New Curcuminoids Isolated from *Zingiber cassumunar* Protect Cells Suffering from Oxidative Stress: A Flow-Cytometric Study Using Rat Thymocytes and H_2O_2

Takayuki Nagano[1,#], Yasuo Oyama[1,*], Noriko Kajita[1], Lumi Chikahisa[1,2], Mami Nakata[1], Eisuke Okazaki[1] and Toshiya Masuda[1]

[1]*Laboratories of Cellular Signaling and Bioorganic Chemistry, Faculty of Integrated Arts and Sciences, The University of Tokushima, Tokushima 770, Japan*
[2]*Cancer Research Laboratory, Taiho Pharmaceutical Co., Ltd., Hanno 357, Japan*

Received April 24, 1997 Accepted September 5, 1997

ABSTRACT—Effects of new complex curcuminoids (cassumunin A and cassumunin B) isolated from tropical ginger, *Zingiber cassumunar*, were examined in dissociated rat thymocytes suffering from oxidative stress induced by 3 mM hydrogen peroxide by using a flow cytometer and ethidium bromide. The effects were compared with those of curcumin, a natural antioxidant, whose chemical structure is included in those of cassumunins A and B. Pretreatment of rat thymocytes with the respective cassumunins at concentrations ranging from 100 nM to 3 μM dose-dependently prevented the hydrogen peroxide (H_2O_2)-induced decrease in cell viability. It had the same action, although less effective, against the treatment with cassumunin A or B (3 μM) immediately after or 60 min after start of the oxidative stress. Respective potencies of cassumunins A and B in protecting the cells suffering from H_2O_2-induced oxidative stress were greater than that of curcumin. It is suggested that cassumunins A and B may possess a potent protective action on living cells suffering from oxidative stress.

Keywords: Curcumin, Curcuminoid, *Zingiber cassumunar*, Flow cytometry, Oxidative stress

Zingiber cassumunar is a medical ginger that is also used as a spice like turmeric. Cassumunins A and B (1), new complex curcuminoids isolated from the rhizomes of *Z. cassumunar*, are expected to exert protective actions on living cells suffering from oxidative stress because they were estimated to have antioxidant activity in an ethanol-water system using linoleic acid as the substrate for auto-oxidation by the TLC method and the thiocyanate method (2). However, there is no information concerning their protective actions on living cells suffering from the oxidative stress. In this study, we first examined the effects of cassumunins A and B on living cells under oxidative stress induced by hydrogen peroxide (H_2O_2) by using a flow cytometer and ethidium bromide (3–5) to determine if they exert protective actions on living cells. Secondly, we have compared the potencies of cassumunins in protecting the cells against the oxidative stress with that of curcumin because curcumin, a compound abundant in ginger rhizomes, is known to have antioxidant activity (6, 7) and its chemical structure is contained in those of cassumunins A and B (see Fig. 1). Furthermore, to characterize the protective actions of cassumunins, we have also compared the actions of cassumunins on living cells under oxidative stress with those of deferoxamine, ebselen and dimethyl sulfoxide.

MATERIALS AND METHODS

Preparation

Experiments were performed on thymocytes dissociated from thymus glands of 4-week-old Wistar rats (Nissin, Tokushima). Thymocytes were chosen for the experiments for the following reasons. First, the cells can be dissociated without the treatment with proteolytic enzymes that may compromise the membranes of some cells during digestion. Cell viability of dissociated thymocytes was greater than 90% under the control condition. Second, thymocytes are suitable for flow cytometric analysis

Present address: Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565, Japan
* To whom correspondence should be addressed.

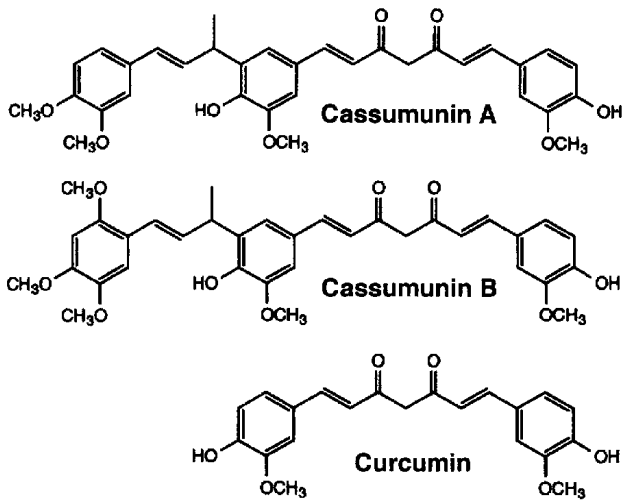

Fig. 1. Chemical structures of cassumunin A (upper), cassumunin B (middle) and curcumin (lower).

because of their spherical shape, size and homogeneity. The technique for dissociation of rat thymocytes was similar to that previously described (3). In brief, thymus glands dissected from the rats were sliced at a thickness of 400 to 500 μm. Thereafter, the slices were gently triturated in chilled Tyrode's solution to dissociate single thymocytes. Tyrode's solution containing dissociated thymocytes was passed through a mesh (a diameter of 53 μm) to remove residues.

Fluorescence measurements and analysis

To estimate the viability of thymocytes in the cell suspension, ethidium bromide (Katayama Chemical Industries, Osaka) was used. Ethidium bromide was added into the cell suspension to achieve a final concentration of 10 μM. Ethidium, which is highly impermeant to intact membrane, can not stain live cells while it can stain dead or damaged thymocytes because of their compromised membranes (3, 4, 8). Therefore, the measurement of ethidium fluorescence from thymocytes provides a clue to estimate the viability of thymocytes in the cell suspension. Fluorescence from thymocytes incubated with ethidium bromide was measured at 2–3 min after the dye application because ethidium slightly stained some of live cells during a prolonged exposure and 2–3 min was enough time to stain dead cells. Measurement of ethidium fluorescence from thymocytes was made by a flow cytometer (Cyto ACE-150; Japan Spectroscopic Co., Tokyo). The excitation wavelength for ethidium was 488 nm produced by an argon laser. Emission was detected at the wavelength of 600±20 nm. The fluorescence histogram obtained from a programmed number of thymocytes was analyzed by using software (Jasco Ver.3XX, Japan Spectroscopic Co.) and a personal computer (PC-9801RX; NEC, Tokyo). Statistical analysis was performed with two sample *t*-tests. A P value of <0.05 was considered significant.

To monitor the changes in $[Ca^{2+}]_i$ of thymocytes with intact membranes, two fluorescent probes, fluo-3-AM (Dojindo Laboratory, Kumamoto) and ethidium bromide were used. The respective dye was added into the cell suspension to achieve a final concentration of 500 nM for fluo-3-AM or 5 μM for ethidium bromide. The cells were incubated with fluo-3-AM for 60 min at least before measurement of fluo-3 fluorescence. Fluo-3 fluorescence is predominantly used for monitoring the changes in $[Ca^{2+}]_i$ because this dye would be fluorescent if bound to intracellular Ca^{2+}. Ethidium that is highly impermeant to intact membrane can not stain live cells. Therefore, live neurons show strong fluorescence of fluo-3 while damaged or dead neurons show ethidium fluorescence. 2′,7′-Dichlorofluorescin diacetate (DCFH-DA; Molecular Probe Inc., Eugene, OR, USA) was used to estimate the cellular status of oxidation by H_2O_2. DCFH-DA is hydrolyzed to DCFH by cellular esterases. DCFH is oxidized by H_2O_2 to 2′,7′-dichlorofluorescin (DCF), a fluorescent form. DCF fluorescence was measured at least 60 min after application of 100 μM DCFH-DA. Measurements of fluo-3 and DCF fluorescences from thymocytes was made by a flow cytometer (Cyto ACE-150). The excitation wavelength for ethidium was 488 nm produced by an argon laser. Emission was detected at the wavelength of 530±20 nm.

RESULTS

Permeation of cassumunins A and B into living cells

Both cassumunins A and B stained thymocytes, resulting in increased intensities of cell fluorescences. As shown in Fig. 2, the intensities of fluorescences in thymocytes treated respectively with cassumunins A and B time-dependently increased, suggesting the time-dependent increases in cellular concentrations of the agents. The intensities of fluorescences seemed to reach the respective steady-state levels within 45 to 60 min after the start of application. Therefore, in order to ensure dose-dependent effects of the agents, the treatment of thymocytes with cassumunin A or cassumunin B started at 60 min before the challenge of oxidative stress. Since maximum intensities of fluorescences recorded from the cells treated respectively with cassumunins A and B were much lower than that of ethidium fluorescence, which was used for detecting dead cells and cells having compromised membranes, in the following experiments, the fluorescences of cassumunins A and B did not affect any measurements of ethidium fluorescence.

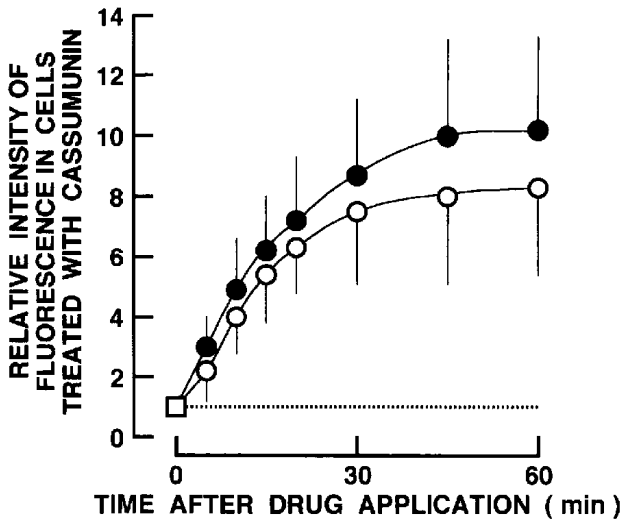

Fig. 2. Time-dependent increases in fluorescence intensities of thymocytes treated with 10 μM cassumunin A (○) and 10 μM cassumunin B (●). Each symbol and bar show the average and S.D. of 4 experiments, respectively. Dotted line indicates the level of fluorescence intensity before the respective applications of cassumunins A and B. The sensitivity of the detector for fluorescence produced by cassumunins was greater (>4) than that for the ethidium fluorescence.

Effect of H_2O_2 on cell viability of thymocytes

Effect of H_2O_2 at concentration of 1, 3 and 10 mM on thymocytes was examined (Fig. 3). Thymocytes were tolerant to the oxidative stress induced by 1 mM H_2O_2 during a period of 3 hr after adding H_2O_2 into cell suspension (Fig. 3). However, it was likely that H_2O_2 at 3 to 10 mM was lethal for thymocytes during exposure lasting for 2 hr or longer (3, 4) since the prolonged exposure of thymocytes to 3 mM or 10 mM H_2O_2 produced a time-dependent increase in the number of thymocytes stained with ethidium, indicating a time-dependent decrease in the number of intact cells (Figs. 3 and 4). The time-dependent change in the cell viability (or the population of cells without ethidium fluorescence) in the presence of 10 mM H_2O_2 was more rapid than that in the presence of 3 mM H_2O_2 (Fig. 4). Therefore, the effects of agents used in this study were examined on the cells under oxidative stress induced by 3 mM H_2O_2 to make certain that we test the effects of the agents applied at 1 hr after the start of oxidative stress on the cell viability.

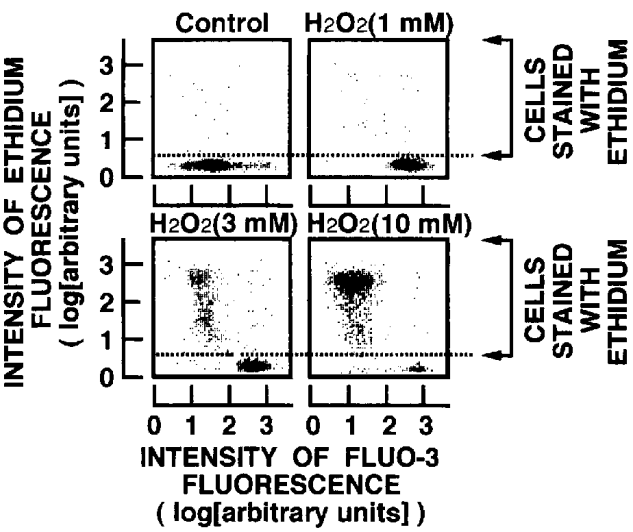

Fig. 3. Effect of H_2O_2 on the cytogram consisting of fluo-3 and ethidium fluorescences obtained from a programmed number of thymocytes. Effects were examined at 3 hr after the start of oxidative stress induced by H_2O_2 at the respective concentrations (1, 3 and 10 mM). The cells under the dotted line were not stained with ethidium, indicating the population of intact cells.

Dose-dependent effects of cassumunins A and B on H_2O_2-induced decrease in cell viability

Cassumunins A and B were respectively applied to the cell suspension containing thymocytes at 1 hr before the start of oxidative stress induced by H_2O_2. Effects of cassumunins A and B on the cell viability were examined at 3 hr after the start of oxidative stress. As shown in Fig. 5, the pretreatment of 3 μM cassumunin A seemed to greatly increase the survival of the cells even at 3 hr after the start of oxidative stress because the H_2O_2-induced increase in the number of cells stained with ethidium was greatly suppressed in the presence of cassumunin A. Effects of cassumunins A and B on the time-dependent changes in the H_2O_2-induced decrease in cell viability (H_2O_2-induced increase in number of thymocytes stained with ethidium) are summarized in Fig. 6.

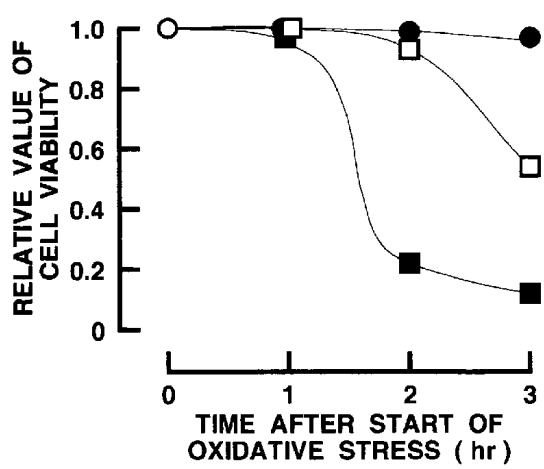

Fig. 4. Dose- and time-dependent change in the cell viability (the population of intact thymocytes) in the presence of H_2O_2 at the respective concentrations (1 mM ●, 3 mM □, 10 mM ■). Result shows an example of those obtained in three experiments.

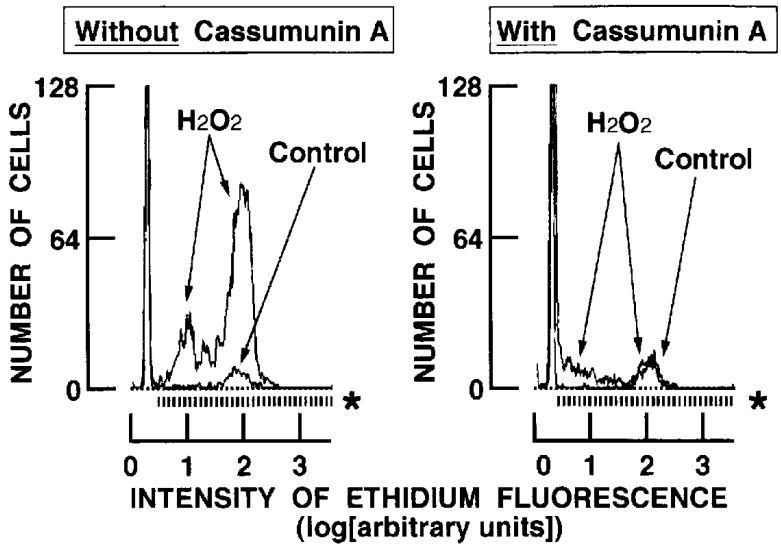

Fig. 5. Effect of cassumunin A on histogram of ethidium fluorescence obtained from 3000 thymocytes. The concentration of cassumunin A was 3 μM. The cells were treated with (right panel: with Cassumunin A) and without (left panel: without Cassumunin A) cassumunin A before the start of oxidative stress induced by 3 mM H_2O_2. The control histogram (as indicated with the arrow and Control) was superimposed with that obtained at 3 hr after the start of oxidative stress (as indicated with the arrows and H_2O_2). Dotted line with an asterisk under the histogram indicates the cells stained with ethidium. Note that the intensity of fluorescence of the cells that were not stained with ethidium in the presence of cassumunin A was similar to that in the absence of cassumunin B, indicating no disturbance of the measurement of ethidium fluorescence by the fluorescence produced by cassumunin A shown in Fig. 2.

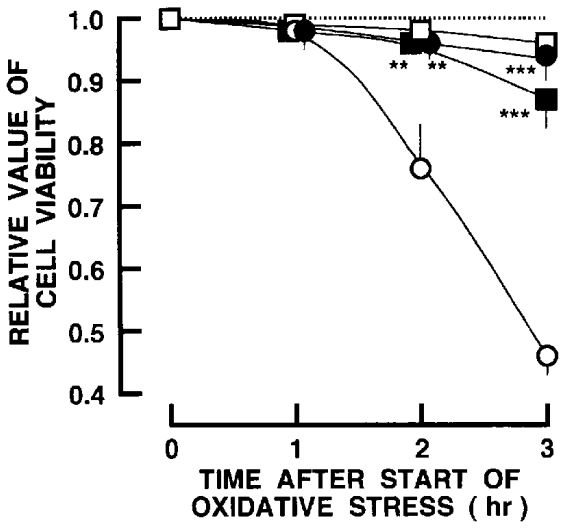

Fig. 6. Time-dependent changes in the cell viabilities of thymocytes subjected to oxidative stress induced by 3 mM H_2O_2 in the absence or presence of the respective cassumunin. Cassumunins A and B at a concentration of 3 μM were applied to cell suspensions at 1 hr before adding 3 mM H_2O_2. □, Control; ○, 3 mM H_2O_2; ●, cassumunin A and 3 mM H_2O_2; ■, cassumunin B and H_2O_2. Each symbol and bar show the mean value and S.D. in 4 to 6 experiments, respectively. Values on the ordinate are relative to the control (as indicated with a dotted line) just before the start of oxidative stress (at 0 hr on the abscissa). Asterisks indicate significant differences between groups subjected from oxidative stress with and without the respective pretreatments with cassumunins A and B (**P<0.005, ***P<0.001).

While cassumunins A and B at the concentrations of 10 μM or less did not affect the cell viability under the control condition, curcumin at a concentration of 10 μM slightly decreased the cell viability during 4 hr after appli-

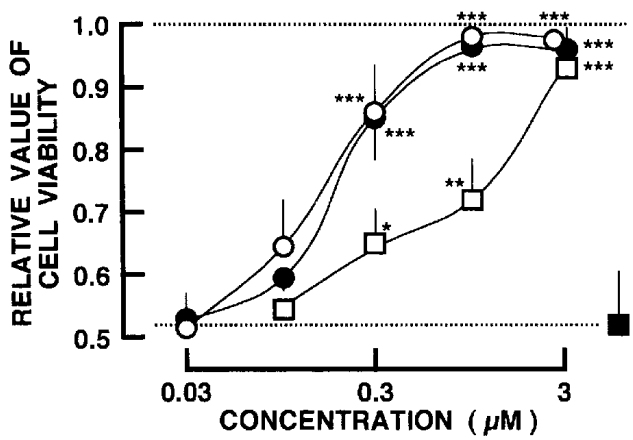

Fig. 7. Dose-dependent effects of cassumunin A, cassumunin B and curcumin on the cell viability of thymocytes subjected to oxidative stress induced by 3 mM H_2O_2. Upper dotted line shows the control cell viability. Lower dotted line and filled square (■) show the cell viability at 3 hr after the start of oxidative stress. ○, cassumunin A; ●, cassumunin B; □, curcumin. Each symbol and bar show the mean value and S.D. in 4 to 6 experiments, respectively. Asterisks indicate significant differences between H_2O_2-subjected groups with and without the respective pretreatments with test agents (*P<0.01, **P<0.005, ***P<0.001).

cation, suggesting that curcumin was cytotoxic. Therefore, the dose-dependent effects of these agents on the cells subjected to H_2O_2-induced oxidative stress were examined at concentrations ranging from 30 nM to 3 μM (Fig. 7). The threshold concentration of cassumunins A and B for protective action on the cells was 100 nM. Increasing concentrations (up to 3 μM) of the respective cassumunin dose-dependently prevented the decrease in cell viability. Cassumunins A and B at concentrations of 1 and 3 μM almost completely blocked the H_2O_2-induced decrease in cell viability. Pretreatment with curcumin at concentrations ranging from 100 nM to 3 μM also dose-dependently prevented the H_2O_2-induced decrease in the cell viability. Results are summarized in Fig. 7.

Time-dependent effects of new curcuminoids on H_2O_2-induced decrease in cell viability of thymocytes

It is very important to see if the treatments of thymocytes with cassumunins after the start of oxidative stress are also effective in protecting the cells. We have examined the effects of cassumunins A and B, respectively, applied at 1 hr before, immediately after and 1 hr after adding 3 mM H_2O_2. The concentration of cassumunins A and B was 3 μM because the cassumunins at this concentration applied at 1 hr before the start of oxidative stress almost completely suppressed the cell death induced by 3 mM H_2O_2. Their effects on the cell viability were examined at 3 hr after the start of oxidative stress. Although cassumunins A and B respectively applied after applying H_2O_2 were also effective in protecting the cells against oxidative stress, the protective actions of cassumunins A and B applied after the start of oxidative stress were less potent than those in the case of pretreatment. Results are summarized in Fig. 8.

Effects of cassumunins A and B on H_2O_2-induced changes in 2',7'-dichlorofluorescin and fluo-3 fluorescences

To characterize the protective actions of cassumunins A and B on the cells with H_2O_2-induced oxidative stress, the effects of cassumunins on H_2O_2-induced changes in DCF and fluo-3 fluorescences of the cells were determined for a number of reasons. First, the intensity of DCF fluorescence reflects the oxidation of intracellular DCFH (a reduced form of DCF) by applied H_2O_2. Therefore, if cassumunins directly affect H_2O_2 or inhibit the oxidation of cellular DCFH by H_2O_2, the intensity of DCF fluorescence in the presence of cassumunins should be lower than that in the absence of cassumunins. However, H_2O_2-induced increase in the intensity of DCF fluorescence in the presence of cassumunins was similar to that in the absence of cassumunins (Fig. 9). Results indicate that cassumunins A and B did not affect the content of H_2O_2 and the oxidation of cellular components by H_2O_2. Secondly, H_2O_2 increases intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) in thymocytes (4). Since a sustained increase in $[Ca^{2+}]_i$ is well-recognized to be linked to cell death and cell injury, if cassumunins A and B inhibit the H_2O_2-induced increase in $[Ca^{2+}]_i$, the cassumunins could reduce the cytotoxicity of H_2O_2. However, as shown in Fig. 9, cassumunins did not affect the H_2O_2-induced increase in the intensity of fluo-3 fluorescence. These results indicate that cassumunins did not affect the H_2O_2-induced

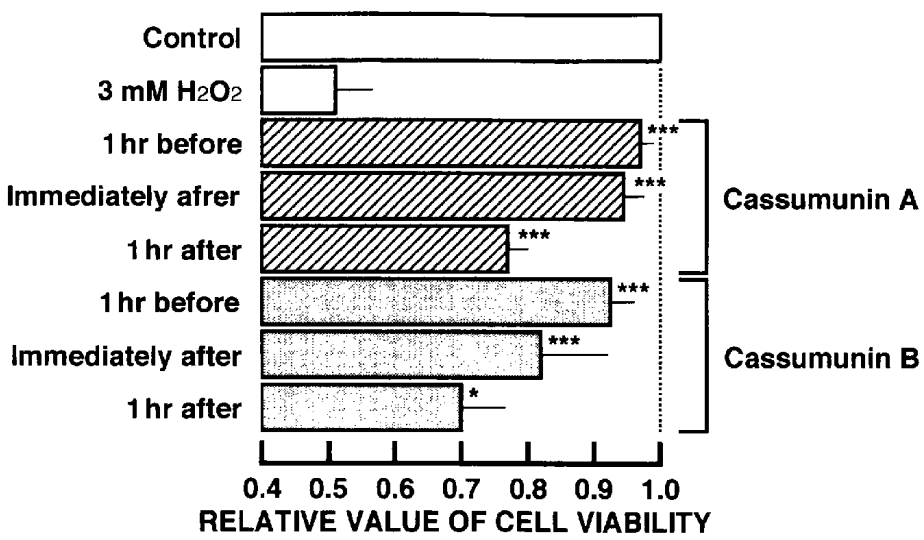

Fig. 8. Effects of cassumunins A and B applied immediately after and at 1 hr after the start of oxidative stress on the cell vialibility of thymocytes. The concentration of each cassumunin was 3 μM. Each column and bar show the average and S.D. of 4 to 6 experiments, respectively. Asterisks indicate significant differences between groups subjected to oxidative stress with and without the respective treatments with cassumunins A and B (*$P<0.01$, ***$P<0.001$).

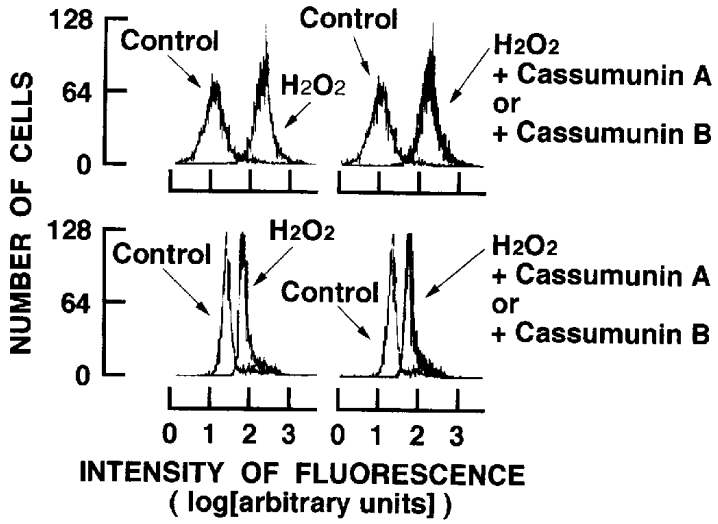

Fig. 9. Effects of cassumunins A and B (3 μM) on the H_2O_2-induced changes in 2′,7′-dichlorofluorescin (upper histograms) and fluo-3 (lower histograms) fluorescences of thymocytes that were not stained with ethidium. Cassumunins were applied to the cells at 1 hr before the start of H_2O_2-induced oxidative stress. Effects were examined at 2 hr after the start of oxidative stress.

increase in $[Ca^{2+}]_i$.

Comparison with actions of deferoxamine, ebselen and dimethyl sulfoxide

Since there are many protective agents for the cells subjected to oxidative stress, we have compared the protective action of cassumunin A on thymocytes with H_2O_2-induced oxidative stress with those of 10 mM deferoxamine (Ciba-Geigy Japan, Takarazuka), 3 μM ebselen (Sigma, St. Louis, MO, USA) and 200 mM dimethyl sulfoxide (DMSO, Katayama Chemical). As shown in Fig. 10, deferoxamine, ebselen and cassumunin A exerted protective action on the cells subjected to oxidative stress, DMSO did not.

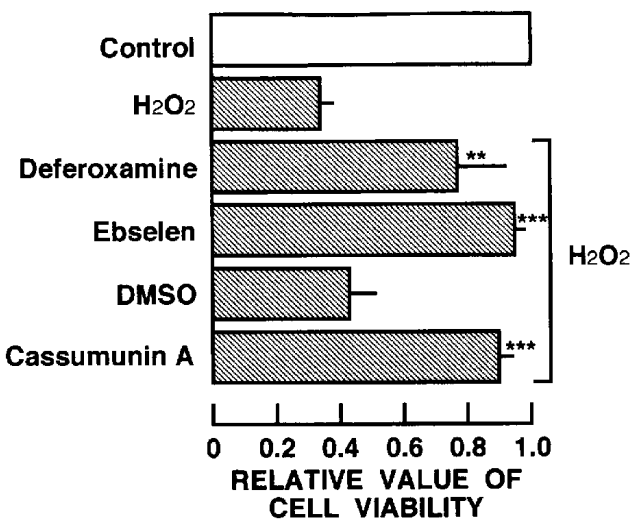

Fig. 10. Comparison of the protective actions of deferoxamine, ebselen and dimethyl sulfoxide (DMSO) on the thymocytes subjected to the oxidative stress induced by 3 mM H_2O_2. Test agents were applied to the cells at 1 hr before the start of H_2O_2-induced oxidative stress. Effects were examined at 3 hr after the start of oxidative stress. Each column and bar show the average and S.D. of 4 to 6 experiments, respectively. Asterisks indicate significant differences between H_2O_2-subjected groups with and without the respective pretreatments with test agents (**$P<0.005$, ***$P<0.001$).

DISCUSSION

Curcumin, whose chemical structure is included in those of cassumunins A and B (Fig. 1), has been known to exert inhibitory actions on lipid peroxidation and DNA damage induced by reactive oxygen species (9–13). In this study, the treatment with curcumin at concentrations ranging from 300 nM to 3 μM significantly prevented thymocytes from cell death or injury caused by H_2O_2-induced oxidative stress (Fig. 7). However, curcumin at concentrations of 10 μM or greater was cytotoxic to thymocytes, while cassumunins A and B were not. Furthermore, the potency of curcumin in protecting the cells against the oxidative stress induced by H_2O_2 seemed to be less than those of cassumunins A and B (Fig. 7).

The respective pretreatments of thymocytes with cassumunins A and B exerted potent protective actions on living cells with oxidative stress (Fig. 7). Cassumunins A and B at micromolar concentrations (1 and 3 μM) almost completely suppressed the H_2O_2-induced decrease in cell viability (Figs. 6 and 7). Furthermore, cassumunins A and B applied immediately after or 1 hr after the start of

oxidative stress were also effective in the prevention of H_2O_2-induced decrease in the cell viability (Fig. 8). This is important because of their clinical applications. The peroxidation reactions induced by reactive oxygen species are involved in many pathological events (14). Since the compounds possessing antioxidant actions are expected to be useful for prevention or treatment of diseases resulting from such pathological events, cassumunins A and B may possess considerable potential because of their protections of living cells subjected to oxidative stress (Figs. 6, 7 and 8).

The mechanism for the protective actions of cassumunins A and B on the cells with oxidative stress induced by H_2O_2 was not elucidated in this study. However, it was unlikely that cassumunins A and B directly affected H_2O_2 because H_2O_2 oxidized cellular DCFH, resulting in an increased intensity of DCF fluorescence, even in the presence of cassumunins (Fig. 9). Cassumunins A and B have been proven to possess activity aganist the oxidation of linoleic acid in chemical assays (2). Other natural curcuminoids have been reported to inhibit nitrite-induced oxidation of hemoglobin (15), copper-mediated oxidation of low density lipoprotein (16), iron-induced lipid peroxidation (17) and H_2O_2-induced hemolysis of erythrocytes (18). Since deferoxamine, a chelator for iron ion, greatly attenuated the H_2O_2-induced decrease in the cell viability (Fig. 10), hydroxyl radical may be involved in cell death or cell injury induced by H_2O_2 in this preparation. Therefore, it may be plausible that cassumunins A and B inhibit the lipid peroxidation of membranes in living cells. However, as shown in Figs. 3 and 9, the application of 3 mM H_2O_2 to the cells also increases $[Ca^{2+}]_i$ (4). The sustained increase in $[Ca^{2+}]_i$ is one of common features in the processes leading to cell death and cell injury (19–25). Although cassumunins A and B did not inhibit the H_2O_2-induced increase in $[Ca^{2+}]_i$ of thymocytes (Fig. 9), one can not neglect the possibility that cassumunins A and B block the processes after H_2O_2-induced increase in $[Ca^{2+}]_i$. We have proposed that an increased $[Ca^{2+}]_i$ induces the formation of reactive oxygen species (5). Therefore, cassumunins A and B would exert the protective actions if it is true for the preparation used in this study.

Acknowledgment

This study was supported by a Grant-in-Aid (1996) from the Ciba-Geigy Foundation (Japan) for Promoting Sciences.

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