Prevention of Murine Acquired Immunodeficiency Syndrome (MAIDS) Development by Oriental Herb Extracts

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Received November 21, 2005; Accepted December 12, 2005

Oriental medicinal herb extracts (OHE) showing anticancer activities were investigated for effectiveness as antiviral drugs. Infection of MuLV to cell line resulted in formation of giant syncytia. Number of giant syncytia in culture treated with OHE decreased by 40% compared to that of non-OHE-treated cell culture. To determine OHE effects on progeny release, RT-PCR was performed. In vivo animal studies demonstrated effectiveness of OHE as antiviral drug when administered orally. After OHE administration, viral cytopathic effects decreased. Infected mice showed splenomegaly and over-proliferation of lymphocytes with decreased CD4+ cell counts. These symptoms decreased in OHE-treated mice, indicating OHE maybe useful therapeutics against MuLV/MAIDS as Human Immunodeficiency Virus (HIV)/AIDS animal model. Results show XC plaque assay and in vivo MAIDS model using MuLV are suitable tools for screening antiretroviral drug candidates.

Key words: Murine Leukemia Virus, Murine Acquired Immunodeficiency Syndrome, Oriental herbal extracts, Human Immunodeficiency Virus, AIDS

Murine Leukemia Virus (MuLV) originates from sarcoma 37, a transplantable connective tissue neoplasm of mice. Infection with a mixture of MuLV causes severe lymphoproliferation and immunodeficiency known as Murine Acquired Immunodeficiency syndrome (MAIDS), which is characterized by an array of symptoms affecting many types of lymphocytes including splenomegaly, lymphadenopathy, and hypergammaglobulinemia. Advanced stage of this symptom is associated with an enhanced susceptibility to the infection and the development of B cell lineage lymphoma. During the final stage of MAIDS, mice are severely immunocompromised, often developing B and/or T cell lymphomas within 16-20 week after infection.

As the clinical importance of retroviruses as causative agents of severe diseases in humans and animals is accentuated, researches aimed at developing specific antiretroviral drugs become more extensive. This necessity has led to the development of a wide range of alternative antiviral strategies, including immunomodulating agents and drugs targeting the course of Human Immunodeficiency Virus (HIV) infection pathways. From this perspective, an ideal anti-HIV-1 therapy should include drugs or agents protecting naive cells from infection and reducing the pool of latently infected cells. Therefore, researchers have evaluated the efficacy of new reagents and drugs, which have other therapeutic abilities in an MAIDS.

Recently, the food industry has been using various natural agents from plant origin as remedies, including oriental herb extracts (OHE), which have been shown to have strong antioxidative characteristics. OHE responsible for alleviating the carcinogenic effect have been identified and tested for their chemopreventive actions. The pertinent investigations include analysis of some active ingredients and major components of the medicine, treatment of diseases, and the search for alternative drugs.

Based on the antioxidant and anticancer activities of OHE, XC plaque assay, a screening system, and in vivo administration of OHE were performed to examine the antiviral effect, which downregulates the MuLV-induced cytopathic effects. In vitro evaluation of the antiviral effects of OHE employed XC plaque assay for enumeration of syncytia, which is one of the viral cytopathic effects, genomic DNA PCR to detect the presence of MuLV-provirus, and RT-PCR for quantification of viral progeny. Moreover, in vivo oral administration of OHE to MuLV-infected female mice relieved viral symptoms, suggesting that OHE could serve as antiretroviral drugs.

Materials and Methods

Cell culture. An SC-1 cell line (ATCC CRL-1404) was grown in Eagle’s minimum essential medium (MEM; SIGMA, US) supplemented with 10% fetal bovine serum (FBS; Sigma, US), 100 µg/ml penicillin & streptomycin (Gibco, US) (MEM10A). An XC cell line (ATCC CCL-165) was also grown in MEM10A. All cell growth and plaque assays were performed at 37°C in a humidified 5% CO2 incubator.

Preparations of virus. MuLV LP-BM5 was obtained from NIH AIDS Research and Reference Reagent Program. Spleens
after UV irradiation, 3 ml cultures were exposed for 45 s to UV irradiation. Immediately after innoculation, the medium was removed, and the cultures were incubated 2 additional days. The cultures were washed once with phosphate-buffered saline, fixed with 100% methanol for 5 min, and stained with the Giemsa stain for 1 h. The Giemsa stain was then discarded, and the cultures were washed twice with tap water, and the plaques were counted under a microscope.

**RT-PCR.** Virion-associated RNA was extracted from both MuLV-infected and non-infected SC-1 cell culture supernatants using QIAGEN Viral RNA Mini kit (QIAGEN, Germany). After RNase-free DNase I digestion, the RNAs were eluted and reverse-transcribed with Molony Murine Leukemia Virus (M-MLV)-reverse transcriptase. The cDNAs were synthesized from 1 µl total RNA, random primers (Promega, US), RNase inhibitor (Promega, US), and reverse transcriptase in a 20 µl reaction mix. A 2 µl volume of the cDNA was used per PCR. Forward primer MuLV-gag (5'CCC ATA TAT CAT CCG TCC CTC TGG-3') and reverse primer MuLV-gag (5'CCT CAT CGT TCC CTC TGG-3') were used to detect the quantity of viral loads. β-Actin primers, internal controls, were also used (forward primer, 5'TCC TCC CTG GAG AGC ACT GTG TTG GC-3'; reverse primer, 5'CCA GAC AGC ACT GTG TTG GC-3'). As negative controls, no-template and no-RT samples were included. The mixtures were processed for 35 cycles consisting of 94°C for 1 min, 50°C for 1 min, and 72°C for 30 s. Following the final cycle, an extension step for 10 min at 72°C was performed. A 10 µl volume of each PCR mixture was analyzed on a 2% agarose gel with ethidium bromide. The density of the PCR products was compared using a Quant-it (BioRad, US) software.

**Genomic DNA extraction.** Total genomic DNA was extracted from MuLV-infected and non-infected SC-1 using QIAGEN DNA Mini Kit (QIAGEN, Germany). Splenocytes harvested from the mouse injected with MuLV were used for genomic DNA extraction. A series of PCR was performed as described before.

**In vivo study.** Female BALB/C mice (6-8 weeks of age) purchased from Orient Inc. (Korea) were divided into six groups of five in each. One group was given oral administration of 5% OHE during the first week before MuLV inoculation, and three groups were administrated with 0.5, 1, or 5% OHE for 3 weeks after virus inoculation. The remaining two groups were negative controls: no OHE treatment group and no MuLV inoculation group. After adaptation period, 4 groups of mice (n = 5) were given intraperitoneal (i.p.) inoculation of MuLV (pfu/ml = 2.21×10^4) and euthanized under anesthesia 4 weeks after MuLV-inoculation. The spleens were then harvested, cut, and weighed. They were homogenized with a plunger in 5% RPMI, and cells without debris were collected. The homogenate was centrifuged at 1200 rpm for 5 min, and the supernatant was collected and filtered through a 0.22-µm filter for the further experiments. The pellets from centrifugation were treated with the Gey’s medium (138 mM NaCl, 6 mM KCl, 1 mM MgSO4, 1.1 mM CaCl2, 0.1 mM EDTA, 1 mM NaHPO4, 5 mM NaHCO3, 5.5 mM
Flow cytometry analysis. Splenocytes (2 x 10^6 cell) were washed twice with FACS buffer (PBS containing 1% bovine serum albumin and 0.1% sodium azide). Washed cells were incubated in 1 ml cold FACS buffer containing PE-Cy5-conjugated rat anti-Mouse CD3 monoclonal antibody (BD Pharmingen, US), fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 antibody (BD Pharmingen, US) at 1:100 dilutions, and appropriate isotype controls were also made. After washing, the cells were fixed with 0.2 ml of 2% paraformaldehyde and analyzed by flow cytometry. Surface-staining was analyzed on an FACSCalibur flow cytometer (Becton Dickinson, US) equipped with a single argon laser (488 nm) for excitation and standard emission optics. Each counted sample was for 10,000 events of each sample. Forward and side scatters were used to measure the cell size and granularity to gate out cell debris. Data are expressed as relative mean fluorescence intensities (MFI). The flow cytometry data are presented in two-dimensional dot plots showing representative results of 10,000 events. The quadrants of all plots were defined on the basis of appropriate isotype controls measured in parallel to determine the background fluorescence levels.

Results

OHE was analyzed systematically for effectiveness against MuLV infection: (i) inhibition of plaque formation in the XC plaque assay (ii) down-regulation of the viral propagation using viral progeny detection, (iii) suppression of splenomegaly and immunodeficiency causing a depletion of CD4+ T cells induced by MuLV infection in BALB/c mice, (iv) inhibition of viral progeny replicate in spleen of the MuLV infected BALB/c mice.

Inhibition of plaque formation by MuLV in the presence of OHE. Because OHE has antioxidative and antitumor activities, we determined whether OHE can inhibit MuLV replication through a slightly modified classical XC plaque assay (Fig. 1). The syncytia formed by MuLV infection was enumerated from the culture treated with or without OHE. One of the murine SC-1 cells was pretreated with OHE, while other cells were post-treated with OHE, infected, and grown to confluency in the presence of the reagent. The SC-1 cells were killed with UV irradiation prior to the addition of XC cells. In the post-treated group, OHE were added before scoring the plaques (Fig. 2B). In parallel dishes, untreated SC-1 cells were grown to determine cytoxicity. The syncytia containing more than five nuclei are counted as a plaque (Fig. 2A). The cells infected in the absence of OHE yielded a viral titer of 2.25 x 10^4 pfu/ml. When pre-treated with OHE, the titer decreased to 1.00 x 10^4 pfu/ml. In the post-treated group, the titer further decreased to 0.75 x 10^4 pfu/ml (Fig. 2C).

Effect of OHE on the viral replication during life cycle in vitro. To determine which stage of viral life cycle was targeted by OHE, we investigated the amplified gag gene in the virus-infected SC-1 cells. Culture supernatants of the SC-1 cells infected with MuLV were assayed for the quantification...
of viral progeny counts. In the XC plaque assay, culture supernatants before and after UV irradiation were used to confirm at which stage more viral progenies were released. However, no differences were observed in the amounts of viral progeny from the cells with/without OHE-treatments as well as pre- and post-treatments of UV irradiation (Fig. 3). Moreover, the effect of OHE on the transcript for MuLV gag was examined in the SC-1 cells infected with MuLV. The mRNAs of virus-infected and OHE-treated SC-1 cells had the same viral RNA count (data not shown), which indicates that OHE cannot block the viral load but instead obstruct the viral cytopathic effect. Genomic DNA of SC-1 cells infected with the virus was used to confirm whether OHE inhibit the post-entry stage of the MuLV infection. The amplified gag gene from an integrated provirus showed no difference among other groups (Fig. 4). It is assumed that the post entry stage of the virus replication is not targeted by OHE, and this reagent does not have an inhibitive role on the integration of the viral genome in the host genome.

**Inhibition of splenomegaly and severe lymphoproliferation by oral administration of OHE.** A total of 25 mice were treated with either virus alone or virus plus OHE, and 5 untreated mice were used as controls. A 35% reduction in the virus-induced splenomegaly was seen at oral post-administration of OHE given for 3 weeks, whereas oral administration of OHE before MuLV infection failed to decrease the spleen weight (Fig. 5A, B). However, this is not surprising, as shown by the invulnerable provirus that was measured in PCR of genomic DNA after virus infection in the presence of OHE. Furthermore, oral administration of OHE after virus infection also decreased the number of the splenocytes in the virus-infected mice (Fig. 5C). However, there were no dose-dependent effects by OHE on the weight of spleen and the count of splenocytes. The biological effect of these different OHE does not accurately correlate with the in vitro analysis discussed above. Because we confirmed that the post-long period of the OHE treatment was more effective than the short period of pre-treatment, the in vivo and in vitro results could be agreed upon.

**Effect of the OHE on a composition of leucocytes in virus infected mice.** OHE had a destructive role in spreading the virus not only in CNS but also in the blood. Generally,
MuLV-infected mice had high counts of white blood cell (WBC) and its components also increased. The proportion of lymphocytes significantly increased due to a severe lymphoproliferation. However, the groups of mice administrated with OHE showed significantly the decline in WBC counts, an indication that OHE alleviates the pathogenesis of MuLV/MAIDS. Furthermore, a neutrophil count in the mice treated with OHE decreased compared to that of untreated mice (Table 1). Because the neutrophils play a role in the innate immune response mediator and operates the inflammatory response, OHE could be an anti-inflammatory agent.\(^{[12]}\)

**Table 1. Total cell counts**

<table>
<thead>
<tr>
<th>Group</th>
<th>WBC (\pm SD)</th>
<th>NE (\pm SD)</th>
<th>LY (\pm SD)</th>
<th>MO (\pm SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>36.9 ± 19.6</td>
<td>31.3 ± 2.5</td>
<td>48.6 ± 7.7</td>
<td>8.3 ± 0.7</td>
</tr>
<tr>
<td>2</td>
<td>37.2 ± 3.1</td>
<td>16.1 ± 2.7</td>
<td>73.8 ± 3.6</td>
<td>8.9 ± 0.9</td>
</tr>
<tr>
<td>3</td>
<td>31.3 ± 9.9</td>
<td>13.1 ± 7.2</td>
<td>78.6 ± 10.4</td>
<td>5.9 ± 0.5</td>
</tr>
<tr>
<td>4</td>
<td>30.5 ± 4.0</td>
<td>9.5 ± 8.2</td>
<td>82.2 ± 9.9</td>
<td>6.9 ± 2.3</td>
</tr>
<tr>
<td>5</td>
<td>30.1 ± 10.5</td>
<td>11.7 ± 2.4</td>
<td>80.6 ± 2.6</td>
<td>6.9 ± 0.2</td>
</tr>
<tr>
<td>6</td>
<td>30.1 ± 10.5</td>
<td>11.7 ± 2.4</td>
<td>80.6 ± 2.6</td>
<td>6.9 ± 0.2</td>
</tr>
</tbody>
</table>

Mice were treated either with OHE before virus inoculation (6) or with different doses of OHE after virus inoculation: 5% OHE (3), 1% OHE (4), 0.5% OHE (5). Control mice were virus non-inoculated (1) and non-inoculated/untreated (2). Only white blood cells (WBC), specifically neutrophil (NE), lymphocyte (LY), and monocyte (MO), were detected. The virus-inoculated/OHE-untreated group have TMTC values (a, b, c, and d), which indicate that, due to many cell counts in the sample, the values could not be shown as numbers; b, c, and d have more than 70, 80, and 19% cells in the sample blood, respectively. Values are Avg. ± SD of five animals.

**Fig. 5. Splenomegaly.** (A) Pictures of spleen from control (1), virus-inoculated and OHE-non-treated mice (2), virus-inoculated and OHE-treated mice (3). (B) Reduction of splenomegaly in mice infected with MuLV before and after administration of OHE. Mice were treated either with OHE before virus inoculation (6) or with different doses of OHE after virus inoculation: 5% OHE (3), 1% OHE (4), 0.5% OHE (5). Mice were virus non-inoculated (1) or non-inoculated/untreated (2). (C) Reduction of severe lymphoproliferation in mice inoculated with MuLV after administration of OHE. Mice were treated with the same method as described in (B).

The prevention of CD4+ cells decline resulted from the OHE effect. Spleens of mice with MAIDS normally contain a small number of CD4+ cells, readily identified by the reduced expression of CD4 (Fig. 6A, Table 2). Although the proportions and total numbers of CD4+ cells in the spleen were not significantly recovered by the OHE treatment, administration of a high dose of OHE promoted approximately 20% restoration of CD4+ cells. Furthermore, the rate of restoration of CD4+ cells was affected by the dose of the administrated OHE (Fig. 6B).

The effect of the OHE on the viral stage in their life cycle in vivo. To determine if the orally administrated OHE suppresses the expression of the viral progeny release, splenic fluid was analyzed for virion-associated RNA quantification using RT-PCR (Fig. 7). Studies of the spleens from the infected mice treated with OHE after the virus inoculation revealed reduced release of the viral progeny, whereas OHE pre-treatment had no effect on the viral load (Fig. 7). Genomic DNA of splenocytes in the virus-infected mice was also used to confirm whether OHE inhibit the post-entry stage of the MuLV replication. No difference was observed in the amplified gag gene count in the integrated viral genome as compared among treatment of different dose of the OHE (Fig. 8), presumably because the post entry stage of virus is not influenced by OHE and this reagent does not play an...
inhibitory role on the integration of viral genome in the host genome.

**Discussion**

The spreading epidemics of AIDS call for therapeutic interventions. To date, no specific therapy is available for the prevention or treatment of HIV viremia. Our findings demonstrate that OHE could be effective in suppressing MuLV pathogeneses such as splenomegaly and severe lymphoproliferation. Furthermore, the orally administered OHE showed a curative activity in the virus-infected mice; unlike other reagents, which can lose their curative properties during the passage through the digestive organ, effectiveness of OHE was not on the decrease. OHE consist of a variety of Chinese herbs, Agastache rugosa Kuntze, Glycyrrhiza Radix, Crataegus Pinnatifida Bunge, Foeniculum vulgare Mill, and Aurantii nobilis pericarpium, which are used by many Oriental countries as reagents for folk remedies, among which G. Radix was found to be effective in mice infected with herpes simplex virus type 1. These mixed reagents showed anti-inflammatory and analgesic activities, showing potentials as curative drugs (Table 3).

The MuLV system described above is ideally suited as a model system for what. Candidate drugs or drug combinations can be tested for their ability to suppress plaque formation in the XC plaque assay. Inhibition of the infection of cultured murine splenocytes by MuLV was analyzed in parallel. Drugs or drug combinations with promising therapeutic indices can then be analyzed for their ability to prevent HIV-induced cytopathic effects in vitro.

Candidate drugs following these first series of tests are selected for in vivo testing using the MAIDS system for expeditious determination of the effective antiretroviral dosages. Due to the rapid onset of MuLV-induced erythroleukemia, results can be obtained within 3 weeks. The ability of the same drug dosage to suppress the infection of splenocytes, particularly T cell in vivo, can be studied next using strains of mice susceptible to MuLV. Drug levels in the mice are determined to facilitate subsequent clinical trials of promising therapeutic agents. The major advantage of such a system is the rapidity with which results can be obtained. Unlike HIV, which has an incubation period of up to 5 years, MuLV...
induces disease in about 60-90 days. The continuing rapid spread of HIV infection and epidemiology requires development of a more efficient drug screening system. In this report, we showed that our MAIDS model system is highly effective for the screening of potential anti-viral therapeutics.

Acknowledgments

The authors acknowledge Dr. K.-M. Park for providing OHEs, and Dr. C.-E. Lee for valuable advice, Drs. S.-H. Kim, Y.-J. Kim for analysis, Drs. M.-S. Kwon, S.-T. Kwon, D.-H. Keum, T. Chung, and S. Hong for their support. This work was supported by Korea Research Foundation Grant KRF-2003-005-F00007.

Table 3. General functions of individual OHE

<table>
<thead>
<tr>
<th>OHE</th>
<th>Function</th>
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<tbody>
<tr>
<td>Agastache rugosa Kuntze</td>
<td>cancerocidal, the curative influenceable of chollera, emesis, pyrexia, headache, dyspepsia, halitosis and stomachache</td>
</tr>
<tr>
<td>Glycyrrhizae Radix</td>
<td>cancerocidal, cytotoxicity, promotion of liver function, blood glucose descent, immune enhancement</td>
</tr>
<tr>
<td>Crataegus Pinnatifida Bunge</td>
<td>the curative influenceable of dyspepsia, leukosis, liver diseases</td>
</tr>
<tr>
<td>Foeniculum vulgare Mill</td>
<td>tranquiliser, tonic and soporific drug</td>
</tr>
<tr>
<td>Aurantii nobilis pericarpium</td>
<td>spasmylosis, anti-exocelosis, NK (Natural Killer) cell activity, anti-allerdisos, cancerocidal</td>
</tr>
</tbody>
</table>