Ephedrae Herba, a Component of Japanese Herbal Medicine Mao-to, Efficiently Activates the Replication of Latent Human Immunodeficiency Virus Type 1 (HIV-1) in a Monocytic Cell Line

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The persistence of latent human immunodeficiency virus type 1 (HIV-1)-infected cellular reservoirs, despite prolonged treatment with highly active antiretroviral therapy (HAART), represents a major hurdle to virus eradication. In this study, we evaluated the effect of Japanese herbal medicine on the induction of HIV-1 replication in latently infected monocytic cell line, U1, in order to eradicate virus efficiently. We found that Mao-to was able to induce HIV-1 replication either alone or in combination with tumor necrosis factor-alpha (TNF-α). Among the four components of Mao-to, only Ephedrae herba had strong effects in inducing HIV-1 replication. Analysis by Western blotting revealed that Ephedrae herba induced the nuclear translocation of nuclear factor-kappa B (NF-κB). Reporter assay data also showed that Ephedrae herba and, slightly, Mao-to activated the NF-κB promoter, indicating that these herbal agents may induce HIV-1 replication through NF-κB activation. These findings suggest that Mao-to and its component, Ephedrea herba, may be good candidates to augment HAART by inducing the expression of latent HIV-1 with the ultimate goal of eliminating persistent viral reservoirs in individuals infected with HIV-1.

Key words human immunodeficiency virus type 1; latent infection; Mao-to; Japanese herbal medicine; Ephedrae herba; nuclear factor-kappa B

Human immunodeficiency virus type 1 (HIV-1) infection is characterized by a period of clinical latency before the development of symptoms and HIV-related disease.1,2 Although highly active antiretroviral therapy (HAART) has been successful in controlling HIV-1 infection, replication-competent HIV-1 persists in resting memory cluster of differentiation 4 (CD4+ T cells and macrophages.3,7 Since current anti-HIV drugs are HIV-1 life cycle dependent, they have little effect on the latent form of HIV-1. It has been suggested that activation of latently infected cells in the presence of HAART shortens the half-life of HIV-1 reservoir because, presumably, reactivation of latent (or integrated) HIV-1 is followed by host cell death.8,9 Therefore, activation of HIV replication in latently infected cells is important to promote more efficient anti-HIV therapy.

Pre-integration and post-integration latency has been described in HIV-1 in the cellular level.10,11 The U1 cell line derived from U937 promonocytic cell line is one of the most thoroughly characterized models of post-integration latency. To activate latently infected HIV-1 in vitro, proinflammatory cytokines such as granulocyte macrophage colony stimulating factor (GM-CSF), interleukin (IL)-3, IL-6, and tumor necrosis factor-alpha (TNF-α) have been used.12,13 These agents have been shown to activate HIV-1 expression in U1 cells by affecting distinct steps of the virus life cycle, including nuclear factor-kappa B (NF-κB)-dependent transcription in the case of TNF-α and phorbol 12-myristate 13-acetate14,15 or a posttranscriptional event in cells stimulated with IL-6.16

In search for clinically relevant adjuvant drugs for HAART, we focused on the Japanese traditional herbal medicine “Kampo” to activate latently infected HIV more safely. Kampo is now widely used in Japan and is fully integrated into modern Japanese medical system.17 We demonstrate here that Mao-to, a herbal medicine used in Japan for the treatment of influenza-like illness (high fever, headache, pain and cough),18 and Ephedrae herba, one of the component herbs of Mao-to, activate HIV-1 replication in latently infected U1 cells and synergize with TNF-α to enhance HIV-1 gene reactivation via induction of NF-κB nuclear translocation in U1 cells.

MATERIALS AND METHODS

Cells and Reagents HIV-1-infected U1 cell line was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Health (Rockville, MD, U.S.A.). U1 is a cloned cell line derived by limiting dilution cloning of U937 cells surviving an acute infection with HIV-1 (LAV-1 strain). U1 cells were maintained in RPMI1640 (Sigma, St. Louis, MO, U.S.A.) supplemented with 10% (v/v) fetal calf serum (FCS, Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin. HeLa cells were maintained in minimum essential medium (MEM) supplemented with 10% (v/v) FCS and antibiotics. Recombinant TNF-α was purchased from PeproTech (Rocky Hill, NJ, U.S.A.). Japanese herbal medicines.17 Sho-saiko-to (TI-9), Mao-to (TI-27), Hotyu-ekki-to (TI-41), JuZen-Taiho-to (TI-48), Ninjin-Youei-to (TJ108), Armeniaca cortex powder, Ephedrae herba (Mao) powder, Cinnamomi cortex powder and Glycyrrhizae radix powder were obtained from Tsumura (Tokyo, Japan). Mao-to (TI-27) has the following components: Ephedrae herba (5.0 g), Armeniaca cortex (5.0 g), Cinnamomi cortex (5.0 g) and Glycyrrhizae radix (1.5 g).
Induction of HIV-1 Replication in U1 Cells and Detection of Intracellular p24 by Flow Cytometry

U1 cells (10^6 cells per well) in 12-well plates were incubated in triplicate with Kampo compounds with or without TNF-α. U1 cells were treated for 24 h with the indicated concentration of Mao-to or Ephedrae herba, fixed with 4% paraformaldehyde (Sigma) for 10 min, permeabilized with 0.1% saponin (Sigma) for 10 min, and stained with FITC conjugated anti-HIV-1 p24 mAb (Beckman Coulter, Fullerton, CA, U.S.A.) for 30 min on ice. After washing, the cells were analyzed by FACSCalibur (BD Bioscience, San Jose, CA, U.S.A.). Data were analyzed with FlowJo software (Tree Star, San Carlos, CA, U.S.A.).

Western Blot Analysis

U1 cells were treated with Mao-to (100 μg/ml) or Ephedrae herba (10 μg/ml) for 0.5, 2, 4 h, and nuclear protein was recovered using the Schreiber’s method as previously described. The amount of nuclear protein was measured by Bradford assay. Ten micrograms of nuclear protein was loaded onto a 10% SDS-polyacrylamide gel and subsequently transferred onto a PVDF membrane. Membranes were probed with anti-p50 (Sc-8414) or anti-p65 (Sc-8008) monoclonal antibodies (mAbs) and anti-γ-tubulin (Sc-7396) or anti-Sp1 (Sc-59-G) polyclonal antibodies as internal control for nuclear proteins. All antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, U.S.A.). Detection was performed using Chemiluminescence Western Blotting Detection System (ECL, GE Healthcare Bio-Science, U.K.).

Luciferase Assay

For transient transfection, HeLa cells were plated in 24-well dishes, and at subconfluent were transfected with 0.5 μg NF-κB luciferase reporter plasmid (Stratagene, La Jolla, CA, U.S.A.) and 0.05 μg Renilla luciferase construct using 2.5 μl/well HilyMax reagent (Dojindo, Kumamoto, Japan). After 24 h, cells were treated with Mao-to or Ephedrae herba in the presence or absence of TNF-α for 5 h. The cells were harvested for luciferase assay as described previously.

Statistical Analysis

Statistical significance of differences observed between experimental groups was determined using one-way ANOVA. p values less than 0.05 were considered significant.

RESULTS

Mao-to Activates HIV-1 Replication in the Chronically Infected Promonocytic Cell Line, U1

U1 cells, an HIV-1 latently infected cell line, harbor two integrated provirus DNA copies, and are characterized by low constitutive levels of virus expression that can be upregulated by several cytokines including TNF-α. The viral replication in U1 cells was considered significant. The activation of HIV-1 more than five fold at 100 μg/ml compared with untreated control in the absence of TNF-α (Fig. 1A). TNF-α alone also induced HIV-1 replication in latently infected U1 cells compared with the unstimulated control (Fig. 1B), and Mao-to enhanced the TNF-α-induced HIV-1 replication in a dose-dependent manner.

Ephedrae Herba Is the Main Component in Mao-to That Activates Latent HIV-1

Mao-to is a mixture of spray-dried powdered extract from 4 herbs (see Materials and Methods). We next investigated which component herbs activated the latent HIV. As shown in Fig. 2A, Ephedrae herba was the component that induced HIV replication. To confirm the effect of Ephedrae herba, U1 cells were treated with increasing concentration of Ephedrae herba in the presence or absence of TNF-α for 24 h. Ephedrae herba dose-dependently upregulated HIV-1 replication both in the presence or absence of TNF-α (Fig. 2B).

Ephedrae Herba Activates Latent HIV-1 via Induction of NF-κB Nuclear Translocation and Activation of NF-κB Promoter

The NF-κB elements in the HIV LTR are important for regulating HIV gene expression in monocyctic cell lines as well as other cell types. NF-κB is a heterodimeric protein complex consisting of 50 kDa and 65 kDa subunits which exist in a covert cytoplasmic form bound to an inhibitor I-κB. Treatment of cells with several cytokines or phorbol ester induces nuclear translocation of NF-κB which enhances the transcription of cellular and viral genes. To determine whether the activation of HIV-1 observed in U1 cells treated with Mao-to or Ephedrae herba is due to increased NF-κB translocation, we prepared nuclear extracts from treated and untreated cells and analyzed the nuclear expression of NF-κB. TNF-α induced the nuclear translocation of both components of NF-κB, p50 and p50,
and Mao-to modestly enhanced the effect of TNF-α (Fig. 3A). Moreover, Ephedrae herba also notably induced the nuclear translocation of NF-κB (p65 and p50) (Fig. 3B). These observations suggest that Mao-to and its component, Ephedrae herba, activate latent HIV in U1 cells via induction of NF-κB nuclear translocation.

To confirm the importance of NF-κB for the activation of latent HIV-1 by Mao-to and Ephedrae herba, we analyzed the NF-κB promoter activity using luciferase assay. NF-κB reporter gene construct was transiently transfected into HeLa cells, and luciferase activity in these transfectants was analyzed 5 h after stimulation with TNF-α, Mao-to or Ephedrae herba. As shown in Fig. 4, the luciferase activity in cells treated with Mao-to or Ephedrae herba and unstimulated by TNF-α was increased 2—3 fold. Importantly, Ephedrae herba increased the TNF-α-induced luciferase activity. Mao-to slightly, but not significantly, enhanced the NF-κB promoter activity. These data suggest that Ephedrae herba and, to a certain extent, Mao-to activate latent HIV-1 replication via NF-κB signaling pathway in cooperation with TNF-α.

**DISCUSSION**

In the present study, we have demonstrated that Mao-to and one of its components, Ephedrae herba, can activate HIV-1 replication in latently infected U1 cells and that co-stimulation of Mao-to or Ephedrae herba with TNF-α resulted in synergistic effects on HIV-1 viral expression. The existence of viral reservoirs that harbor latent forms has limited the success of HAART. It has been suggested that reactivation of the latent reservoirs could allow effective targeting and possible eradication of virus. Clinical efforts aimed at eliminating the pool of latently infected cells have been attempted with IL-2 or anti-CD3 antibodies, but resulted in regrettable outcome. Recently, prostratin, a non-tumor-promoting phorbol ester, was proven to be an effective activator of latent virus. Prostratin was observed to lack toxicity for short-term application, however, long-term side effects are yet unknown. Since the reversal of HIV-1 latency will require repeated administration of antagonists, such latent virus inducers must exhibit relatively low toxicity, permitting patients to withstand long-term, multi-round treatments.

Traditional Japanese herbal medicine, Kampo, has been used for the treatment of various diseases for more than 1500 years, and is still widely practiced in Japan. It is now for-
mally approved by the Ministry of Health, Labour and Welfare of Japan, and Japanese practitioners mainly use the freeze-dried herbal extracts produced in factories. Kampo drugs are used for long-term treatment and have been proven to have minimal side effects. In addition, several Kampo were shown to have immune-modulatory and anti-viral effects both in vitro and in vivo. Hence, we attempted to search among the Kampo medicines for an effective inducer of latent HIV-1. Among five Kampo drugs tested, we found that Mao-to dose-dependently activated latent HIV-1 in U1 cells with synergistic effects with TNF-α (Fig. 1). Mao-to includes Glycyrrhiza radix, which has been widely used in East Asia for health promotion. Since Glycyrrhiza radix is known to have cytoprotective effect and inhibitory effect on NF-κB activation in macrophages, it may dampen the effect of Ephedrae herba. The evidence that Glycyrrhizic acid suppresses UV-induced HIV-1 gene expression supports our presumption. This may also explain our observations that the effect of Mao-to on NF-κB promoter activity was lesser compared with Ephedrae herba (Fig. 4). However, it may be preferable to use Mao-to rather than Ephedrae herba for long-term treatment because Glycyrrhiza radix also works to strengthen body function and may provide protection from side effects.

NF-κB is an inducible cellular transcription factor that regulates a wide variety of cellular and viral gene expression, and is known to play a major role in regulating HIV-1 gene expression. In HIV-1 latently infected cells, activation of NF-κB could trigger the transcription of viral genes resulting in an explosive increase in HIV-1 replication. We observed that Ephedrae herba efficiently induced NF-κB nuclear translocation (Fig. 3) and activated the promoter activity (Fig. 4) cooperatively with TNF-α. However, since most of the herbal medicines have diverse pharmacological effects and NF-κB-independent mechanisms appear to be operating in chronic HIV-1 infection, further investigations are required to understand the activation mechanisms by Ephedrae herba. These studies will be helpful for the design and development of strategies for eliminating HIV from its reservoirs. In conclusion, our work suggests that Ephedrae herba is a potent inducer of latent HIV-1 from myeloid lineages and development of strategies for eliminating HIV from its reservoirs. In conclusion, our work suggests that Ephedrae herba is a potent inducer of latent HIV-1 from myeloid lineages and development of strategies for eliminating HIV from its reservoirs.

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