BIOMEDICAL AND ENVIRONMENTAL SCIENCES **17**, 153-164(2004)

Ampelopsin, a Small Molecule Inhibitor of HIV-1 Infection Targeting HIV Entry[1]

DE-YU LIU[*,2], JIAN-TAO YE[*], WEN-HUI YANG[*], JIN YAN[#], CHANG-HONG ZENG[#], AND SA ZENG[*]

[*]*Department of Chemistry, Zhongshan Medical College, Sun Yat-sen University, Guangzhou 510089, Guangdong, China;* [#]*Guangdong Provincial Center for Disease Prevention and Control, Guangzhou 510300, Guangdong, China*

Objective To investigate the anti-HIV effects of ampelopsin and its interaction with HIV-1 coreceptor CXCR4. **Methods** Through anti-virus experiments *in vitro*, the inhibitory effect of ampelopsin on HIV-1 infection was verified. Chemotaxis assay was performed to show the ability to induce PBMCs migration by ampelopsin, RANTES and SDF-1α. Fluorescence labelling monoclonal antibody was utilized to observe the interaction of ampelopsin and CXCR4. Mice immunosuppressant model was also established to detail the role ampelopsin played in regulating cellular immunological functions. **Results** Ampelopsin could protect sensitive cells against HIV-1 infection and dramatically reduce HIV-1 antigen P24 expression. HIV-1_{SF33} attaching to MT-4 cells was interfered by ampelopsin, and the EC_{50} was 0.175 mg/mL for cellular protection and 0.024 mg/mL for P24 inhibition. At co-cultivating phase, EC_{50} was 0.229 mg/mL and 0.197 mg/mL respectively. Furthermore, the EC_{50} was 0.179 mg/mL and 0.348 mg/mL in acute infection. Human PBMCs migration was induced after being challenged with ampelopsin or chemokines, and synergistic action was observed during co-treatment. Ampelopsin alone resulted in maximal chemotaxis at 1 mg/mL. HIV-1 co-receptor CXCR4 on the surface of PBMCs was decreased by internalization, which indicated the effect of ampelopsin on CXCR4. About 70% CXCR4 was reduced by ampelopsin at 1 mg/mL. Ampelopsin also augmented cellular immunological functions in immunosuppressive mice. **Conclusion** Ampelopsin displays a strong inhibitive role during HIV-1 absorption, incubation and acute infection. These results are coincident with its immune enhancement.

Key words: Ampelopsin; HIV-1; Chemokine receptors; Cellular immunity

INTRODUCTION

The evidence that nonhuman CD4-positive cell lines are insensitive to HIV-1-induced syncytium or infection, indicates that CD4 alone is not sufficient for HIV-1 invasion[1]. As it is now known, CCR5 is the most important co-receptor for the M-tropic (also designated as R5) strains that are commonly transmitted between individuals[2], and CXCR4 is the most relevant co-preceptor for the T-tropic isolates (also referred to as X4) that emerge after several years of HIV-1 infection[3]. Prevention of HIV-1 infection and inhibition of HIV-1 replication by chemokines, antagonists of chemokine receptors, or mAbs to chemokine

[1]This work was supported by Science Foundation of Guangdong Province (No.97002).

[2]Correspondence should be addressed to De-Yu LIU, Fax: 86-20-87331476. E-mail: liudy@gzsums. edu.cn

Biographical note of the first author: De-Yu LIU, male, born in 1953, professor, graduated from Sun Yat-sen University, published 29 papers in scientific journals, main research field is natural medicine.

0895-3988/2004
CN 11-2816

receptors, which induce down-regulation of chemokine receptors and/or directly block HIV-1 interaction with the co-receptors, have been demonstrated[4,5]. Therefore, the HIV-1 co-receptors CXCR4 and CCR5 represent exciting new therapeutic targets for the development of novel antiretroviral agents.

Although chemokines and their derivatives have been under intense investigations, it would, in principle, be desirable to have small molecule inhibitors that are amenable to bulk synthesis by traditional chemical techniques, and orally available. Ampelopsin, belonging to flavonoid, is an active component extracted from the root of Chinese medicinal herb *Radix Ampelopsis cantoniensis* or *Ampelopsis grossedentata*. In the classical Chinese traditional medication, ampelopsin has the functions of detoxication and anti-inflammation. Since the 1970s, the structural analysis, extraction and purification of ampelopsin have been well established[6]. Our previous studies have found that ampelopsin possesses anticancer effects on various human cancer cell lines *in vitro* and on transplant B16 mouse melanoma *in vivo*[7]. However, its effect on immune system and its value in AIDS have not been documented. This study was carried out in an attempt to evaluate the activities of ampelopsin against HIV-1 infection. The underlying mechanism was also discussed. In addition, the effects on mice immunosuppressive model were investigated to detail the role ampelopsin played *in vivo*. Our results demonstrated that ampelopsin was proved to be efficient against HIV-1 infection, and could profoundly augment the cellular immunological functions in mice. The anti-HIV-1 effect of ampelopsin is partly due to down-regulation of CXCR4 on the surface of target cells. Since ampelopsin abounds in South China, it is likely to become a new anti-AIDS agent with a plentiful resource and at a cheaper cost.

MATERIALS AND METHODS

Materials

Ampelopsin was prepared in our laboratory and its purity was ＞98%. Recombinant human RANTES, SDF-1α was purchased from Sigma Chemical Co. FITC-mouse anti human CXCR4 monoclonal antibody was from R&D Systems, and FITC-mouse IgG_{2a} as isotype control was from BD Biosciences. Azidothymidine (AZT), MTT, Coomassie brilliant blue, bovine serum albumin (BSA), phytahematoagglutinin P (PHA-P), concanavalin A (Con-A), and hydrocortisone (HC) were products of Sigma. RPMI 1640 and fetal bovine serum (FBS) were from GIBCO BRL. Lymphocyte separating medium was a product of TBD Technologic Centre. Recombinant human interleukin 2 (IL-2) was from Pepro Tech.

The 24-well transwell was purchased from Corning Costar. HIV-1 P24 antigen test kit was from Organon Teknika. IL-2 test kit was from JingMei Biotech. Healthy human blood was from Guangzhou Blood Center. MT-4 cell line, H9 cell line, $HIV\text{-}1_{SF33}$ strain and $HIV\text{-}1_{GD\text{-}1}$ strain were generous gifts from Guangdong Provincial Center for Disease Prevention and Control. BALB/C mice and YAC-1 mice cell line were provided by Animal Center of Sun Yet-sen University.

Methods

Extraction of ampelopsin radix *Ampelopsis cantoniensis* or *Ampelopsis grossedentata* was crushed to pieces, and extracted three times by 95% ethanol. Afterwards, ethanol was retrieved and deposit was solved by water. Two steps of extraction were performed in turn, using petroleum ether and chloroform respectively. The water layer was stilled in a

decompressor till precipitation of solid. After static stay overnight, the sedimentation was dissolved by hot water and boiled with active carbon. Subsequently, the liquid was filtered while hot and recrystallized. Ampelopsin, a white needle crystal, was obtained after dehydration[8].

Ampelopsin, a white needle crystal with a melting point of 246℃-247℃, was easy to dissolve in hot water or hot ethanol, and could be dissolved in methanol, ethanol or acetone, while it was poorly dissolved in water or ethyl acetate and difficultly dissolved in petroleum ether or chloroform.

PBMCs purification Human peripheral blood mononuclear cells (PBMCs) were prepared from freshly heparinized whole blood. In brief, the healthy blood was diluted 1:1 in PBS, and layered onto lymphocyte separating medium, then centrifuged for 25 min at 400 g. The white interphase was harvested and thrombocytes depleted by three subsequent washing and centrifuging steps. PBMCs were suspended in complete RPMI 1640 medium containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L glutamine, and cultured at 37℃[9].

Viruses preparation HIV-1_{SF33} was seeded to MT-4 cells. After co-incubation for 2 h, unabsorbed virus was separated by centrifugation. The infected cells were cultured until syncytium emerged. Serial tenfold dilutions of the supernatant were added to uninfected MT-4 cells in a 96-well plate. On day 4, the P24 antigen levels of each well were tested using a HIV-1 P24 antigen test kit (ELISA). The half-maximal tissue culture infectious dose ($TCID_{50}$) was calculated by Spearman-Karber equation[10]. HIV-$1_{GD\text{-}1}$ strain was propagated in PHA plus IL-2 stimulated PBMCs, and estimated in the same way[11].

Antiviral assays To estimate the effect on HIV-1 absorbing process, ampelopsin at indicated concentrations and 200 $TCID_{50}$ virus was added into cells simultaneously. Virus positive control (PC) and normal cell control (NC) were performed as well. After 1.5 h incubation, drug and virus in culture supernatants were removed by centrifugation. Four days later, MTT reduction assay was carried out[12], and the absorbance (A) was measured at 540 nm using a microplate reader (MBX-Ⅱ, Bio-Rad). The cellular protective rate was reflected as $(A_{Drug}\text{-}A_{PC})/(A_{NC}\text{-}A_{PC})\times 100\%$[13]. P24 antigen in culture supernatants was detected using ELISA kit, and the P24 inhibitive rate was calculated as $(A_{PC}\text{-}A_{Drug})/A_{PC}\times 100\%$[14].

To investigate the protection in the co-cultivating phase[15], cells were pretreated with ampelopsin for 2 h, and AZT (10 μg/mL) was used as positive drug control. After that, cells were incubated in the presence of 200 $TCID_{50}$ HIV-1. On day 4, cellular protective rate and P24 inhibitive rate were evaluated respectively as described above.

Correspondingly, the effect on acute HIV-1 infection was determined[16]. Cells were added to 200 $TCID_{50}$ HIV-1 in volume ratio 1:1, and co-cultured in incubator for 2 h. After centrifugation, cells were washed and challenged with ampelopsin. On day 4, protective rate and P24 inhibitive rate were both examined.

Chemotaxis assay Freshly separated PBMCs were suspended, and then 0.1 mL cells at 1×10^6/mL were added to the top chamber of a 24-well transwell (6.5 mm diameter, 5 μm pore size, polycarbonate filter). Test compounds at different concentrations were added to the lower chamber. The chambers were incubated for 2 h at 37℃. Cells passed through the membrane and were attached to the lower surface of the membrane. The membranes were fixed and stained using 1% Coomassie brilliant blue. Migrated cells were counted microscopically in five oil immersion fields at a 500× magnification. The chemotactic potency of a sample was expressed as the chemotactic index (CI), which was the value of the amount of cells migrated towards the test sample divided by the amount of randomly

migrated cells[17].

Flow cytometry PBMCs were suspended with PBS containing 1% BSA at 1×10^6/mL, and then incubated for 30 min at 37℃ with various concentrations of ampelopsin or SDF-1α. Afterwards, the drugs were washed out with ice cold PBS. The cells were divided into two equal portions. One portion was stained with FITC-labelled CXCR4 antibody, and the other was stained with FITC-labelled isotype control. Cells in the absence of drugs were used as the normal control (NC). The staining procedure was performed on ice for 1 h, and washed twice with ice cold PBS containing 1% BSA and 0.05% NaN_3. Finally, the cells were analyzed on a FACScan analyzer (Epics-Elite, Beckman), at least 10 000 events were accumulated for each sample and mean fluorescence intensity (MFI) was calculated. CXCR4 expression inhibitive rate was reflected as $[1-(MFI_{Drug}-MFI_{Isotype})/(MFI_{NC}-MFI_{Isotype})]\times100\%$. To investigate the mechanism underlying the inhibition, the similar experiment was performed at 4℃[18].

In vivo studies BALB/C mice were selected to establish an immunosuppressive model achieved by muscle injection of hydrocortisone (HC) at a dose of 25 mg/kg. Briefly, BALB/C mice were randomized into 5 groups: ampelopsin at high dose (400 mg/kg), medium dose (200 mg/kg), low dose (100 mg/kg), hydrocortisone (HC) and normal saline (NS). After consecutive injection of hydrocortisone for 5 days, the ampelopsin groups were treated by abdominal injection at the indicated dosage, while HC and NS were given normal saline. All the mice were sacrificed on day 11, and splenic cells were separated and cultured in RPMI 1640[19].

The splenic cells were used as effective cells (E), YAC-1 cell line was used as target cells (T). The two kinds of cells were seeded in a 96-well plate at a ratio of 20:1. After 24 h incubation, MTT assay was performed, and NK cell activity was defined as $[1-(A_{E+T}-A_E)/(A_T)]\times100\%$. On the other hand, the splenic cells challenged with ConA at a concentration of 10 μg/mL, were considered as positive control (PC). Cells in the absence of ConA were used as negative control (NC). After 48 h incubation, MTT assay was carried out, and the T-lymphocyte proliferation was estimated as the difference between A_{PC} and A_{NC} (ΔA)[20]. The IL-2 level in the supernatants of ConA stimulated cultures was measured using an IL-2 kit. IL-2 concentration was calculated by linear regression.

RESULTS

Ampelopsin as a Potent HIV-1 Inhibitor

The 50% cytotoxic concentration (CC_{50}) of ampelopsin was determined using MTT reduction assay. The survival rate of cells increased with decreasing of ampelopsin concentration. The CC_{50} for MT-4 and PBMCs cells was 8.36 mg/mL, and 6.15 mg/mL respectively (data not shown).

HIV-1_{SF33} attaching to MT-4 cells was interfered with ampelopsin as shown in Table 1. When virus was seeded in the presence of ampelopsin, cellular protection and P24 inhibition were both strong. MTT assay displayed that ampelopsin, at the concentrations from 0.05 mg/mL to 1 mg/mL, could protect more than half cells against infection, while AZT (10 μg/mL) had only a mild effect of 39.8%. The difference was significant ($P<0.01$). Ampelopsin at concentrations above 0.25 mg/mL could inhibit over 60% antigen expression, while AZT was far less effective ($P<0.01$). The EC_{50} of ampelopsin was 0.175 mg/mL for cellular protection, and 0.024 mg/mL for antigen inhibition.

TABLE 1

Effects of Ampelopsin on HIV-1_{SF33} Absorbing MT-4 Cells

Groups	Drug Concentration	MTT Assay		ELISA Assay	
		Absorbance $\bar{x} \pm s$	Cells Protection Rate/%	Absorbance $\bar{x} \pm s$	Cells Protection Rate/%
Ampelopsin	1.000 mg/mL	0.947±0.017	87.3	0.255±0.015	80.1
	0.500 mg/mL	0.923±0.025	80.3	0.426±0.010	66.6
	0.250 mg/mL	0.904±0.052	74.9	0.475±0.034	62.8
	0.125 mg/mL	0.859±0.032	62.1	0.789±0.153	38.2
	0.050 mg/mL	0.832±0.034	54.7	1.012±0.088	20.8
AZT	10.00 μg/mL	0.780±0.052	39.8	0.739±0.090	42.1
PC	–	0.639±0.022	0.00	1.277±0.036	0.00
NC	–	0.992±0.047	100.0	0.033±0.007	–

At the co-cultivating phase, ampelopsin and AZT both manifested potent cellular protection and P24 inhibition as shown in Table 2. The protective rate and inhibitive rate were 73.7% and 80.2% for 1 mg/mL ampelopsin, and 62.4% and 63.0% for 10 μg/mL AZT. For ampelopsin, EC_{50} was 0.229 mg/mL and 0.197 mg/mL respectively.

TABLE 2

Effect of Ampelopsin on MT-4 Cells Co-Cultivation With HIV-1_{SF33}

Groups	Drug Concentration	MTT Assay		ELISA Assay	
		Absorbance $\bar{x} \pm s$	Cells Protection Rate/%	Absorbance $\bar{x} \pm s$	Cells Protection Rate/%
Ampelopsin	1.000 mg/mL	1.058±0.128	80.2	0.920±0.086	73.7
	0.500 mg/mL	0.943±0.093	68.2	0.977±0.084	72.1
	0.250 mg/mL	0.772±0.047	50.2	1.503±0.088	57.1
	0.125 mg/mL	0.698±0.034	42.5	2.143±0.162	38.8
	0.050 mg/mL	0.582±0.038	30.3	2.406±0..191	31.2
AZT	10.00 μg/mL	0.894±0.040	63.0	1.318±0.064	62.4
PC	–	0.293±0.052	0.00	3.500±0.000	0.00
NC	–	1.247±0.103	100.0	0.043±0.007	–

Ampelopsin and AZT both displayed powerful cellular protection and P24 inhibition in acute infection as shown in Table 3. The EC_{50} of ampelopsin for MT-4 cells was 0.179 mg/mL for cellular protection, and 0.348 mg/mL for antigen inhibition. AZT at 10 μg/mL also resulted in 68.1% protection and 72.5% inhibition.

TABLE 3

Effects of Ampelopsin on Acute Infected MT-4 Cells With HIV-1_{SF33}

Groups	Drug Concentration	MTT Assay		ELISA Assay	
		Absorbance $\bar{x} \pm s$	Cells Protection Rate/%	Absorbance $\bar{x} \pm s$	Cells Protection Rate/%
Ampelopsin	1.000 mg/mL	1.071±0.041	83.2	0.348±0.014	72.9
	0.500 mg/mL	1.002±0.051	71.8	0.490±0.053	61.9
	0.250 mg/mL	0.927±0.042	59.4	0.752±0.066	41.5
	0.125 mg/mL	0.812±0.027	40.2	0.875±0.023	31.9
	0.050 mg/mL	0.775±0.052	34.2	0.957±0.146	25.5
AZT	10.00 μg/mL	1.006±0.078	72.5	0.410±0.070	68.1
PC	–	0.570±0.026	0.00	1.286±0.053	0.00
NC	–	1.172±0.071	100.0	0.028±0.006	–

Note. Tables 1-3. Anti-HIV-1 effects of ampelopsin. Ampelopsin was added at different phases: at the time of HIV-1 absorbing MT-4 cells (Table 1), at the same time of viral inoculation (Table 2), at the time of cell acutely infected by HIV-1 (Table 3). Cellular protection was tested by MTT assay, and the absorbance (A) was recorded. The protective rates were presented as $(A_{Drug}-A_{PC})/(A_{NC}-A_{PC})\times100\%$. P24 antigen in culture supernatants was detected using ELISA kit, and the P24 inhibitive rate was referred to $(A_{PC}-A_{Drug})/A_{PC}\times100\%$. Data were expressed as $\bar{x} \pm s$ of two independent experiments performed in triplicate.

Moreover, when we used PBMCs as target cells, ampelopsin yielded similar outcomes. For HIV-1_{SF33} attaching, co-cultivating and acute infection, the EC_{50} of cellular protection was 0.146 mg/mL, 0.183 mg/mL and 0.100 mg/mL respectively, while the EC_{50} of P24 inhibition was 0.169 mg/mL, 0.321 mg/mL and 0.249 mg/mL respectively. Ampelopsin had the same effects on HIV-1_{GD-1} or H9 cell line (data not shown).

Ampelopsin Enhanced Chemokine-induced Chemotaxis in PBMCs

PBMCs showed a classic bell-shaped chemotactic response upon exposure to increasing concentrations of either RANTES (Fig.1a) or SDF-1α (Fig.1b), and both concentration-response curves reached maximum at 100 ng/mL of chemokine (Figs.1 a and b). Ampelopsin could affect chemotaxis, reaching maximum at 1 mg/mL, and co-treatment of 1 mg/mL ampelopsin with RANTES or SDF-1α strongly increased cell migration induced by either chemokines (to ~200% at chemokine concentrations of 100-1000 ng/mL, $(P < 0.01)$. RANTES or SDF-1α could also significantly augment ampelopsin-stimulated chemotaxis (Fig.1c, $P < 0.01$).

Ampelopsin Down-regulated CXCR4 on Surface of PBMCs

CXCR4 down-regulation on the surface of PBMCs was monitored by pre-incubating the cells at various concentrations of SDF-1α and ampelopsin for 30 min at 37℃ or 4℃. The cells were then stained with FITC-labeled monoclonal antibody, which could specifically identify the coreceptors on target cells. The mean fluorescence intensity (MFI), detected by flow cytometry, indicated the quantity of receptors on cellular surface[21]. At 37℃, SDF-1α at a concentration above 100 ng/mL could significantly eliminate the MFI on PBMCs ($P < 0.01$). Ampelopsin at a concentration over 0.1 mg/mL had the similar effects

(Fig. 2a, $P<0.01$). However, at 4℃ the MFI was not affected by either SDF-1α or ampelopsin (Fig. 2b, $P>0.05$). The loss of surface CXCR4 induced by ligand binding at 37℃ was presumed to be due to its down-regulation rather than altered receptor accessibility to the antibody, since ligand binding was not affected when measured at 4℃, a temperature at which receptor internalization was prevented[22].

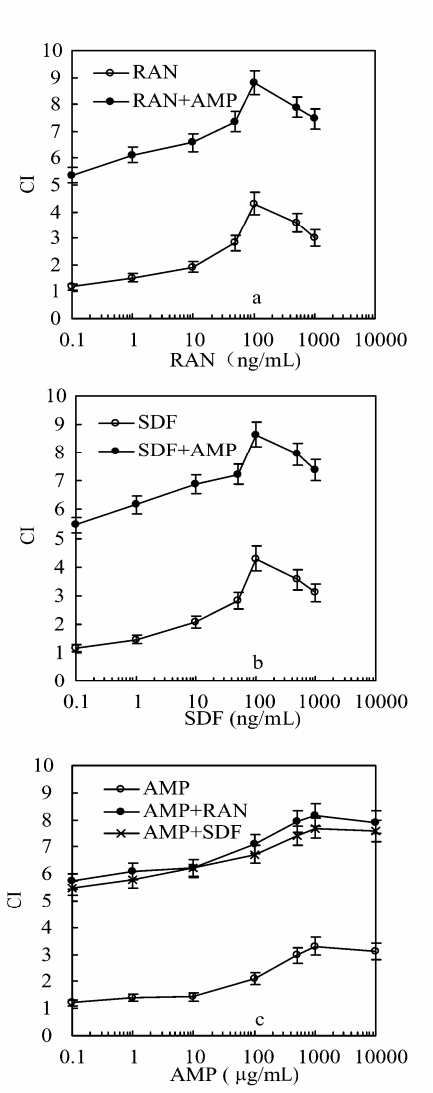

FIG.1. Enhanced RANTES and SDF-1α-induced chemotaxis by ampelopsin in PBMCs. Cells were challenged with RANTES (RAN, a) or SDF-1α (SDF, b) at concentrations indicated in the presence or absence of 1 mg/mL ampelopsin at 37℃ for 2 h, and the chemotaxis was determined by cell counting. The ability to induce cell migration was expressed as CI, which means the value of the amount of cells migrated towards the test sample divided by the amount of randomly migrated cells. PBMCs were incubated at different concentrations of ampelopsin (AMP, c) in the absence or presence of 100 ng/mL RANTES or SDF-1α at the same condition. Data were expressed as $\bar{x} \pm s$ of two independent experiments performed in triplicate.

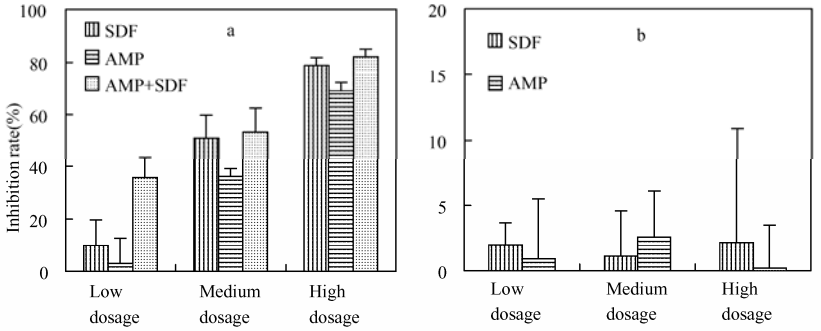

FIG. 2. Ampelopsin down-regulated CXCR4 on surface of PBMCs. PBMCs were incubated at various concentrations of SDF-1α (10, 100, 1 000 ng/mL from low dosage to high dosage) and ampelopsin (10, 100, 1 000 μg/mL from low dosage to high dosage with or without 10 ng/mL SDF-1α) for 30 min at 37℃ (Fig. 2a) or 4℃ (Fig. 2b). The cells were then stained with FITC-labeled CXCR4 monoclonal antibody. The mean fluorescence intensity (MFI) was detected by flow cytometry. CXCR4 expression inhibitive rate was calculated as [1- (MFI_{Drug}-$MFI_{Isotype}$) / (MFI_{NC}-$MFI_{Isotype}$)]×100%. Data were expressed as $\bar{x} \pm s$ of two independent experiments performed in triplicate.

Ampelopsin Augmented Cellular Immune Functions of Mice

When the immunosuppressive model was established, all mice were listless and shed, and those in the contrast NS group were devoid of these effects. After ampelopsin was administrated, the body conditions of mice were obviously improved, while those in the HC group were even worse. The splenic T-lymphocyte proliferation, NK cell activity and IL-2 level in all groups were tested (Figs. 3 a, b, and c). The results revealed that a medium dosage of ampelopsin was proved to be most effective. The splenic T-lymphocyte proliferation, NK cell activity and IL-2 level, were ameliorated by ampelopsin, however there was not a dose-effect relationship. All observed values in the HC group were significantly lower than those in the NS group ($P<0.01=$, indicating the success of mice model. The data of high, medium and low dose ampelopsin groups were higher than those of the HC group ($P<0.01=$. Especially, the medium dose group had a stronger proliferative effect and a higher IL-2 level, while its NK cell activity had no significant difference in comparison with the high dose group ($P>0.05$).

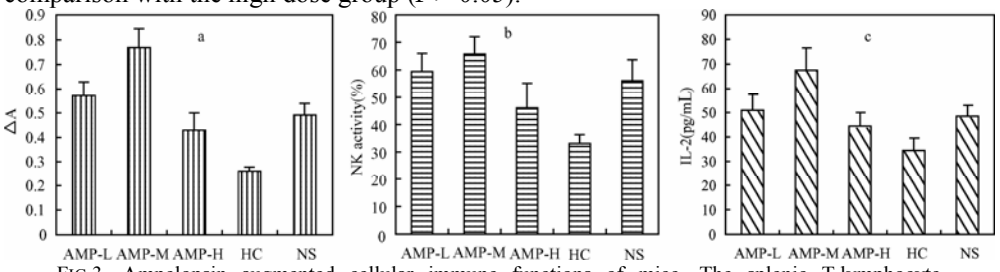

FIG.3. Ampelopsin augmented cellular immune functions of mice. The splenic T-lymphocyte proliferation (Fig. 3a), NK cell activity (Fig. 3b) and IL-2 level (Fig. 3c) were tested. AMP-L, AMP-M and AMP-H were ampelopsin at concentrations of 100 mg/kg, 200 mg/kg and 400 mg/kg respectively. MTT assay was performed respectively, and absorbance (A) was recorded. The T-lymphocyte proliferation was estimated as the difference between A_{PC} and A_{NC} (ΔA). NK cell activity was defined as [1-（A_{E+T}-A_E）/（A_T）]×100%. IL-2 concentration (pg/mL) was calculated by linear regression. Data were expressed as $\bar{x} \pm s$ of two independent experiments performed in triplicate.

DISCUSSION

An increased rate of $CD4^+$ T-cell decline exemplified a more rapid disease course, thus the protection of HIV-1 sensitive cells is one of the indexes to evaluate antiviral effects of test compounds[23]. P24, belonging to the proteins that construct the core of HIV-1, plays an important role in stabilizing viral RNA chain. The level of P24 antigen *in vitro* can be used to indicate viral replication, which is one of the makers to detect anti-HIV agents[24]. In our research, three types of $CD4^+$ T-lymphocytes were utilized as target cells: human PBMCs, MT-4 and H9 cell lines. The results showed that the cytotoxic effect increased along with the dosage, but ampelopsin displayed a relatively low toxicity at the concentration of 1 mg/mL.

The international strain HIV-1_{SF33} and the local strain HIV-$1_{GD\text{-}1}$ are SI viruses with a fast replication rate and a high titer. The antiviral experiments were carried out in three phases: viral entry, co-cultivation and acute infection. Ampelopsin was co-incubated with HIV-1 for 1.5 h, which was the crucial time for viral attaching, and then removed from the culture after subsequent centrifugation. If factors interfering with viral entry existed, the cells infected and viral replication would be lower compared with positive control groups. The data confirmed that after challenged with ampelopsin the cell survival was improved, P24 level was reduced and syncytium was also suppressed. Syncytium was induced by cellular fusion of HIV-1 infected CD_4^+ cells and uninfected ones, so had a close relation with cell lesion and death caused by virus[25]. Our experiments revealed that ampelopsin had a potent inhibition against HIV-1 entry. The results indicated the incapacity of AZT during virus entry, since AZT, as a nucleoside analogue targeting the reverse transcriptase of HIV-1, played a chief role in HIV-1 replication[26].

Chemokine receptors on target cells, integral membrane proteins with seven-transmembrane spanning segments, are the co-receptors for HIV-1 entry. Their physiological function is to bind to chemokines to trigger intracellular cascade through G-proteins[27]. It is known that, HIV-1 entry process involves interactions between HIV-1 envelop protein gp120, CD4 molecule and co-receptors on the surface of target cells, mainly CCR5 and CXCR4[28]. Therefore, drugs that target chemokine receptors, the co-receptors of HIV-1, have a great potential in AIDS therapy. Chemotaxis is the prototypic function of chemokines, and thus serves as a biological relevant function *in vitro* assay for chemokine receptor activation[29]. Ampelopsin could induce PBMCs migration, and yield synergism with RANTES or SDF-1α, which gives a cue that ampelopsin interacts with chemokine receptors. Thus, chemotaxis assay can lead us to relate its antiviral effects with HIV-1 co-receptors. Using FITC-labeled antibody, we observed the effect of ampelopsin on CXCR4, the main co-receptor utilized by T-tropic HIV-1 isolates. Since the internalization of receptors on the surface of cells was prevented at 4℃, ampelopsin lost its ability to reduce the binding of antibody. This outcome indicated that the effect of ampelopsin was to downregulate CXCR4, and this effect could be used to prevent HIV-1 entry. CXCR4 plays an important role in HIV-1 infection, because the principal co-receptors used by HIV-1 change from CCR5 to CXCR4 when the M-tropic strains at early stage are transformed to T-tropic strains afterwards. The SDF-1 gene deletion or CXCR4 gene knockout leads to lethal outcome in mice[30], however, drugs that block CXCR4 have not been found to show hazards in the human body[31]. It seems that CXCR4 can be the target to prevent virus entry. So that, ampelopsin with inhibitive effect on CXCR4 expression, can be developed as a new agent targeting early HIV-1 invasion.

Cellular immunity is the earliest immunologic response occurring at HIV-1 primary

infection, and has a crucial role in the elimination and inhibition of viral propagation at an earlier period[32]. After infection, the functions of NK cells decrease, so do the $CD8^+$ cells. The γ interferon secreted by NK cells can keep the activity of $CD8^+$ cells, the antiviral reaction of which is important for controlling viral load[33]. Human $CD4^+$ cells can be classified into two subgroups: TH1 and TH2. The cytokines produced by both groups mediate the immune functions and influence disease process. Among the TH1-type cytokines, IL-2 is the essential factor to keep the growth and functions of $CD4^+$ as well as $CD8^+$ cells[34]. Moreover, IL-2 maintains the activity of NK cells, and a high IL-2/IL-10 ratio foretells a good prognosis[35]. Thus, the effects of ampelopsin on cellular immunity in mice could be observed. T-lymphocyte proliferation, NK cell activity and IL-2 level were chosen as the major indexes in this study. To simulate the immune functions of HIV-1 infected individuals, an immunosuppressive model in mice was established. Statistical analysis showed that ampelopsin did not have a classic dose-effect response. T-lymphocyte proliferation and IL-2 concentration were increased most dramatically in the medium dosage groups while a similar effect was observed on NK cell activity. So, ampelopsin can be used to ameliorate situations under HIV infection. Our experiment indicates that the dosage plays an important role in its effects. If it is out of range, the advantage may be compromised.

At an early stage of infection, HIV-1 is M-tropic virus, the major co-receptor used by CCR5. This type of HIV-1 can invade macrophages and primary $CD4^+$T cells, but can not replicate in transformed T cells. Therefore, it will be necessary to investigate the preventive effect of ampelopsin in an earlier period using M-tropic virus and sensitive cell lines. Since the co-receptors of HIV-1 belong to G protein coupling receptor family, to estimate the regulation of ampelopsin on G protein activity and intracellular signal transduction pathway, will be the key to explain its antivirus and immunoloregulation mechanisms. Furthermore, the binding ability of ampelopsin to CXCR4 or CCR5 and its structure-effect relationship also wait for clarification. Model of big animals, such as rhesus monkey, which is more similar to the condition of human body, is needed to detail the influence of ampelopsin on immune system during HIV-1 infection.

In general, the discovery of chemokine receptors as HIV co-receptors, has not only delineated the mechanism underlying virus infection, but also shed light on prevention of AIDS at its early stage. Researches focusing on chemokines and their receptors have made great achievements in the past several years, and many agents targeting HIV co-receptors have entered clinical phases. Ampelopsin showed potent anti-HIV-1 effects *in vitro*, and could strengthen immune system *in vivo*. Its effects seemed to be mediated by HIV-1 co-receptors. With abundant resource and low cost, ampelopsin is a promising new agent against HIV infection and in AIDS therapy.

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(*Received March* 23, 2003 *Accepted December* 19, 2003)