayed kinetics of 1,25(OH)2D3-induced viral production contrasted with the detection of significant amounts of viral particles in the culture supernatants in the first 8 hours after stimulation with TNF-α (Fig 4B). In studies of the dose dependence of viral production, 1,25(OH)2D3 at 0.1 μmol/L induced substantial accumulation of virus in the culture supernatants (Fig 4C). Maximal virus production was observed at 1 μmol/L 1,25(OH)2D3, with an ED50 of 0.3 μmol/L. Further analysis indicated that virus production in cells treated with 1,25(OH)2D3 was specifically suppressed at least 60% by anti–TNF-α antibodies (Fig 5A), which suggests a role for TNF-α in the action of 1,25(OH)2D3. To rigorously prove that TNF-α was involved in the production of virus induced by 1,25(OH)2D3, culture supernatants of cells incubated in the presence of 1,25(OH)2D3 for variable times were analyzed for the presence of soluble TNF-α. As shown in Fig 5B, TNF-α was detected in the culture supernatants of cells incubated for at least 2 to 3 days in the presence of 1,25(OH)2D3. The concentrations of TNF-α detected were sufficiently elevated as to be able to induce viral production (see for comparison Fig 2).

We also observed a small but measurable amount of virus produced by J22-HL-60 cells cultured in the absence of any external stimulus, and the capacity of the unstimulated cells to produce virus decreased with the number of cell passages (Fig 6A). Thus, no virus was detected in the supernatant of cells continuously cultured for at least 3 months in the absence of any stimulus (Fig 6A). However, these cells were able to respond to TNF-α with the production of virus at levels equivalent to those observed with fresh cultures (Fig 6B). Also, there was no change in the kinetics or the dose dependence of viral production induced by TNF-α. Maximal viral production was observed 24 hours after initiating the culture in response to 0.1 ng/mL of TNF-α (data not shown). We then tested the effect of anti–TNF-α antibodies on the production of virus by unstimulated cells. As shown in Fig 7A, anti–TNF-α antibodies suppressed production of virus by at least 60%. These observations prompted us to test for the presence of TNF-α in the culture supernatants of cells cultured for different periods of time. As shown in Fig 7B, soluble TNF-α was observed in the culture supernatants of J22-HL-60 cells maintained in culture for less than 2 months. Decreased levels of TNF-α were detected in the supernatant of cells maintained in continuous culture for longer periods of times (Fig 7B), an observation that correlates well with the lack of viral production by these cells.

**DISCUSSION**

TNF and TPA are strong differentiating agents for myelomonocytic leukemic cell lines.41-43 Cytochemical differentiation into monocytes and macrophages is detectable after treatment with TNF-α or TPA for 3 days or 24 hours, respectively.34,42,43 In our studies, TNF-α and TPA were found to enhance virus production in J22-HL-60 cells within 12 hours. This result suggests that the effect of TNF-α on virus production may not be related solely to cell differentiation. Although it has been reported that HIV expression increases during monocyte maturation in association with an increase in expression of the transcription factor NFκB,20 it is clear that TNF-α or TPA may increase expression of NFκB without cell maturation. Expression of NFκB mRNA in HL-60 cells was enhanced 1 hour after exposure to TNF-α or TPA.44 Our data indicate that TNF-α or TPA stimulates
TNF, VITAMIN D₃, AND HIV PRODUCTION

Fig 4. (A) Kinetics of HIV-1 production in response to 1,25(OH)₂D₃. J₂,-HL-60 cells were cultured in the presence of 0.1 μmol/L of 1,25(OH)₂D₃ (●) or medium alone (○). Culture supernatants were harvested at 0, 6, 12, 24, 48, 72, 96, and 120 hours and analyzed for the presence of the HIV-1 p24 core antigen. (B) Short-term analysis of HIV-1 production in response to 1,25(OH)₂D₃ or TNF-α. J₂,-HL-60 cells were cultured in the presence of 0.1 μmol/L 1,25(OH)₂D₃ (●) or 0.1 ng/mL of TNF-α (○). Culture supernatants were harvested at 0, 3, 4, 6, 8, 10, 12, and 18 hours and analyzed for the presence of the HIV-1 p24 core antigen. (C) Dose response of HIV-1 production to 1,25(OH)₂D₃. J₂,-HL-60 cells were cultured for 48 (●) and 72 (○) hours in the presence of the indicated concentrations of 25(OH)₂D₃, and culture supernatants were harvested and analyzed for the presence of the HIV-1 p24 core antigen. Data are presented as nanograms of p24 core antigen per milliliter of supernatant and correspond to the mean of triplicate experiments.

HIV production early in the differentiation process of myeloid precursors along the mononuclear phagocyte lineage.

1,25(OH)₂D₃ also induces differentiation of immature HL-60 cells along the myelomonocytic pathway.⁴⁻⁶ Viral production induced by treatment with 1,25(OH)₂D₃ required more than 48 hours, which suggests a role for cell differentiation in this process. This delay in viral production is consistent with our previous study indicating that virus elaboration was associated primarily with the mature, differentiated population of monocytes and was dissociated from cell proliferation.⁴ Surprisingly, a substantial proportion (>60%) of the effect of 1,25(OH)₂D₃ on HIV-1 production was blocked by TNF-α antibodies, and TNF-α accumulated in a time-dependent fashion in the supernatant of cells stimulated with 1,25(OH)₂D₃. In addition, the kinetics of accumulation of TNF-α preceded the kinetics of viral production. These results strongly suggest that production of TNF-α is an important step in the induction of HIV-1 production after treatment of J₂,-HL-60 cells with 1,25(OH)₂D₃.

We also examined the effect of an antiviral agent, AZT, on viral production. AZT had no effect on HIV production in J₂,-HL-60 cells. This result is consistent with the concept that AZT, a specific inhibitor of the viral reverse transcriptase,⁴⁵ is not effective once the HIV provirus is integrated into host cell chromosomal DNA. Thus, our data suggest that treatment of the HIV-infected cells with TNF-α or 1,25(OH)₂D₃ increases viral production via enhanced provirus transcription as opposed to viral replication.

Our data also indicate that the basal production of virus in cells not subjected to any external stimulus is because of an autocrine mechanism involving the intrinsic production of TNF-α by the infected cells. It was also possible to main-

Fig 5. (A) Effect of anti–TNF-α antibodies on HIV-1 production in response to 1,25(OH)₂D₃. J₂,-HL-60 cells were cultured in medium alone (○), in medium containing 0.1 μmol/L 1,25(OH)₂D₃ (●), or in medium containing 0.1 μmol/L 1,25(OH)₂D₃ and 1 μg/mL of anti–TNF-α antibodies (●). Culture supernatants were harvested at the times indicated and assayed for the presence of the HIV-1 p24 core antigen. Data are presented as nanograms of p24 core antigen per milliliter of supernatant and correspond to the mean of triplicate experiments. (B) Production of TNF-α in response to 1,25(OH)₂D₃. J₂,-HL-60 cells were cultured for variable periods of time in the presence (●) or the absence (○) of 0.1 μmol/L 1,25(OH)₂D₃, and the respective supernatants were assayed for the presence of TNF-α by ELISA. Data are presented as nanograms of TNF-α per milliliter of supernatant and correspond to the mean of two experiments.

Fig 6. (A) Production of HIV-1 by unstimulated J₂,-HL-60 cells. Cells maintained in continuous culture for different periods of time (from 1 week to 6 months) were fed fresh medium and incubated for an additional 24 hours. Culture supernatants were harvested and assayed for the presence of the HIV-1 p24 core antigen. (B) Production of HIV-1 by J₂,-HL-60 cells stimulated with exogenous TNF-α. Cells maintained in continuous culture for different periods of time were fed fresh medium containing 0.1 ng/mL of TNF-α and incubated for an additional 24 hours. Culture supernatants were harvested and assayed for the presence of the HIV-1 p24 core antigen. Data are presented as nanograms of p24 core antigen per milliliter of supernatant and correspond to the mean of triplicate experiments.
described that signaling through the TNF receptor involves a multiprotein complex and a cascade of phosphorylation-dephosphorylations. Additional evidence for the existence of a sphingomyelin pathway and its involvement in the activation of NFKB has been recently provided. We have also obtained evidence indicating that a pathway able to recapitulate the basic characteristics of the mechanism of action of TNF (involving hydrolysis of sphingomyelin to ceramide and the production of TNF) is active in inducing viral production in the J22–HL-60 cells (Rivas et al, submitted). The availability of the J22–HL-60 cells offers a unique opportunity to identify intermediary molecules involved in the pathway that leads from TNF to activation and production of HIV under controlled experimental conditions.

Fig 7. (A) Effect of anti–TNF-α antibodies on the production of HIV-1 by unstimulated J22–HL-60 cells. J22–HL-60 cells were incubated for 24 hours in the presence of increasing concentrations of anti–TNF-α antibodies, and culture supernatants were harvested and assayed for the presence of the HIV-1 p24 core antigen. Data are presented as picograms of p24 core antigen per milliliter of supernatant and correspond to the mean of triplicate experiments. (B) Production of TNF-α by unstimulated J22–HL-60 cells. Cells maintained in continuous culture for different periods of time (from 1 week to 6 months) were fed fresh medium and incubated for an additional 24 hours. Culture supernatants were harvested and assayed for the presence of TNF-α.

Two different TNF receptors have been described in mammalian cells, and both receptors can be activated by binding to TNF-α or TNF-β. It has been shown that the mechanism of action of TNF-α on HIV production in infected cells involves the translocation-activation of the transcription factor NFB. The molecular basis of NFB activation has been studied in detail and it is known that it involves a multiprotein complex and a cascade of phosphorylation-dephosphorylations. On the other hand, no clear information is available regarding the possible role of each individual TNF receptor in the signaling mechanism that leads to viral production. In addition, relatively little is known about the identity of other components involved in cell signaling through the TNF receptor(s). HL-60 cells are an exception to this rule, as in these cells it has been described that signaling through the TNF receptor involves the activation of a sphingomyelinase with concomitant production of ceramide and activation of a ceramide-dependent serine-threonine kinase. We have shown that sphingomyelinase and ceramide cause activation of mitogen-activated protein kinase and NFB in HL-60 cells, suggesting that the sphingomyelin pathway is important for TNF signaling, at least in HL-60 cells. We recently developed direct evidence for HIV proviral activation in J22–HL-60 cells induced by sphingomyelinase and ceramide. Although these studies illuminate a sphingomyelin-mediated TNF signaling pathway for HIV activation, the data presented are limited to HL-60 cells.

This pathway may also be activated by 1,25(OH)2D3 in HL-60 cells, although with a different kinetics and an observation consistent with our data indicating that TNF-α is involved in the production of virus induced by treatment of the J22–HL-60 cells with 1,25(OH)2D3. These findings are consistent with an autocrine role for TNF-α in the induction of HIV expression in the J22–HL-60 cells, as has been shown in other cellular systems. Additional evidence for the existence of a sphingomyelin pathway and its involvement in the activation of NFB has been recently provided. We have also obtained evidence indicating that a pathway able to recapitulate the basic characteristics of the mechanism of action of TNF (involving hydrolysis of sphingomyelin to ceramide and the production of TNF) is active in inducing viral production in the J22–HL-60 cells (Rivas et al, submitted). The availability of the J22–HL-60 cells offers a unique opportunity to identify intermediary molecules involved in the pathway that leads from TNF to activation and production of HIV under controlled experimental conditions.

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