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Benzylideneascorbate Induces Apoptosis in L929 Tumor Cells

Sei-ichi Tanuma[1,2], Daisuke Shiokawa[3], Yutaka Tanimoto[1], Masahiko Ikekita[1], Hiroshi Sakagami[4], Minoru Takeda[4], Satoru Fukuda[5] and Mutsuyuki Kochi[6]

[1]*Department of Biochemistry, Faculty of Pharmaceutical Sciences, Science University of Tokyo, Shinjuku-ku, Tokyo, Japan*

[2]*Research Institute for Bioscience, Science University of Tokyo, Noda, Chiba, Japan*

[3]*Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Bunkyo-ku, Tokyo, Japan*

[4]*First Department of Biochemistry, School of Medicine, Showa University, Shinagawa-ku, Tokyo, Japan*

[5]*Laboratory of Electron Microscopy, University of Tokyo, Bunkyo-ku, Tokyo, Japan*

[6]*Ichijoukai Hospital, Ichikawa-shi, Chiba, Japan*

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Summary : Sodium 5,6-benzylideneascorbate (SBA), which is known to be an antitumor substance, was found to induce apoptotic cell death of L929 tumor cells directly in a concentration- and time-dependent manner. The dying cells exhibited cell shrinkage, disappearance of cell surface microvilli, chromatin condensation and DNA fragmentation into nucleosomal oligomers characteristic of apoptosis. These results indicate a possible mechanism by which SBA induces tumor regression *in vivo*.

Benzaldehyde derivatives such as β-cyclodextrin benzaldehyde inclusion compound, 4,6-benzylidene-α-D-glucose and SBA have been shown to have antitumor activity on chemically-induced rat tumors and human cancers (1~8). We previously reported that intravenous administration of SBA was effective clinically against in inoperable tumors of the ovary, stomach, pancreas,

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uterus, bile duct and lung (1~3, 7). However, the mechanism by which SBA exerts its antitumor activity is still unknown.

We recently found that SBA has no apparent immunopotentiation activity such as for stimulating the productions of cytokines (tumor necrosis factor, and interleukin-1 and -2) *in vitro* or *in vivo* (7, 8). This finding led us to suppose that SBA might induce apoptotic cell death (10~12) of tumor cells directly. To test this possibility, we investigated the effect of SBA on cultured L929 tumor cells. We demonstrate here that death of tumor cells treated with SBA is associated with various features of apoptosis. Our findings indicate a possible biochemical mechanism for the antitumor activity of benzaldehyde derivatives *in vivo*.

Materials and Methods

Materials —— SBA (see Fig. 1 for structure) was provided by ChemiScience Ltd., Tokyo. Its purity was more than 95% as judged by NMR analysis. RNase A and proteinase K were purchased from Sigma. All other reagents were of analytical grade.

Cell culture —— The C3H mouse fibrosarcoma cell line L929 was maintained in monolayer culture in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories). L929 cells were cultured for 2 days prior to SBA-treatment. Cell viability was assessed by testing exclusion of 0.2% trypan blue.

Microscopic analysis —— Cells were fixed and embedded as described previously (9). Ultrathin sections stained with uracyl acetate were examined in a H-7000 electron microscope (Hitachi, Japan). The frequency of apoptotic cells was determined by light microscopy.

DNA preparation —— After SBA-treatment, the detached (nonadherent) L929 cells were collected by centrifugation at 400 x g for 5 min, and the attached (still adherent) cells were harvested with a cell scraper and centrifuged at 400 x g for 5 min. Both resulting pellets were lysed in lysis buffer [50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroyl sarcosinate]. The solutions were incubated sequentially with 0.5 μg/ml of RNase A for 60 min at 50 °C, and 0.5 μg/ml of proteinase K for 60 min at 50 °C. The resulting DNA preparations were analyzed by 1.8% agarose gel electrophoresis.

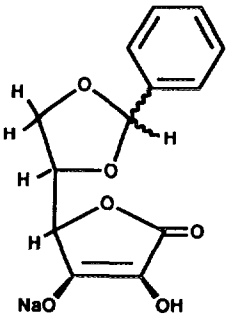

Fig. 1. Chemical structure of SBA.

Agarose gel electrophoresis —— Electrophoresis of the fragmented DNA was carried out in 1.8% agarose gels in TBE buffer [89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA (pH 8.0)] containing 0.5 μg/ml ethidium bromide at 100 V for 1.5 hr. DNA molecular weight markers (Takara 1-Eco T14 I digest No.3401) were used for calibration. The DNA fragmentation pattern was examined in photographs taken under UV illumination. Percent fragmentation was determined by densitometry of DNA fragments with molecular weights of less than 7 kb.

Results

After continuous treatment with 1000 μg/ml SBA for 24 and 48 hr, approximately 10 and 40% of the L929 cells, respectively, were detached from the culture dishes. After 48 hr, approximately 30% of the detached cells had died, whereas the still adherent (attached) cells remained viable as judged by dye exclusion. Light microscopic analysis showed that the detached cells and 10~15% of the attached cells were shrunken after SBA treatment for 24 and 48 hr. To investigate the possible induction of apoptosis by SBA, we examined two different criteria of apoptosis (10-12).

The first criterion of apoptosis was cell morphology, which we examined in detail by electron microscopy. Fig. 2 shows that morphological alterations that were typical of various steps of apoptotic cell death occurred in both detached and attached cells treated with 1000 μg/ml SBA for 48 hr. Significant chromatin condensation was observed even in the nuclei of attached cells (Fig. 2b). In the detached cells, disappearance of cell surface microvilli and nuclear fragments with dense

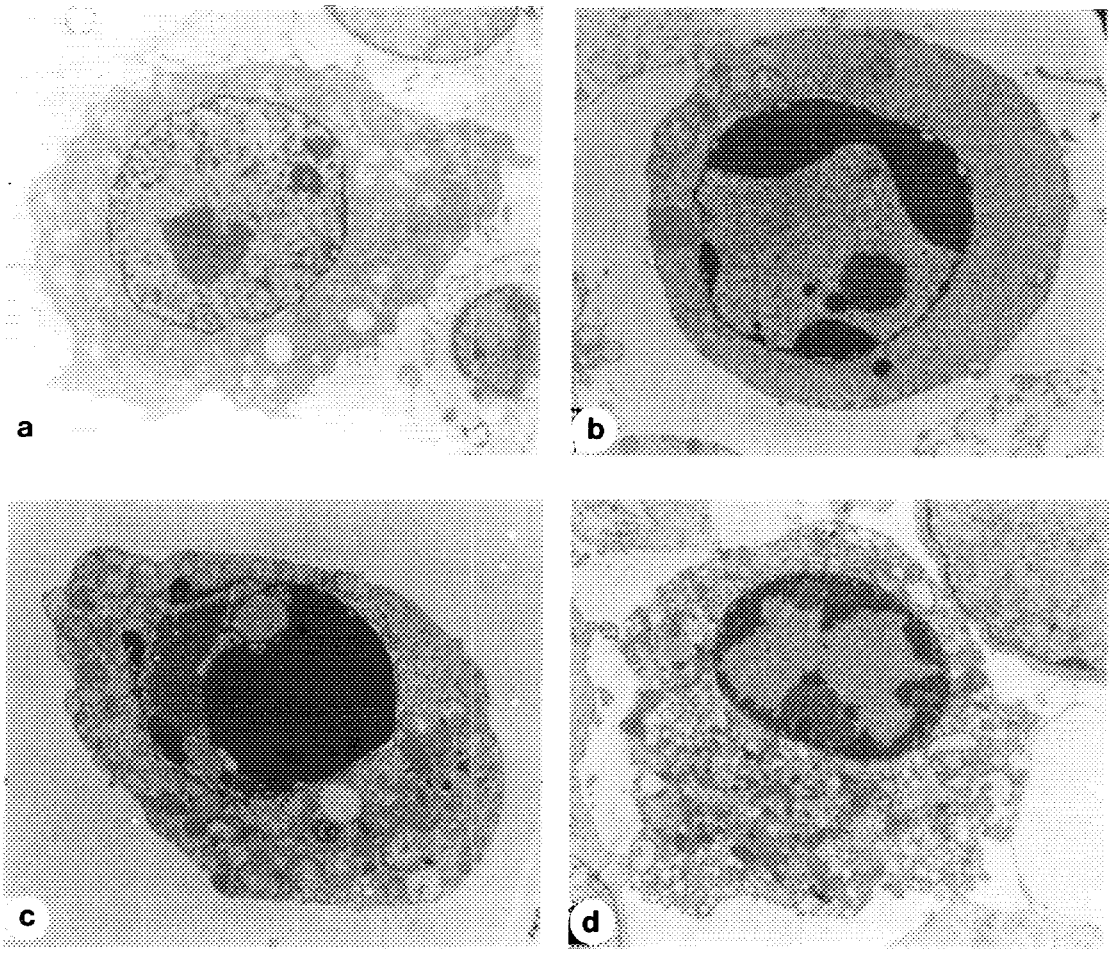

Fig. 2. Electron microscopic appearance of L929 cells treated with SBA at 1000 µg/ml for 48 hr. Control (a), attached (b) and detached (c,d) cells , magnification, x 4700.

chromatin were observed (Fig. 2c). Swollen cells undergoing secondary necrosis were also present among the detached cells (Fig. 2d).

As a second criterion of apoptosis, we examined whether SBA-induced cell death was associated with DNA fragmentation in the nucleus. Evidence that SBA induced DNA fragmentation was demonstrated by agarose gel electrophoresis (Fig. 3). In SBA-treated detached cells, a substantial amount of DNA was cleaved into a ladder of discrete fragments of nucleosomal DNA corresponding to multiples of about 180 bp. The extent of DNA fragmentation increased with the SBA concentration and

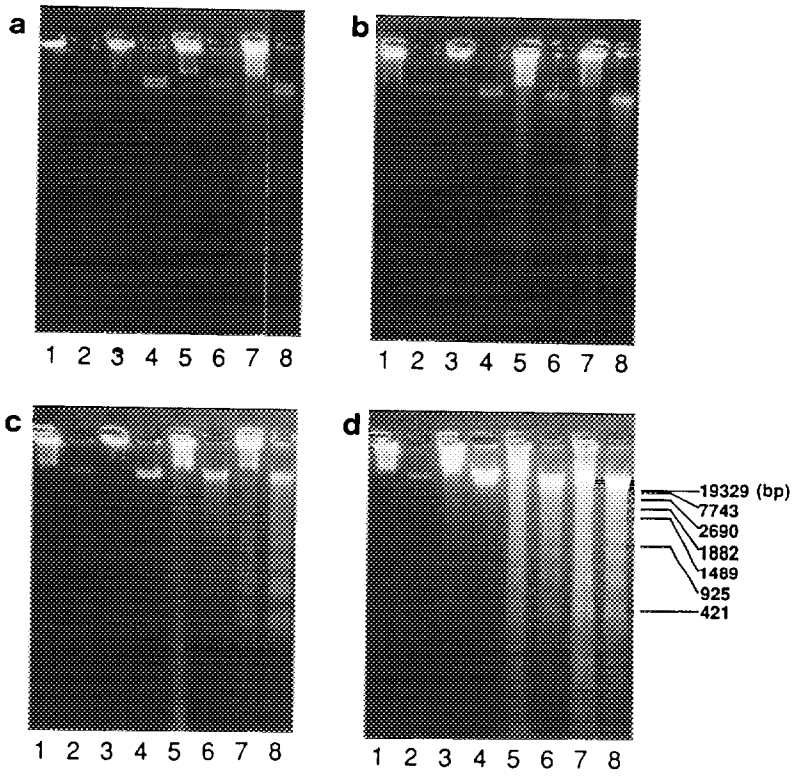

Fig. 3. Agarose gel electrophoresis of DNA from SBA-treated L929 cells. Concentration of SBA (μg/ml) used for treatment : 0 (a), 30 (b), 300 (c), and 3000 (d). Lanes 1, 3, 5 and 7, and 2, 4, 6 and 8 show the patterns of DNA from attached and detached cells (2 x 10^6 cells each), respectively, cultured for 0 (lanes 1 and 2), 1 (lanes 3 and 4), 2 (lanes 5 and 6), and 3 (lanes 7 and 8) days.

incubation time. In contrast, essentially no fragmentation was observed in untreated (control) cells during culture. Fig. 3 also shows that DNA fragmentation in the attached cells preceded cell detachment.

In a kinetic study on the attached cells incubated with 300 and 3000 μg/ml of SBA, DNA cleavage was first seen after 2 days and 1 day, respectively (Fig. 4a). The extents of DNA fragmentation in the detached cells exceeded those in the attached cells (Fig. 4b). In both attached and detached cells, DNA fragmentation was greater with the higher concentration of SBA.

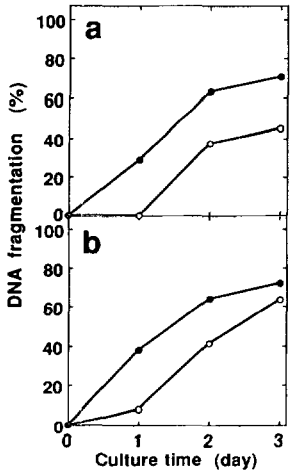

Fig. 4. Kinetics of DNA fragmentation in SBA-treated L929 cells. Percentage DNA fragmentation is plotted as a function of the incubation time with 300 (-○-) or 3000 (-●-) μg/ml of SBA. Attached (a) and detached (b) cells.

Discussion

Our previous studies have suggested that the antitumor activity of SBA might be due to its direct cytocidal action on tumor cells rather than its an immunopotentiation of the host (7, 8). The present study showed that SBA killed L929 tumor cells *via* events characteristic of apoptosis. Although we cannot rule out the possibility that SBA may activate other cytotoxic mechanisms, its induction of apoptosis seems to be the primary cause of cell death. This assumption is supported by the following observations. Slight but significant DNA fragmentation was first detected in the attached cells, and after their detachment, the extent of DNA fragmentation increased. This implies the SBA-induced DNA fragmentation began before cell detachment and increased in the detached cells, a time frame that preceded the cell death. Furthermore, the DNA fragmentation was associated with significant chromatin condensation, seen by electron microscopy. Similarly, apoptotic cell death by SBA was observed in human promyelocytic leukemia HL-60, human histiocytic lymphoma U-937 and mouse null cell lymphoma KML_1-7 (data not shown). This indicates that the phenomenon is not peculia to the L929 cell line. Taken together, these findings

suggest that SBA triggers apoptosis in the tumor cells. This is consistent with the hypothesis that tumor regression after *in vivo* administration of SBA is due, at least in part, to a direct cytocidal action on tumor cells. We are now investigating how SBA induces apoptosis in tumor cells.

References

1. Kochi, M., Takeuchi, S., Mizutani, T., Mochizuki, K., Matsumoto, Y. and Saito, Y. (1980) Cancer Treat Rep. 64 21-23.
2. Kochi, M., Isono, N., Niwayama, M. and Shirakabe, K. (1985) Cancer Treat Rep. 69,533-537.
3. Kochi, M., Ueda, S. and Hagiwara, T. (1988) Progress in Cancer Research and Therapy. 35, 338-343.
4. Tatsumura, T., Tsujimoto, M., Koyama, S., Furuno, T., Komori, Y., Sato, H., Yamamoto, K., Kitagawa, M. and Kagamimori, S. (1990) Br. J. Cancer. 62,436-439.
5. Pettersen, E. O., Dornish, J. M. and Rønning, O. H. (1985) Cancer Res. 45, 2085-2091.
6. Watanuki, M. and Sakaguchi, K. (1980) Cancer Res. 40, 2574-2579.
7. Sakagami, H., Asano, K., Fukuchi, K., Gomi, K., Ota, H., Kazama, K., Tanuma, S. and Kochi, M. (1991) Anticancer Res. 11,1533-1538.
8. Sakagami, H., Takeda, M., Utsumi, A., Fujinaga, S., Tsunoda, A., Yasuda, N., Shibusawa, M. Koike, T., Ota, H., Kazama, K., Shiokawa, D., Tanuma, S. and Kochi, M. (1993) Anticancer Res., in press.
9. Fukada, S., Seki, A., Matsuo, Y., and Watari, N. (1991) J. Clin. Electron Microscopy 24, 5-6.
10. Kerr, J. F. R., Wyllie, A. H. and Currie, A. R. (1972) Br. J. Cancer. 26, 239-257.
11. Wyllie, A. H., Kerr, J. F. R. and Currie, A. R. (1980) Int. Rev. Cytol. 68, 251-306.
12. Wyllie, A. H., Morris, R. G., Smith, A. L. and Dunlop, D. (1984) J. Pathol. 142, 67-77.