APOPTOSIS OF HIV-INFECTED CELLS FOLLOWING TREATMENT WITH SHO-SAIKO-TO AND ITS COMPONENTS

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SUMMARY: Baicalein and baicalin are components of Sho-saiko-to (SST), a Chinese medical drug which is claimed to be therapeutically effective in treating HIV-infected patients. Although 20 μg/ml of baicalin was not cytotoxic to CEM cells, a cultured T cell line, it proved to be cytotoxic to HIV-infected CEM cells (CEM-HIV) with a higher HIV-releasing capacity and DNA fragmentation was detected within 24 hr of incubation. However, after incubation of CEM-HIV with a lower dose of baicalin (0.1, 0.3 and 2 μg/ml) for 24 and 48 hr, the viable cell number increased by about 25% and the p24 release into the medium was 25% lower than that of the control. After further incubation in the presence of the agent for 6 and 9 days, only cells with a lower HIV-releasing capacity survived. Baicalin might selectively induce apoptosis of CEM-HIV cells which have a high virus-releasing capacity, and stimulate proliferation of CEM-HIV which have a relatively lower capacity of HIV-production.

INTRODUCTION

A traditional Chinese medicinal (Kampo) drug, Sho-saiko-to (SST) is reported to be therapeutically effective in the treatment of HIV-infected patients (1-4). Baicalein and baicalin, which are components of SST, have been shown to inhibit reverse transcriptase (RT) of murine leukemia virus (MLV) and type 1 hu-
man immunodeficiency virus (HIV-1) (1). SST and baicalein are reported to inhibit the RT activity by about 50% and 90% at concentrations of 100 μg/ml and 2 μg/ml, respectively (2). When normal lymphoblasts stimulated with PHA were cultured with lymphocytes from HIV-positive patients (an HIV primary infection model) in the presence of SST or baicalein, these agents also suppressed HIV RT activity and production of p24 (3). Furthermore, it has been demonstrated that SST enhances the mitogenic response of peripheral mononuclear cells (5), stimulates production of polyclonal antibody and interleukin 1 (6), and induces proliferation of T helper/inducer lymphocytes (7). These results indicate that SST contains factors which not only possess antiretroviral ability but also can act as modulators of the immune system.

In addition, we report here that SST and its components, baicalein and baicalin, induce and/or enhance apoptosis of HIV-infected CEM cells with a high expression of HIV genes and also stimulate proliferation of HIV-infected CEM cells with a high expression of HIV genes and also stimulate proliferation of HIV-infected CEM cells with a low expression of these genes.

MATERIALS AND METHODS

Flavonoids and chemicals: SST, 5,6,7-trihydroxyflavone (baicalein) and baicalein-7-D-glucuronide (baicalin) were obtained from Tsumura & Co., Tokyo. Each of these drugs was dissolved in dimethylsulfoxide (DMSO) (SST, 200 mg/ml; baicalein and baicalin, 20 mg/ml). The solutions were stored at room temperature in the dark, and diluted with PBS or medium just before use.

Monoclonal antibodies (mAb): 0.5 β, a mouse mAb (8) reactive with V3 loop in gp120 of cytopathic retrovirus (HTLV-IIIB) (9), was kindly provided by Dr. S. Matsushita of Kumamoto University. F105 (10) is a human mAb to the CD4-binding site of HIV-1 gp120 and was a kind gift of Dr. Marshall Posner of Harvard Medical School.

Preparation of chronic HIV-infected cells: CEM cells (11) were cultured in RPMI-1640 supplemented with 10% fetal calf serum and infected with HTLV-IIIB as described (11). The infected cells were cultured 2 months or more before use and more than 90% of the HIV-infected CEM cells (CEM-HIV) expressed stable gp120, as detected with 0.5β mAb by flow cytometric analysis with a FACScan (12).
Detection of apoptosis: HIV-CEM and CEM cells (5 × 10^6/ml) were incubated with various concentrations of baicalin (0.2 to 40 μg/ml) for 24 hr. The viable cell numbers were counted microscopically in a hemocytometer by the trypan blue exclusion method, then the cells were pelleted by centrifugation and lysed with a lysis buffer (50 mM Tris-HCl, 10 mM EDTA-Na and 0.5% w/v sodium-N-lauroylsarcosinate). After digested with RNAsase A for 30 min and proteinase K for 60 min at 50 C to remove RNA and protein, the DNA was analyzed by electrophoresis in 2% agarose gel.

Assay for viable cells and HIV p24 antigen: One hundred microliters of washed CEM-HIV or CEM cells (2 × 10^5/ml) was seeded into wells of a 96-well flat-bottom plate containing 100 μl of various concentrations of SST, baicalein or baicalin in complete medium. After 24 and 48 hr, each culture supernatant was harvested for determination of p24. HIV p24 antigen was detected and semi-quantified with a HIVAGNEIA II (Abbott) Kit (Dainabot Corp., Tokyo). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (13) was used to count of viable cells as follows. First, 20 μl of 7.5 mg/ml MTT in PBS was added to each well and mixed thoroughly by repeated pipetting. The absorbance of each mixture was determined on an ELISA plate reader at a wavelength of 550 nm and a reference wavelength of 630 nm.

Detection of gp120 on CEM-HIV cells: CEM cells infected with HTLV-IIIB (2 × 10^5/ml) were cultured with varying concentrations of baicalin. Every 3 days, the viable cells were counted microscopically in a hemocytometer by the trypan blue exclusion method, washed three times with PBS and adjusted to 2 × 10^5/ml with fresh medium containing baicalin. The gp-120 density on cells was determined with F-105 (0.1 mg/ml). FITC-conjugated mouse anti-human Ig was used as the second Abs. At last, 50 μl of propidium iodide (PI) (1 μg/ml) was added to stain dead cells. After washing, cells were fixed with 1% paraformaldehyde and analyzed in the FACScan. PI-positive dead cells were excluded from calculation of fluorescence intensity.

RESULTS

When CEM-HIV cells were incubated with baicalin for 24 hr, at doses of 20 and 40 μg/ml, electrophoresis of DNA of the cells revealed DNA fragmentation bands, whereas, under the same conditions, normal CEM cells did not (Fig. 1A).
Fig. 1. Baicalin induced apoptosis of CEM-HIV cells. CEM-HIV and normal cells (1 × 10^6) cells were cultured with various concentrations of baicalin for 24 hr at 37 °C. The cells were lysed and digested with RNAase A and Proteinase K and then analyzed by electrophoresis in a 2% agarose gel (A), the viable cell numbers were also counted microscopically in a hemocytometer by the trypan blue exclusion method and expressed in percentage to those of control cells with no baicalin (B). CEM-HIV (1 × 10^6) cells were cultured with 20 μg/ml of baicalin for 24 hr at 37 °C, then, gp120 was detected with F-105 mAb and dead cells were excluded by PI stain (C).
Fig. 2. Effect of a low dose of baicalin on viable cell number and virus antigen. CEM (A) or CEM-HIV (B) cells (2 x 10^5) were incubated at 37 C in the presence of various concentrations of baicalin. After 24 (○) and 48 hr (□), the number of viable cells were counted by the MTT method. p24 antigen in supernatant of cultured CEM-HIV cells was also determined and the relative dose of p24/2 x 10^5 viable cell was calculated (C). CEM-HIV cells (2 x 10^5) were cultured in the presence of 0.1 (○), 0.3 (□) and 2 μg/ml (■) baicalin. After 3, 6 and 9 days, gp-120 was detected with F-105 mAb followed by indirect immunofluorescence staining and flow cytometric analysis with a FACScan. The mean fluorescence intensity was then calculated and expressed in the percentage to that of control cells with no baicalin (D).

This DNA fragmentation is a typical phenomenon of apoptosis. Figure 1B shows the viable cell number in this time. Baicalin of 20 μg/ml slightly affected normal CEM cells but was cytotoxic to CEM-HIV. Figure 1C shows that after incubation of CEM-HIV cells with 20 μg/ml of baicalin for 24 hr, the fluorescence intensity of gp120 on survived cells detected with F-105 mAb was lower than that of the con-
Because F-105 has been reported to react only with gp120 of virus-budding cells (14), it is thought that these survived cells have a lower HIV-releasing capacity, and baicalin can selectively induce apoptosis of CEM-HIV cells which have a high virus-releasing capacity. The growth rate of CEM-HIV cells was about 10% lower than normal CEM cells. However, the addition of a low dose of baicalin (0.1 to 5 μg/ml) to the culture medium of these cells restored their growth potential to the level of uninfected CEM cells (Figs. 2A, B).

A low dose of baicalin did not influence the viable cell number of normal CEM cells after incubation for 24 or 48 hr (Fig. 2A), although the number of CEM-HIV cells was shown to have increased (Fig. 2B). Moreover, a low dose of baicalin suppressed release of p24 antigen into the culture supernatant in a dose-dependent manner (Fig. 2C). SST and baicalein had the same effects as baicalin (data not shown).

The mean fluorescence intensity of gp120 on CEM-HIV cells detected with F-105 mAb was also lower than that of the control by about 15% when CEM-HIV cells had been cultured in the presence of a low dose of baicalin for a longer period (6 or 9 days) (Fig. 2D).

**DISCUSSION**

In the present study, we confirmed that a high dose of baicalin (20 μg/ml) can selectively induce apoptosis of CEM-HIV cells which have a high virus-releasing capacity. Although no DNA fragmentation was detected after cultivation of CEM-HIV cells with low dose of baicalin for 24 hr, only a low capacity of virus-budding cells survived after relatively a long period (6 or 9 days). This is just the same as the effect of a high dose of baicalin. On the other hand, cultivation of CEM-HIV cells with a low dose of baicalin for 24 and 48 hr increased the viable cells in number and resulted in a decreased amount of p24 antigen released into the supernatant. In populations of CEM cells chronically infected with HIV (CEM-HIV), most of the cells were infected with this virus, and the provirus of HIV had been incorporated into chromosomal DNA. Therefore, the decrease in p24 may not have been due to inhibition of the RT function by baicalin. Baicalin may have caused apoptosis of the cells which had been releasing large amounts of p24 antigen or instead have stimulated the growth of the cells releasing low levels of this antigen. Although there may be other mechanisms by which baicalin
can suppress synthesis or release of p24 antigen, apoptosis induced by this drug must have some role in the suppression of p24 antigen generation. It has been reported that in vitro CD4+ cells from AIDS patients respond normally to pokeweed mitogen (PWM), a mitogen which induces proliferation of both B lymphocytes and helper/inducer T lymphocytes without stimulation of suppressor/cytotoxic T lymphocytes (15). However, in the presence of SST (20 μg/ml), the proliferative response to PWM was found to be enhanced (+23%) in a group of patients with relatively high counts of CD4+ cells (average CD4+/CD4- = 0.092) but was suppressed (−14%) in another group of patients with relatively low CD4+ cell counts (average CD4+/CD4- = 0.070) (4). These results with lymphocytes of HIV-infected patients are similar to the results reported here.

Although the precise mechanism by which baicalin-induced apoptosis of CEM-HIV cells is unknown, it seemed not via the Fas antigen system (16), because after treatment of CEM-HIV cells with 20 μg/ml of baicalin for 24 hr, Fas antigen on CEM-HIV could not be enhanced (data not shown).

In conclusion, the present findings suggest that SST and its components have the ability to induce apoptosis of HIV heavily infected cells and the capacity to induce proliferation of HIV-infected cells with a low expression of the HIV-genome. Therefore, administration of these agents to HIV-infected patients might prolong their life span.

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REFERENCES


