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Suppression of cytokine production and neural cell death by the anti-inflammatory alkaloid cepharanthine: a potential agent against HIV-1 encephalopathy

Mika Okamoto[a], Minoru Ono[b], Masanori Baba[a,*]

[a]*Division of Human Retroviruses, Center for Chronic Viral Diseases, Faculty of Medicine, Kagoshima University, 8–35-1, Sakuragaoka, Kagoshima 890-8520, Japan*

[b]*Research Laboratories, Kaken Shouyaku Co., Mitaka, Tokyo 181-0013, Japan*

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Abstract

Inflammatory cytokines and human immunodeficiency virus type 1 (HIV-1) gp120 are considered to play an important role in the pathogenesis of HIV-1-associated CNS disorders. These substances are produced predominantly by HIV-1-infected or activated macrophages and microglia in the brain and induce neural cell death. Cepharanthine is a biscoclaurine alkaloid isolated from *Stephania cepharantha* Hayata and has been shown to have anti-inflammatory, anti-allergic, and immunomodulatory activities *in vivo*. We previously reported that this compound could inhibit tumor necrosis factor (TNF)-α- or phorbol 12-myristate 13-acetate-induced HIV-1 replication in latently infected U1 cells through the inhibition of nuclear factor-κB, a potent inducer of HIV-1 gene expression. In the present study, we demonstrated that cepharanthine suppresses the production of inflammatory cytokines and a chemokine, i.e. TNF-α, interleukin (IL)-1β, IL-6, and IL-8, in human monocytic cell cultures, including primary monocyte/macrophage cultures. This effect of cepharanthine was concentration-dependent, and significant suppression was observed at 0.1 μg/mL. Furthermore, the compound also inhibited TNF-α- and gp120-induced death of differentiated human neuroblastoma cells at a concentration of 0.04 to 0.2 μg/mL. It penetrates the blood–brain barrier, and a medicine containing cepharanthine as a major component has been used in Japan for the treatment of patients with chronic inflammatory diseases. Thus, cepharanthine should be investigated further for its therapeutic and prophylactic potential in HIV-1-associated CNS disorders.

Keywords: Cepharanthine; Alkaloid; Inhibitor; HIV-1; Inflammatory cytokines; Monocytes; Macrophages; Neural cell death; AIDS dementia

1. Introduction

HIV-1-associated CNS disorders, including encephalopathy, frequently occur in the late stage of HIV-1 infection [1]. The pathological abnormalities of HIV-1-associated encephalopathy are characterized by brain atrophy, multifocal giant cell infiltration, and diffuse leukoencephalopathy [2,3]. These pathological changes are widely diffused in the brain of HIV-1-infected patients, whereas productive HIV-1 infection is detected only in macrophages and microglia. The obvious HIV-1 infection cannot be identified in neurons and oligodendrocytes [1]. Although the pathogenesis of HIV-1-associated encephalopathy has not been fully elucidated, recent studies have demonstrated that some inflammatory cytokines and viral antigens are involved in the neuropathogenesis of HIV-1 infection.

In HIV-1-infected individuals, the levels of inflammatory cytokines, such as TNF-α, IL-1, and IL-6, are elevated significantly [4]. Since these cytokines are released abundantly from HIV-1-infected brain macrophages, microglia, and astrocytes [5,6], it is likely that the levels of inflammatory cytokines are also elevated in the brain [7]. TNF-α has been shown to have neurotoxic effects *in vitro*, and a pos-

* Corresponding author. Tel.: +81-99-275-5930; fax: +81-99-275-5932.

E-mail address: baba@m.kufm.kagoshima-u.ac.jp (M. Baba).

Abbreviations: HIV-1, human immunodeficiency virus type 1; TNF, tumor necrosis factor; IL, interleukin; PMA, phorbol 12-myristate 13-acetate; FBS, fetal bovine serum; ROS, reactive oxygen species; NF-κB, nuclear factor κB; NAC, *N*-acetyl-l-cysteine; M/M, monocyte/macrophage; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; NO, nitric oxide; and HAART, highly active antiretroviral therapy.

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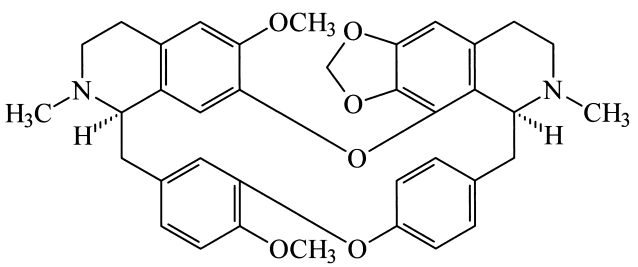

Fig. 1. Chemical structure of cepharanthine.

sible mechanism of its neurotoxicity is the induction of apoptosis by increasing intracellular oxidative stress [8]. In addition, HIV-1-infected macrophages and microglia become reservoirs that continuously produce viral antigens [9]. Among the viral antigens, gp120, gp41, and Tat have proved neurotoxic *in vitro* [10–12]. The mechanism of gp120-induced neural cell death has also been proven to be apoptosis, resulting from the induction of ROS [13,14]. These observations have prompted us to investigate the compounds that inhibit HIV-1 replication in chronically infected macrophages and microglia, suppress the production of inflammatory cytokines from activated immune cells, and antagonize the neurotoxicity of cytokines and viral antigens.

We have recently demonstrated that cepharanthine is a highly potent and selective inhibitor of HIV-1 replication in the chronically infected monocytic cell line U1 [15]. Cepharanthine (Fig. 1) is a biscoclaurine alkaloid isolated from *Stephania cepharantha* Hayata and has been shown to have anti-inflammatory, anti-allergic, and immunomodulatory activities *in vivo* [16–18]. Cepharanthine could inhibit TNF-α- or PMA-induced viral production in U1 cells at low (ng/mL) concentrations. Studies on its mechanism of action revealed that cepharanthine is inhibitory to the activation of NF-κB [15]. We assumed that the compound had potential as a prophylactic and therapeutic agent against HIV-1-induced CNS disorders, yet its inhibitory effects on cytokine production and neural cell death remained to be elucidated. In this study, we have demonstrated that cepharanthine is able to suppress the production of inflammatory cytokines and a chemokine in monocytic cells. In addition, the compound is also inhibitory to the TNF-α- and gp120-induced death of differentiated human neuroblastoma cells.

2. Materials and methods

2.1. Compounds and reagents

Cepharanthine (6′,12′-dimethoxy-2,2′-dimethyl-6,7-[methylenebis(oxy)]oxyacanthan) was derived from *S. cepharantha* Hayata, and its purity (>99%) was confirmed by HPLC analysis. It was dissolved in dimethyl sulfoxide at 20 mg/mL (or higher) and was stored at –20° until used. TNF-α was purchased from Boehringer GmbH; LPS and NAC were from the Sigma Chemical Co. Recombinant HIV-1 gp120 (III_B strain) was obtained from ImmunoDiagnostic Inc. gp120 was produced by Chinese hamster ovary cells, and its purity was more than 95%.

2.2. Cells

The monocytic cell line U937 and human primary M/Ms were used in the inhibition assay for cytokine production. U937 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 U/mL of penicillin G, and 100 μg/mL of streptomycin. M/Ms were isolated from a healthy donor and cultivated according to a procedure described by Perno *et al.* [19]. The isolated cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 10% human AB serum, and antibiotics. The human neuroblastoma cell line SK-N-MC was obtained from the American Type Culture Collection (ATCC HTB-10). The cells were maintained in Eagle's minimum essential medium (Dulbecco's modification) supplemented with 10% FBS and antibiotics. Prior to stimulation with TNF-α or gp120, the cells were differentiated to a neural phenotype by the addition of 5 μM retinoic acid (Sigma) for 4 days. Exposure of SK-N-MC cells to retinoic acid resulted in morphologic changes, including the development of neuritic processes as well as the expression of neural cell markers.

2.3. Cytokine production assay

U937 cells (5×10^5 cells/mL) were incubated at 37° in the absence or presence of cepharanthine for 2 hr, stimulated with PMA (10 ng/mL), and incubated further. For the assay in M/Ms, the isolated monocytes (2×10^5 cells/mL) were cultured for 7 days to differentiate to macrophages. The cells were pretreated with the test compound for 2 hr, stimulated with LPS (10 μg/mL), and incubated further. After a 24-hr incubation, the culture supernatants were collected and examined for cytokine or chemokine levels with a cytokine- or chemokine-detection ELISA kit (Genzyme). The cytotoxicity of cepharanthine was determined by the MTT method [20].

2.4. Cell death inhibition assay

SK-N-MC cells (5×10^4 cells/mL) were grown and differentiated in poly-L-lysine-coated 24-well plates. The cells were pretreated with or without the test compounds (NAC or cepharanthine) for 2 hr in culture medium supplemented with 1% FBS and stimulated with TNF-α (1 and 10 ng/mL) or gp120 (1 nM). After a 2-day incubation at 37°, cell viability was determined by trypan blue exclusion. Cell death was also analyzed by flow cytometry. To this end, the cells were pretreated with or without the test compounds (NAC or cepharanthine) for 2 hr in culture medium supplemented with 1% FBS and stimulated with TNF-α (10 ng/

mL). After a 12-hr incubation, the cells were collected and washed with PBS, fixed with 70% ethanol on ice for 30 min, and washed with PBS again. The cell pellets were then incubated with 0.1 mg/mL of RNase A at 37° for 20 min. After incubation, the cell suspensions were centrifuged (400 *g*) at 4° for 5 min. The precipitates were stained with 25 μg/mL of PI (Sigma) on ice for 20 min, resuspended in PBS containing 0.1% bovine serum albumin, and analyzed by FACScan™ (Becton Dickinson). To morphologically detect the apoptotic nuclei, SK-N-MC cells were grown in poly-l-lysine-coated culture slides (Becton Dickinson). The cells were treated and cultured as described above and subjected to modified TUNEL staining [21] with a DeadEnd™ Colorimetric Apoptosis Detection System (Promega), according to the protocol of the manufacturer.

3. Results

3.1. Suppression by cepharanthine of cytokine and chemokine production

To investigate the activities of cepharanthine, we conducted a series of experiments to determine whether the compound suppressed the production of TNF-α, IL-1β, IL-6, and IL-8, the cytokines and chemokine considered to be involved in the inflammation and death of neural cells. When U937 cells were stimulated with 10 ng/mL of PMA in the absence of cepharanthine, 170-, 5.1-, and 19-fold elevations of TNF-α, IL-1β, and IL-8 levels in the culture supernatants were observed, as compared with those in unstimulated U937 cells, respectively (Table 1). On the other hand, a significant elevation was not observed for IL-6 production (data not shown). Cepharanthine did not reduce the viability and proliferation of PMA-stimulated U937 cells at concentrations up to 1 μg/mL after a 24-hr incubation period (Table 1). However, it did reduce the viable cell number to 63% of the control culture (no compound) at a concentration of 10 μg/mL. Therefore, cepharanthine was tested in subsequent experiments at concentrations of less than 1 μg/mL. The compound suppressed the production of TNF-α, IL-1β, and IL-8 in a concentration-dependent fashion (Table 1). The levels of TNF-α, IL-1β, and IL-8 in the presence of 1 μg/mL of cepharanthine were approximately 48, 49, and 41%, respectively, of those in the absence of the compound.

To confirm the effect of cepharanthine, we examined whether it could suppress cytokine and chemokine production in human primary M/Ms. As shown in Fig. 2, M/Ms produced little, if any, TNF-α, IL-1β, IL-6, and IL-8 without stimulation. However, when stimulated with 10 μg/mL of LPS, abundant levels of TNF-α, IL-6, and IL-8 were detected in the culture supernatants of M/Ms after a 24-hr incubation period. The level of IL-1β was found to be similar to that in PMA-stimulated U937 cells (Table 1). In any case, however, cepharanthine could suppress the production of these cytokines and chemokine in LPS-stimulated M/Ms in a concentration-dependent fashion (Fig. 2). The levels of TNF-α, IL-1β, IL-6, and IL-8 in the presence of 1 μg/mL of cepharanthine were approximately 54, 40, 72, and 53%, respectively, of those in the absence of the compound. Cepharanthine did not alter the morphology or viability of M/Ms at test concentrations (data not shown). Thus, except for IL-6, cepharanthine proved to be a potent inhibitor of inflammatory cytokines and chemokine in monocytic cells.

Table 1
Inhibitory effect of cepharanthine on cytokine and chemokine production in U937 cells

	Cepharanthine concentration (μg/mL)	Cytokine or chemokine (pg/mL)	Cell viability (%)
TNF-α			
NC	0	5 ± 1	ND[a]
PMA	0	850 ± 170	100
	0.01	740 ± 220	98 ± 7
	0.1	510 ± 160*	99 ± 9
	1	410 ± 200**	103 ± 20
	10	ND	63 ± 11**
IL-1β			
NC	0	1.0 ± 0.8	
PMA	0	5.1 ± 0.8	
	0.01	4.8 ± 0.5	
	0.1	3.1 ± 0.6	
	1	2.5 ± 0.9	
IL-8			
NC	0	1,400 ± 300	
PMA	0	27,000 ± 4,000	
	0.01	25,000 ± 5,000	
	0.1	14,000 ± 4,000*	
	1	11,000 ± 3,000*	

U937 cells were incubated in the absence or presence of cepharanthine for 2 hr, unstimulated (NC) or stimulated with PMA (10 ng/mL), and incubated further. After a 24-hr incubation, cytokine or chemokine levels and cell viability were determined by ELISA and the MTT method, respectively. Data are means ± SD of at least three separate experiments. The statistical significance between the control (PMA-treated but compound-untreated) sample and each compound-treated sample was determined by the *t*-test.

[a] ND: not determined.

* $P < 0.01$.

** $P < 0.05$.

3.2. Cepharanthine inhibition of neural cell death

To investigate the effect of cepharanthine on neural cell death, SK-N-MC cells were differentiated by treatment with 5 μM retinoic acid for 4 days. After differentiation to a neural phenotype, the viability of SK-N-MC cells was approximately 80%, as determined by trypan blue exclusion (Fig. 3). When the cells were stimulated with 1 and 10 ng/mL of human recombinant TNF-α for 2 days, their viability was reduced to 56.6 and 51.5%, respectively (Fig. 3), indicating that TNF-α is cytotoxic to the differentiated

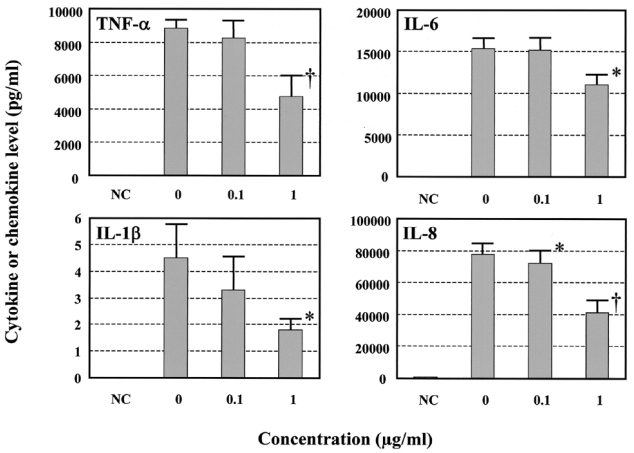

Fig. 2. Inhibitory effect of cepharanthine on cytokine and chemokine production in M/Ms. Human primary M/Ms were incubated in the absence or presence of the test compound for 2 hr, unstimulated (NC) or stimulated with LPS (10 μg/mL), and incubated further. After a 24-hr incubation, the levels of TNF-α, IL-1β, IL-6, and IL-8 were determined by ELISA. Data are mean values ± SD of triplicate experiments. The statistical significance between the control (LPS-treated but compound-untreated) sample and each compound-treated sample was determined by the *t*-test. Key: (*) $P < 0.05$, and (†) $P < 0.01$.

SK-N-MC cells. Since the neurotoxicity of TNF-α seems to be mediated by intracellular ROS production, we examined whether the antioxidant NAC was protective against neural cell death. Treatment with 81.6 μg/mL (500 μM) of NAC increased the cell viabilities to 68.8 and 61.2% in the presence of 1 and 10 ng/mL of TNF-α, respectively (Fig. 3). Cepharanthine could prevent the TNF-α-induced neural cell death at a much lower concentration than NAC. The effect of cepharanthine was concentration-dependent, and some protection against cell death was identified even at 0.008 μg/mL (Fig. 3). Treatment with 0.04 and 0.2 μg/mL of cepharanthine significantly increased the viability of TNF-α-stimulated SK-N-MC cells (Fig. 3). Interestingly, cepharanthine was found to be less protective against cell death at 1 μg/mL than at 0.2 μg/mL, probably due to its potential toxicity for the differentiated SK-N-MC cells (Fig. 3). In addition, the compound was toxic to the undifferentiated SK-N-MC cells at 10 μg/mL (data not shown). Its 50% inhibitory concentration for cell proliferation was 4.6 μg/mL.

It has been demonstrated that HIV-1 gp120 is neurotoxic and that its toxicity is also attributed to the induction of intracellular ROS production [13,14]. Therefore, we investigated the toxicity of human recombinant gp120 to the differentiated SK-N-MC cells. Like TNF-α treatment, a 2-day stimulation with 1 nM gp120 reduced the viability from 61.3% (unstimulated control) to 43.3% (Fig. 4). In contrast, the addition of NAC or cepharanthine significantly protected the cells against the gp120-induced toxicity. Pretreatment with 81.6 μg/mL of NAC and 0.2 μg/mL of cepharanthine increased cell viability up to 63 and 52.6%, respectively (Fig. 4). Again, 1 μg/mL of cepharanthine showed reduced protection against gp120-induced cell death (Fig. 4).

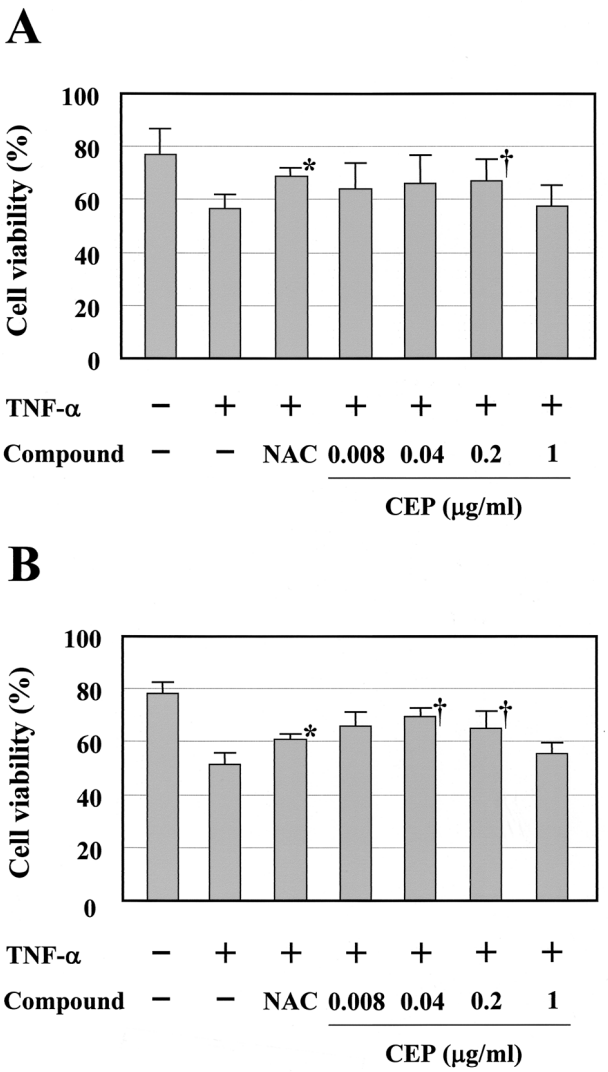

Fig. 3. Suppression of TNF-α-induced neural cell death by cepharanthine. SK-N-MC cells (5×10^4 cells/mL) were grown and differentiated, as described in "Materials and methods." The cells were untreated (–) or treated with 81.6 μg/mL of NAC, or with 0.008, 0.04, 0.2, or 1.0 μg/mL of cepharanthine (CEP). The cells were then unstimulated (–) or stimulated (+) with 1 ng/mL (A) or 10 ng/mL (B) of TNF-α. After a 2-day incubation, cell viability was determined by trypan blue exclusion. Data represent mean values ± SD of at least three separate experiments. The statistical significance between the control (TNF-α-treated but compound-untreated) sample and each compound-treated sample was determined by the *t*-test. Key: (*) $P < 0.05$, and (†) $P < 0.01$.

To confirm the results obtained by trypan blue exclusion and gain an insight into the mechanism of TNF-α or gp120-induced cell death, the cells were stained with PI and analyzed by flow cytometry. The number of dead cells was increased (from 31 to 41%) by stimulation with TNF-α (Fig. 5). However, treatment with NAC reduced the number of dead cells to 36 and 34% at a concentration of 16.3 (data not shown) and 81.6 μg/mL (Fig. 5), respectively. Cepharanthine (0.2 μg/mL) almost completely protected SK-N-MC

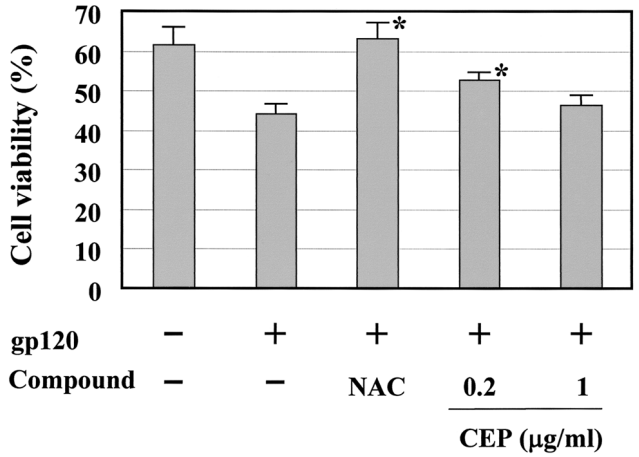

Fig. 4. Suppression of gp120-induced neural cell death by cepharanthine. SK-N-MC cells (5×10^4 cells/mL) were grown and differentiated, as described in "Materials and methods." The cells were untreated (–) or treated with 81.6 μg/mL of NAC, or with 0.2 or 1.0 μg/mL of cepharanthine (CEP). The cells were then unstimulated (–) or stimulated (+) with 1 nM gp120. After a 2-day incubation, cell viability was determined by trypan blue exclusion. Data represent mean values ± SD of at least three separate experiments. The statistical significance between the control (gp120-treated but compound-untreated) sample and each compound-treated sample was determined by the *t*-test. Key: (*) $P < 0.05$.

cells against TNF-α-induced cell death (Fig. 5). Furthermore, similar results were obtained in gp120-stimulated SK-N-MC cells, where the percentages of dead cells were 24% (unstimulated control cells), 32% (stimulated control cells), 26% (stimulated cells in the presence of 81.6 μg/mL NAC), and 25% (stimulated cells in the presence of 0.2 μg/mL of cepharanthine) (data not shown). TUNEL staining of the TNF-α-stimulated cells showed that the cell death was due to apoptosis (Fig. 5), so that cepharanthine and NAC proved to have anti-apoptotic activity in neural cells.

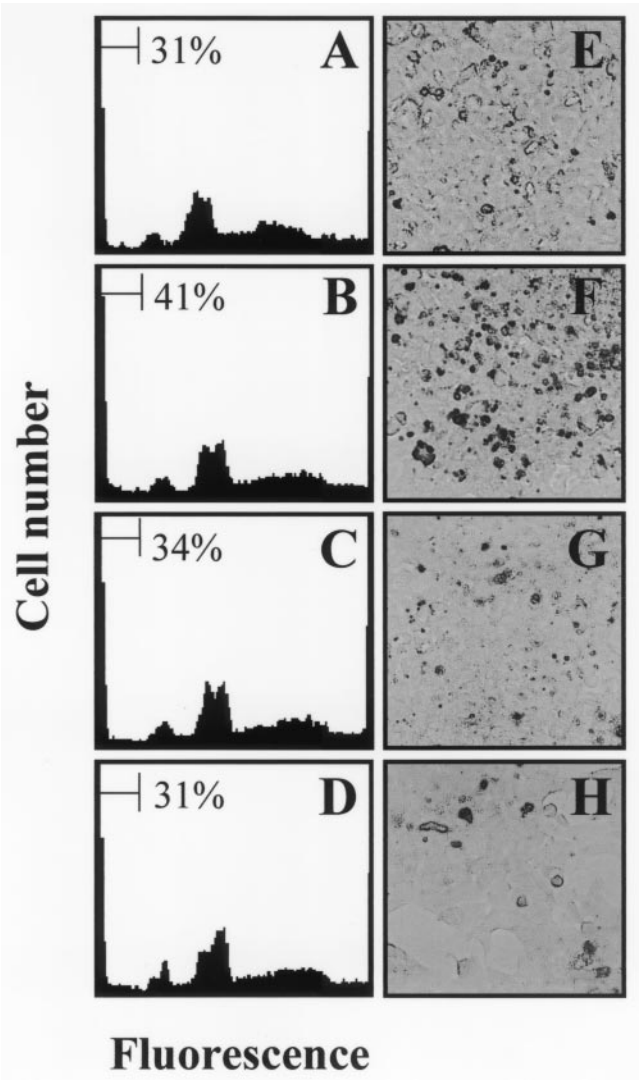

Fig. 5. PI (left panels) and TUNEL (right panels) staining for TNF-α-stimulated neural cells. SK-N-MC cells were grown and differentiated, as described in "Materials and methods." The cells were untreated (A, B, E, and F) or were treated with 81.6 μg/mL of NAC (C and G) or 0.2 μg/mL of cepharanthine (D and H) for 2 hr. The cells were then unstimulated (A and E) or stimulated with 1 ng/mL of TNF-α (B-D and F-H). After a 12-hr incubation, the cells were stained with PI and subjected to laser flow cytometric analysis. The number in each panel indicates the percentage of dead cells. The cells were also subjected to TUNEL staining and observed microscopically (magnification: ×100). Experiments were repeated three times, and representative results are shown.

4. Discussion

In this study, we demonstrated that cepharanthine could suppress the production of TNF-α, IL-1β, and IL-8 in stimulated monocytic cells (Table 1 and Fig. 2). Brain macrophages are known to produce inflammatory cytokines, which could contribute to neural cell death [7,22]. In fact, we have demonstrated that TNF-α induced cell death in the neural cell line SK-N-MC (Fig. 3). The effective concentrations of cepharanthine against cytokine production and neural cell death were less than 1 μg/mL and as low as that against the replication of HIV-1 in chronically infected cells [15].

TNF-α and gp120 have been reported to induce apoptosis in neural cells [8,14], and several studies have suggested that TNF-α is protective for primary neurons [23]. Furthermore, the mechanisms of neural cell apoptosis have not been fully elucidated. Previous studies have demonstrated that the production of intracellular ROS accounts, at least in part, for the apoptosis [8,13]. Since both cepharanthine and NAC have antioxidant properties, they are assumed to suppress ROS production. Furthermore, the neurotoxicity of gp120 is mediated by the induction of the tumor suppressor protein p53 [14]. p53 induces apoptosis through a three-step process: the transcriptional induction of redox-related genes; the formation of ROS; and the oxidative degradation of mitochondrial components, resulting in cell death [24].

Cepharanthine inhibits NF-κB activation in monocytic cells chronically infected with HIV-1, as demonstrated by a gel-mobility shift assay [15]. Although the relation between

NF-κB activation and HIV-1-associated encephalopathy is still unclear, some studies have suggested that the apoptosis of neural cells is mediated, in part, by NF-κB activation [25–27]. It was shown that the neurotoxicity of gp120 is mediated by NO through a pathway involved in superoxide anions [28] and that NO also mediates HIV-1 gp41-induced neurotoxicity [29]. The human inducible NO synthetase promoter requires the activation of phosphatidylcholine-specific phospholipase C and NF-κB [30], and cepharanthine is known to suppress NO production in activated macrophages [31]. Thus, it is possible that the inhibition of NF-κB activation and the subsequent suppression of NO production is, in part, a mechanism of the protective effect of cepharanthine on neural cells.

It has been reported that a high level of HIV-1 RNA in the cerebrospinal fluid is associated with HIV-1 encephalitis [32], which was remarkably improved by HAART. In addition, HAART suppressed the expression of inflammatory neurotoxins in patients with HIV-1-associated dementia [33]. HAART has achieved lasting suppression of the virus, yet the present treatment may not be able to eradicate HIV-1 from chronically infected cells, such as macrophages and resting T cells [34,35]. These cells are likely to migrate through the blood–brain barrier [36]. Furthermore, most of the licensed anti-HIV-1 drugs have a limited capacity to enter the brain [37–39]. In addition, neuro-tropic HIV-1 strains may differ in virological properties from the strains existing in the body [40]. Suboptimal concentrations of the anti-HIV-1 agents for neurotropic strains may easily develop drug-resistant mutants or generate HIV-1 reservoir cells in the brain. Cepharanthine can penetrate the blood–brain barrier [41]. Biscoclaurine alkaloids, containing cepharanthine as a major component, have been widely used in Japan for the treatment of chronic inflammatory diseases, radiation-induced leukopenia, asthma bronchiale, and alopecia areata without serious side-effects. Thus, the compound should be investigated further for its therapeutic and prophylactic potential in HIV-1-associated CNS disorders.

Acknowledgements

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