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SPECIFIC INHIBITORS OF VACUOLAR TYPE H^+-ATPASES INDUCE APOPTOTIC CELL DEATH

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Summary. Concanamycin A and bafilomycin A_1 are known as strong inhibitors of the vacuolar type H^+-ATPases in vitro. These inhibitors exhibited cytotoxic effects on twelve cell lines in cell viability assay. On the other hand, the F_1F_0-type H^+-ATPase inhibitor oligomycin and the E_1E_2-type H^+-ATPase inhibitor vanadate showed no cytotoxic effect. We show here that concanamycin A and bafilomycin A_1 induce a significant increase in the proportion of fragmented DNA in agarose gel electrophoresis. Flow cytometric cell cycle analysis of WEHI 231 cells stimulated with concanamycin A revealed the increased percentage of apoptotic cells with hypodiploid DNA. These findings indicate that cell death induced by specific inhibitors of vacuolar type H^+-ATPases occurs through apoptosis.

Vacuolar type H^+-ATPases (V-ATPases) are found on the membranes of various intracellular compartments of eukaryotic cells such as lysosomes, endosomes, the Golgi complex, and secretory granules (1, 2). V-ATPases have been identified, purified and characterized at the molecular level in recent years (3-6), indicating distinct sensitivities against several inhibitors when compared to F_1F_0-ATPases in the mitochondria and E_1E_2-ATPases in the plasma membranes. Bafilomycin A_1 was found to be a potent selective inhibitor of the V-ATPases derived from *Streptomyces* species (7). Manabe et al. indicate that bafilomycin A_1 is effective not only in vitro but also to intact cells, and could be a very useful tool both in vitro and in vivo to elucidate physiological roles of V-ATPases (8). Umata et al. reported that the acidification of intracellular acidic

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compartments is inhibited when Vero cells are treated with 500 nM bafilomycin A_1, indicating that bafilomycin A_1 effectively inhibits V-ATPase activity when it is added to the culture medium (9). Recent work has also shown that bafilomycin A_1 inhibits acidification and protein degradation in lysosomes of cultured cells (10). It has been reported that concanamycin derivatives inhibit V-ATPase activity to the same extent as does bafilomycin A_1 (11).

Embryonic and postembryonic development is tightly regulated not only by the proliferation and differentiation of the cells but also cell death (12). Apoptosis has been shown to play important roles in many physiological processes including immune, hematopoietic, and developmental systems (12). This is sometimes referred to as programmed cell death, and basically distinct from accidental cell death or necrosis. The cells undergoing apoptosis show morphological changes with loss of plasma membrane of microvilli, chromatin condensation, nuclear fragmentation and shrinkage, and the formation of dense chromatin masses (apoptotic bodies) (12, 13). Its hallmark biochemical feature is endonuclease-mediated cleavage of internucleosomal DNA linker sections (13). It is well known that WEHI 231 cells undergo apoptotic cell death when surface immunoglobulin is crosslinked by anti-IgM antibody (14, 15). In the present study, we report that concanamycin A and bafilomycin A_1, specific inhibitors of the V-ATPases, induce cell death of WEHI 231 cells through apoptosis, as shown by an increased in the proportion of fragmented DNA, and by the typical ladder pattern of DNA fragmentation in agarose gel electrophoresis.

Materials and Methods

Cell lines and culture conditions. P388D_1, J774.1, U937, and EL-4 cells were maintained in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml). WEHI 231 cells, kindly provided by Drs. T. Tsubata and T. Honjo, Kyoto University, were cultured in RPMI 1640 medium supplemented with 10% FCS, 50 μM 2-mercaptoethanol and antibiotics. Mouse hybridoma cell lines HS-72 and LS-6 (16), M1, and Sp2/0-Ag14 were maintained in Iscove's Modified Dulbecco's medium (IMDM; GIBCO) supplemented with 10% FCS and antibiotics. HepG2 cells (RIKEN Cell Bank) and KB cells (Japanese Cancer Research Resources Bank) were maintained in Dulbecco's Modified Eagle Medium (DMEM; GIBCO) supplemented with 10% FCS and antibiotics. MC3T3-E1 cells were propagated in α-MEM (GIBCO) supplemented with 10% FCS and antibiotics.

Cell viability assay. The cells were washed extensively to remove the growth factors and suspended to a density of 4×10^5/ml in media containing 5% FCS and antibiotics.

The cells (2 x 10^4/well of 96-well plate) were cultured with appropriate amounts of concanamycin A and bafilomycin A1 in an atmosphere of 5% CO_2 in air. Stock MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazplium bromide 2.5 mg/ml; Sigma Chemical Co., St. Louis, MO) solution (20 µl/well) was added to the wells, and plates were incubated at 37°C for 4 h. Acid-isopropanol (100 µl of 0.04 N HCl in isopropanol) was added and mixed thoroughly. The plates were read on a Multiskan Bichromatic microplate reader (Labsystems, Helsinki, Finland), using a test wavelength of 570 nm and a reference wavelength of 620 nm (MTT assay) (17). The 50% effective dose (ED_{50}) measure of cell viability was determined to be subject to qualification in that cell viability is also indicated by the slope of the dose-response curve.

DNA fragmentation. To assess the nature of cell death mediated by the V-ATPase inhibitors, we used WEHI 231 B lymphoma cells as a model system, because WEHI 231 cells are well known to undergo apoptosis when treated with anti-IgM antibody (14, 15). WEHI 231 cells were suspended in RPMI 1640 medium containing 5% FCS, 50 µM 2-mercaptoethanol and antibiotics, and cultured for 18 h with appropriate amounts of concanamycin A, bafilomycin A1, and goat anti-mouse IgM antibody (Southern Biotechnology Associates, Inc., Birmingham, AL). The cells were lysed with 10 mM Tris-HCl (pH 7.4)-5 mM EDTA-1% Triton X-100. The lysates were centrifuged to remove integral nuclei. The supernatants were digested with RNase (0.5 mg/ml) for 1 h at 37°C, incubated with Proteinase K (10 mg/ml) for 1 h at 50°C, and extracted with phenol-chloroform (1:1; vol/vol) before precipitation with ethanol. The precipitates were dried and solubilized in 10 nM Tris-HCl (pH 8.0)-1 mM EDTA. Electrophoresis was performed with a 2% agarose gel, which was stained with ethidium bromide (18).

Flow cytometric analysis. WEHI 231 cells were incubated under concanamycin A-treated conditions and anti-IgM-treated conditions for 24 h and 48 h as described above. The cells were harvested, washed and then suspended in ice-cold phosphate buffered saline (PBS; pH 7.2) at a concentration of 2 x 10^6 cells/ml. Apoptosis was quantified by flow cytometric determination of the proportion of the cells with hypodiploid DNA by the method of Perandones et al. (19). The cells were resuspended in 1 ml of hypotonic propidium iodide (PI) solution (3.4 mM sodium citrate, 50 µg/ml PI, 0.1% Triton X-100, 1 mM Tris, and 0.1 mM EDTA) and stored in darkness on ice until they were analyzed by Becton Dickinson FACS scan flow cytometer. Apoptotic nuclei were distinguished by their hypodiploid DNA content compared with the diploid DNA content of normal nuclei.

Results

We examined the effects of the specific V-ATPase inhibitors, concanamycin A and bafilomycin A1, on cell viability of several cell lines. After 48 h of culture, the toxicity of V-ATPase inhibitors to these cell lines were examined by MTT assay. Concanamycin A and bafilomycin A1 were found to be significantly toxic to WEHI 231 cells, Sp2/0-Ag14 cells and mouse hybridomas, whereas these inhibitors had no effect on the cell viability of MC3T3E1 cells in the concentration range up to 200 nM (Table 1).

Table 1. ED_{50} of concanamycin A and bafilomycin A_1 on several cell lines*

Cell lines	Character	ED_{50}(nM)	
		Concanamycin A	Bafilomycin A_1
Mouse			
WEHI 231	B lymphoma	9.4	20.7
EL-4	T lymphoma	13.0	37.5
M1	Myeloblast	21.3	42.3
SP2/0-Ag14	Myeloma	10.7	13.0
HS-72	Hybridoma	8.1	16.9
LS-6	Hybridoma	16.1	18.5
P388D_1	Macrophage	42.3	96.6
J774.1	Macrophage	29.2	37.9
MC3T3-E1	Osteoblast-like	> 200.0	> 200.0
Human			
U937	Histocytic lymphoma	28.7	40.2
KB	Oral epidermoid carcinoma	50.5	58.1
HepG2	Hepatocellular carcinoma	21.2	66.9

*The cells (2×10^4/well) were cultured with various concentrations of concanamycin A or bafilomycin A_1 for 48 h. The ED_{50} was calculated as described in the text.

There are three general groups of proton pumping ATPases with different pharmacological properties, V-ATPases, E_1E_2-type H^+-ATPases, and the mitochondrial type F_1F_0-type H^+-ATPases. We have exploited the different inhibitor sensitivity of the three types of H^+-ATPase to identify its type for cell death of WEHI 231 cells. In the present study, three different inhibitors of H^+-ATPases were cultured with WEHI 231 cells for 48 h. Concanamycin A (12.5 nM) and bafilomycin A_1(50 nM), specific inhibitors of the V-ATPases, were found to be significantly cytotoxic to WEHI 231 cells in MTT assay (Fig. 1). However, 5 μM of vanadate, E_1E_2-type H^+-ATPases inhibitor, had no effect on the cell viability of WEHI 231 cells. F_1F_0-type H^+-ATPase inhibitor oligomycin showed no cytotoxic effect on WEHI 231 cells in the concentration range up to 10 μg/ml.

To examine whether the V-ATPase inhibitors could lead to apoptosis, we monitored two parameters of the process to cell death of WEHI 231 cells. DNA analysis of WEHI 231 cells revealed that the fragmented DNA could be detected 18 h after stimulation of concanamycin A or bafilomycin A_1. The ladder pattern of fragmented DNA in anti-IgM-treated WEHI 231 cells was also detected. DNA was cut into multiple fragments with 180-bp size differences in agarose gel electrophoresis (Fig. 2). We analyzed the percentage of apoptotic WEHI 231 cells with hypodiploid DNA by using FACS scan flow

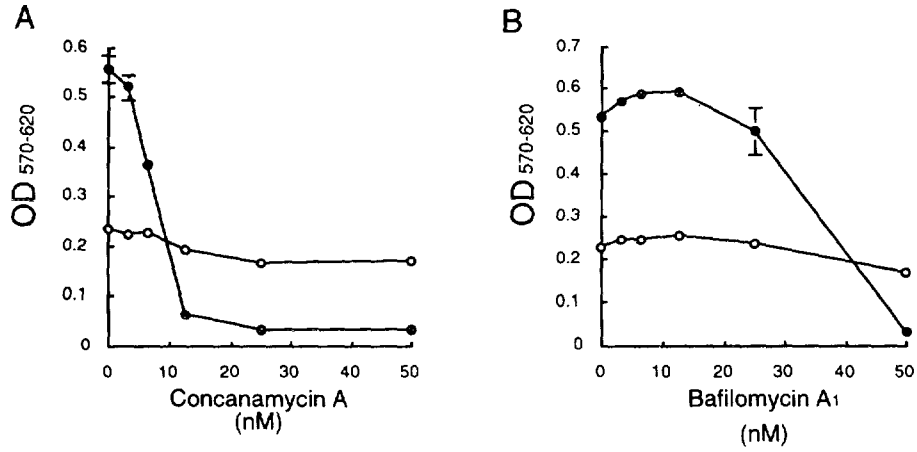

Figure 1. Effects of concanamycin A and bafilomycin A1 on cell viability of WEHI 231 cells. WEHI 231 cells ($2x10^4$/well) were cultured in 96-well culture plates with appropriate amounts of the V-ATPase inhibitors. After being cultured for 24 h (○) and 48 h (●), cell viability was measured as described in the text.

cytometer. The PI histogram clearly distinguished nuclei with normal diploid DNA from apoptotic nuclei with hypodiploid DNA. After being cultured with concanamycin A (10 nM) for 24 h and 48 h, the percentages of WEHI 231 cells with hypodiploid DNA were

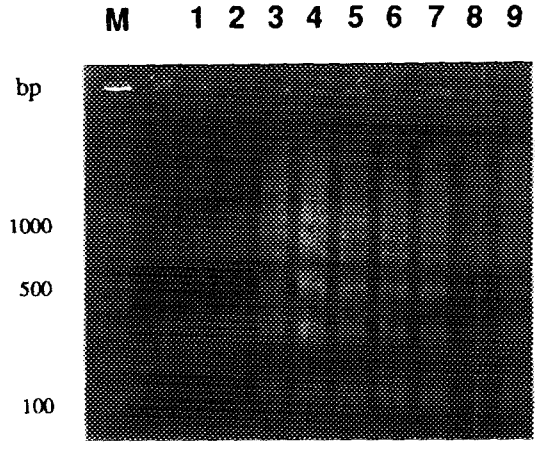

Figure 2. DNA fragmentation of WEHI 231 cells treated with the V-ATPase inhibitors. WEHI 231 cells were suspended in RPMI 1640 medium containing 5% FCS, 50 μM 2-mercaptoethanol and antibiotics with the V-ATPase inhibitors or anti-IgM antibody and cultured for 18 h. The DNA was extracted, electrophoresed, and stained as described in the text. Lane M, Superladder low DNA Marker (Gen Sura Laboratories, Inc., Del Mar, CA). Lane 1; non-stimulated WEHI 231 cells. Lane 2-4; WEHI 231 cells were cultured with bafilomycin A1 (7.5, 15, and 30 nM). Lane 5-7; WEHI 231 cells were cultured with concanamycin A (7.5, 15, and 30 nM). Lane 8 and 9; WEHI 231 cells were cultured with anti-IgM antibody (10 and 20 μg/ml).

35.5% and 57.1%, respectively. After being cultured with anti-IgM antibody (20 μg/ml) for 48 h, the percentage of WEHI 231 cells with hypodiploid DNA were 46.5% (Fig. 3).

Discussion

Apoptosis is a programed process of cell death, in which an endogenous endonuclease cuts DNA in the nucleosomal linker regions (20). Selective activation of an endogenous endonuclease appears to be responsible not only for widespread chromatin cleavage but also for the major nuclear morphologic changes. The activation of the program is regulated by many different signals that derive from both intracellular and the extracellular stimuli. The present experiments have shown that specific inhibitors of V-ATPase, concanamycin A and bafilomycin A1 exhibit cytotoxic effects and provoke apoptosis in several mammalian cell lines at nanomolar concentrations.

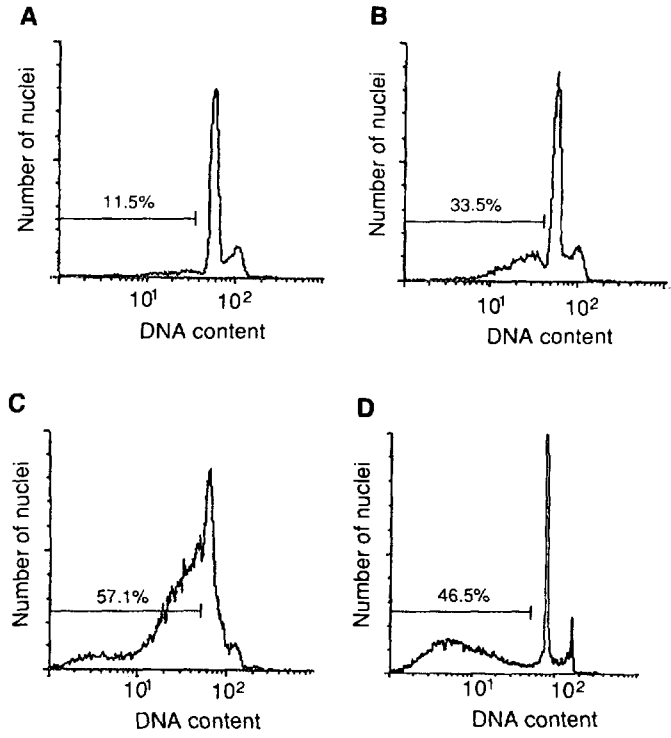

Figure 3. Concanamycin-A-induced apoptosis in WEHI 231 cells. WEHI 231 cells were cultured with or without concanamycin A. The cells were stained with PI and apoptotic nuclei with hypodiploid DNA content were analyzed by FACS scan flow cytometer. Note the percentages of apoptotic WEHI 231 cells with hypodiploid DNA. A; without concanamycin A. B; with concanamycin A (10 nM) for 24 h. C; with concanamycin A (10 nM) for 48 h. D; with anti-IgM antibody (20 μg/ml) for 48 h.

The specific inhibition of V-ATPases by concanamycin A and bafilomycin A1 have been studied by using purified enzymes in vitro. Recent work has shown that two derivatives of concanamycin and bafilomycin A1 strongly inhibit the V-ATPases in vitro and in vivo, as well as cell proliferation (8). Muroi et al. indicated that concanamycin A inhibits V-ATPase activity and acidification of organelles more strongly than bafilomycin A1 (11). This finding is in accord with our results wherein the ED_{50} of concanamycin A was lower than bafilomycin A1 on WEHI 231 cells in MTT assay (Table 1). We demonstrated that the specific V-ATPase inhibitors such as concanamycin A and bafilomycin A1, can induce DNA fragmentation of mouse B lymphoma cell line WEHI 231 cells in agarose gel electrophoresis (Fig. 2), which is then followed by cell death. Tsubata et al. showed that cross-linking of surface immunoglobulin receptors on WEHI 231 cells leads to typical DNA fragmentation and apoptosis, and that the signal for abrogating surface immunoglobulin-mediated apoptosis is generated by association of the CD40 ligand on T cells with the CD40 molecule on WEHI 231 cells (21). In the present study, concanamycin A and bafilomycin A1 were found to induce apoptotic cell death of WEHI 231 cells and seemed to be useful agents for investigating the cellular events of apoptotic cells, suggesting that inhibition of V-ATPase activity is responsible for triggering apoptosis, that is, V-ATPase activity mediates the decision between life and death of a cell.

The actual cause and mechanism of apoptotic cell death induced by the V-ATPase inhibitors are still unknown. It is widely assumed that cells are killed by the endonuclease-mediated cleavage of their DNA. A Ca^{2+}/Mg^{2+}-dependent endonuclease activity capable of cleaving chromatin at internucleosomal sites was first identified in nuclear preparations from thymocytes undergoing apoptosis (22). On the contrary, Barry et al. indicated that increased intracellular Ca^{2+} is not a primary signal for endonuclease activation in apoptosis, and that intracellular acidification is consistent with the involvement of endonuclease activation in apoptosis (23). Regulation of cytoplasmic pH (pHi) within the physiological range is crucial to the maintenance of normal cell function. Recent work has shown the existence of a novel mechanism of pHi regulation in murine peritoneal macrophages, suggesting the existence of an ATP-dependent proton extrusion mechanism in the plasma membrane of mononuclear phagocytes (24, 25). Furthermore, several lines of evidence have indicated that the V-ATPases are the most probable candidate for the generator of the acidity of organelles belonging to the central vacuolar system (1). Taken together, these findings suggest that acid extrusion in the plasma membrane and acidity in the central vacuolar system by means of V-ATPases

might contribute to activation of endogenous endonuclease, resulting in apoptotic cell death. Further work is needed to elucidate the pivotal mechanism of apoptotic cell death induced by the V-ATPase inhibitors. In conclusion, the present results indicate novel roles for concanamycin A and bafilomycin A_1 in a variety of mammalian cells. These findings might give insight into the important physiological and pathological roles of the V-ATPases *in intact cells*.

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