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Sodium benzylideneascorbate induces apoptosis in HIV-replicating U1 cells

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Abstract

U1 cells, a subclone of U937 cells chronically infected with human immunodeficiency virus type 1 (HIV-1), produced HIV-1 only in the presence of inducers such as 12-*O*-tetradecanoxylphorbol 13-acetate (TPA) or tumor necrosis factor (TNF)-α. The expression of HIV-antigen on U1 cells induced by TPA or TNF-α was found to be prevented by sodium 5,6-benzylidene-L-ascorbate (SBA) in a concentration-dependent manner. Treatment of U1 cells with SBA in the presence of inducers resulted in cell death with cell shrinkage, chromatin condensation and DNA fragmentation into nucleosomal oligomers, characteristics of apoptosis. In contrast, SBA had scarcely any apoptotic effect on U1 cells in the absence of inducers. SBA did not also induce apoptosis in parental U937 cells in the presence or absence of inducers. These results suggest that HIV-replicating U1 cells selectively undergo apoptosis on treatment with SBA.

Key words: HIV replication; Apoptosis; Benzylideneascorbate; Anti-HIV drug

1. Introduction

Various benzaldehyde derivatives, such as SBA, 4,6-benzylidene-α-D-glucose (BG) and β-cyclodextrin benzaldehyde inclusion compound (CDBA) are known to be antitumor drugs [1–8]. Especially, SBA has been shown to have antitumor activity not only on chemically-induced rat tumors but also on inoperable human tumors of the ovary, stomach, pancreas, uterus, bile duct and lung [7,8]. We previously showed that SBA had no apparent immuno-potentiation activity such as for stimulating the productions of cytokines, TNF and interleukin-1 and -2 in vitro or in vivo [7,8]. Furthermore, we recently found that death of tumor cells treated with SBA was due to direct induction of apoptosis [9].

Apoptosis is a process of physiological and pathological cell death that occurs during embryogenesis, thymic negative selection, hormone-induced tissue atrophy, normal tissue turnover and tumor regression [10–13]. The most widely accepted criterion of apoptosis is cell morphological alterations. It is characterized by cell shrinkage, fragmentation and condensation of chromatin and disappearance of cell surface microvilli [10,12]. Furthermore, the internucleosomal cleavage of chromosomal DNA is the biochemical characteristic most commonly associated with apoptosis [11,13]. The biological significance of apoptosis stems from its active cell-elimination mechanism in response to wide variety of signals, including hormones, chemotherapeutic agents and ionizing radiation, and its control functions for physiological systems, i.e. neural, endocrine and immune systems.

The National Cancer Institute/the National Institutes of Health has implicated a screening system for discovery of new classes of synthetic and natural compounds with anti-HIV activity. Apoptosis inducible activity of SBA and the possible availability of its derivatives prompted us to explore the potential anti-HIV activity of this chemical class. There is also no information so far about the effect of SBA on HIV replication. We, therefore, investigated the ability of SBA to inhibit HIV-1 replication in cultured U1 cells infected with HIV-1. We demonstrate here that SBA selectively induced apoptotic cell death of U1 cells expressing HIV-1 in the presence of inducers. These findings provide encouragement for its consideration as a new mechanistic type of anti-HIV drug.

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Abbreviations: SBA, sodium 5,6-benzylidene-L-ascorbate; HIV, human immunodeficiency virus; TPA, 12-*O*-tetradecanoxylphorbol 13-acetate; NFκB; nuclear factor kappa B; LTR, long terminal repeat; TNF, tumor necrosis factor; PKC, protein kinase C; bp, base pair.

2. Materials and methods

2.1. Materials

SBA, which is a mixture of diastereomers (S/R = 1/2) (see Fig. 1 for chemical structures) [9], was purchased from ChemiScience Ltd.,

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Tokyo. TPA, RNase A and proteinase K were from Sigma. Recombinant human TNF-α more than 99% pure had a specific activity of 3×10^7 units/mg protein. DNA size markers of φX174 DNA digests with *Hae*III were from Toyobo Co. Ltd., Japan. All other reagents were of analytical grade.

2.2. Cell culture and virus

Human histiocytic lymphoma (U937) cells and a subclone of U937 cells integrated with two copies of HIV-1, U1 cells, were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, penicillin (100 IU/ml) and streptomycin (100 μg/ml) at 37°C in Co2 incubator. Cell viability was measured as exclusion of 0.2% Trypan blue under phase contrast microscopy. The frequency of apoptotic cells was counted under light microscopy after staining with erythrocin B. A strain of HIV-1, HTLV-IIIB, was used in the anti-HIV assay. The virus was prepared from the culture supernatant of Molt-4/HTLV-IIIB cells which were persistently infected with HTLV-IIIB [14].

2.3. HIV expression

HIV-expressing cells were determined by using human polyclonal anti-HIV-1 antibody. U1 or U937 cells were cultured in the presence or absence of an inducer for the indicated times and then successively treated with human polyclonal anti-HIV-1 positive serum and FITC-conjugated rabbit anti-human IgG. Numbers of HIV-1 antigen positive cells were counted in a CytoACE-150 (Japan Spectroscopic Co. Ltd., Japan) equipped with a 488 nm Argon laser and 10 mW light output. FITC emission signals were collected using the standard filter set and amplified logarithmically. Ten-thousand events per sample were collected, stored and analyzed [15].

2.4. DNA fragmentation assay

Cells were harvested and lysed in lysis buffer (50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5% w/v sodium *N*-lauroyl sarcosinate). DNA was prepared from the lysates by successive treatments with 0.5 mg/ml RNase A for 30 min and 0.5 mg/ml proteinase K for 40 min at 50°C. The resultant DNA preparation was subjected to 2% agarose gel electrophoresis. DNA fragmentation detected by ethidium bromide staining was examined in photographs taken under UV-illumination. Percent fragmentation was determined by densitometric analysis of the DNA fragments as described previously [9].

3. Results

HIV replication in U1 cells was detected by the appearance of HIV-1 antigen on the cell surface. As shown in Fig. 2, approximately 25% of the total U1 cells expressed HIV-1 antigen after 2 days treatment with TPA or TNF-α. Interestingly, in both inducers, SBA significantly inhibited the appearance of HIV-1 antigen positive cells. The half-maximal effective concentrations (EC_{50}) of TPA and TNF-α were calculated to be 70 and 40 μg/ml, respectively. SBA itself did not induce HIV-1 replication in the absence of inducers.

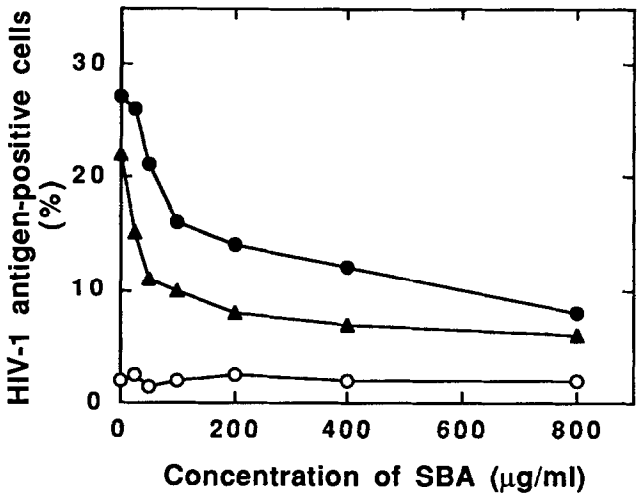

Fig. 2. Inhibitory effect of SBA on U1 cells expressing HIV-1-specific antigen. U1 cells were cultured in the absence (○) or presence of 3 ng/ml TPA (●) or 3 ng/ml TNF-α (▲) with the indicated concentrations of SBA for 2 days. The percentage of HIV-1 antigen-positive cells, determined as described in section 2, is shown as a function of the SBA concentrations. Values are means of three determinations (standard errors of the means ≤ 10%).

We next examined the cytocidal effect of SBA on U1 cells. The viability of U1 cells in the presence of TPA or TNF-α was decreased dose-dependently by SBA treatment, but SBA had essentially no effect on U1 cells not producing HIV (in the absence of an inducer) (Fig. 3a). Interestingly, the percentage decreases of viability (Fig. 3a) were almost the same as those of HIV-antigen positive U1 cells (Fig. 2). The EC_{50} values for the cytocidal effect of SBA in the presence of TPA and TNF-α were 60 and 30 μg/ml, respectively. These values were also the same as those of suppression of HIV-1 antigen production (Fig. 2). On the other hand, SBA had essentially no cytocidal effect on noninfected parental U937 cells (Fig. 4a). Thus the HIV-replicating U1 cells seemed to be selectively affected by SBA.

These findings led us to suppose that SBA might induce apoptotic cell death of U1 cells expressing HIV-1. To test this possibility, we investigated the effect of SBA on cell morphology and DNA fragmentation, which are criteria of apoptosis [10–13], of U1 cells in the presence of TPA or TNF-α. Light microscopic analysis showed that approximately 20% and 10% of U1 cells were shrunken after SBA treatment for 2 days in the presence of TPA and TNF-α, respectively (Fig. 3b). In these cells, chromatin condensation was also observed. In contrast, SBA treatment resulted in little apoptotic effect on U937 cells at concentrations of 0–400 μM (Fig. 4b).

Evidence that SBA induced DNA fragmentation in the

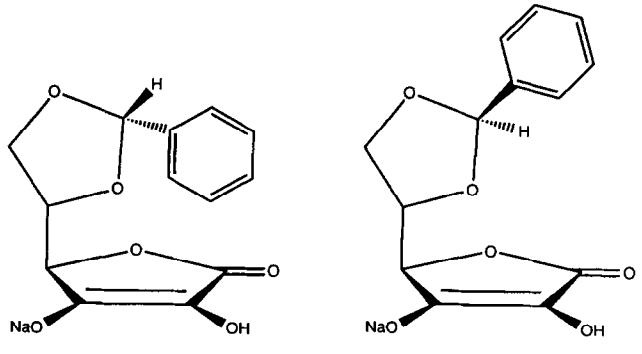

Fig. 1. Structure formulae of diastereomers of SBA.

nucleus was demonstrated by agarose gel electrophoresis (Fig. 5). In SBA-treated U1 cells in the presence of TPA or TNF-α, a substantial amount of DNA was cleaved into a ladder of discrete fragments of nucleosomal DNA (approximately 180 bp). In contrast, little DNA fragmentation was observed in U1 cells producing HIV-1 without SBA treatment (treated with TPA or TNF-α alone). Moreover, U1 cells treated with SBA alone did not show any and DNA fragmentation (Fig. 5) as well as morphological alterations (Fig. 3b), characterisatics of apoptosis.

4. Discussion

Here we report that SBA suppressed HIV-replication in cultured U1 cells induced by TPA or TNF-α. SBA was found to kill U1 cells in the presence of inducers via events characteristic of apoptosis. At concentrations of 25–200 μM of SBA, there was a clear separation between its effects on HIV-producing cells and nonproducing U1 and parental U937 cells indicating its selective induction of apoptosis in HIV-replicating U1 cells. Thus the observed effect of SBA on HIV-replicating U1 cells is suggested to be the results of a direct effect in inducing apoptosis not a cytostatic effect. This assumption is supported by the observation that the percentage reduction of HIV-antigen positive U1 cells by SBA in the presence of TPA or TNF α was almost the same as that of appearance of apoptotic cells. Furthermore, SBA seemed to have a selective action on HIV-replicating U1 cells because treatment of U1 cells with SBA in the absence of inducers resulted in no apoptotic effect. Moreover, even in the presence of inducers, SBA had essentially no effect on parental U937 cells over the range of concentrations tested. These findings are consistent with the hypothesis that the anti-HIV activity of SBA in U1 cells is due to its selective inducibility of apoptosis to HIV-replicating U1 cells. The molecular basis of this selectivity is unknown, but may be related to differences in metabolic activities and homeostatic mechanisms at the cellular level. We are now investigating how SBA selectively induces apoptosis in HIV-replicating U1 cells.

It is noteworthy that suppression effect of SBA on HIV replication induced by TNF-α was observed at lower concentrations of SBA than that by TPA. The signal transduction of TPA is known to be mediated by protein kinase C (PKC) [16–19]. In contrast, activation

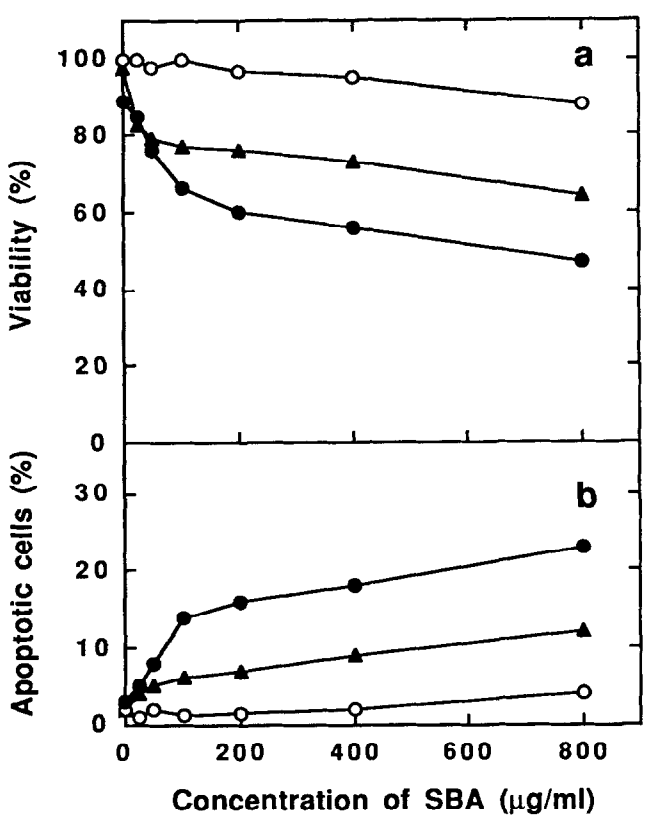

Fig. 3. Induction of apoptosis in U1 cells by SBA. U1 cells were treated with SBA in the absence (○) or presence of 3 ng/ml TPA (●) or 3 ng/ml TNF-α (▲) for 2 days. Cell viability (a) and appearance of apoptotic cells (b) were measured as described in section 2. Values are means of three determinations (standard errors of the means ≤ 8%).

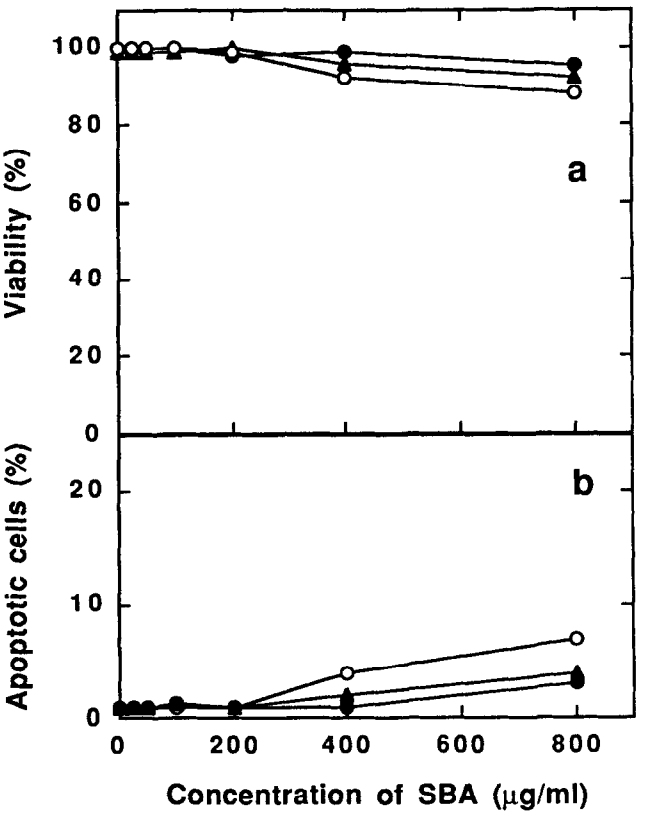

Fig. 4. Effect of SBA on U937 cell viability and apoptosis. U937 cells were treated with SBA in the absence (○) or presence of 3 ng/ml TPA (●) or 3 ng/ml TNF-α (▲) for 2 days. Cell viability (a) and appearance of apoptotic cells (b) were measured as described in section 2. Values are means of three determinations (standard errors of the means ≤ 7%).

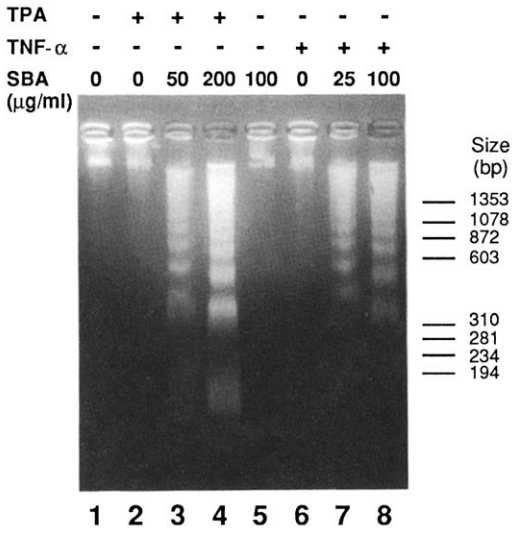

Fig. 5. DNA fragmentation analysis by agarose gel electrophoresis of DNA from SBA-treated U1 cells. U1 cells were treated with the indicated concentrations of SBA for 2 days in the presence or absence of 3 ng/ml TPA or 3 ng/ml TNF-α. Number at right of figure represent the size of DNA standards in base pairs.

of NFκB, which is a transcription factor binding to the κB motif in HIV-long terminal repeat (LTR), by TNF-α can occur independently of PKC [17,18,20]. Although the intracellular mediator of TNF-α is not yet ascertained, production of radical oxigens has been suggested to be involved in NFκB activation [21–23]. Since SBA is known to be an anti-oxidant [24], treatment of SBA might result in inhibition of NFκB activation induced by TNF-α in addition to its triggering apoptosis. So the combined effect is probably the reason why SBA was more suppressive to HIV replication induced by TNF-α than that by TPA. This idea is supported by observations that anti-oxodants could suppress HIV-replication [25,26].

So far a large number of antiretroviral compounds have been proven to be effective against HIV infections [27]. Inhibition of nucleoside reverse transcriptase such as 3′-azido-2′,3′-deoxythymidine (AZT), 2′,3′-dideoxyinosine (ddI) and 2′,3′-dideoxycytidine (ddC) are clinically available, but their efficacies are limited [28]. Thus new types of compounds with anti-HIV activity are required. Agents inducing apoptosis such as SBA and its derivatives may be new types of anti-HIV drugs. Although the molecular mechanism of SBA-induced apoptosis still remains to be determined, we anticipate that selective induction of apoptosis in HIV-expressing U1 cells could lead to new methods of therapy for HIV-related dieseases.

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