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Vesnarinone Causes Oxidative Damage by Inhibiting Catalase Function through Ceramide Action in Myeloid Cell Apoptosis

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ABSTRACT

Vesnarinone is an effective inotropic agent for treating congestive heart failure, but its clinical usage is restricted because of the severe side effect of agranulocytosis. In myeloid HL-60 cells, vesnarinone increased the intracellular content of a pro-apoptotic lipid mediator, ceramide, in a time- and dose-dependent manner. Vesnarinone-induced apoptosis was significantly enhanced by simultaneous treatment with a cell-permeable *N*-acetyl sphingosine (C2-ceramide). Treatment with neither vesnarinone, C2-ceramide, nor simultaneously with vesnarinone and C2-ceramide caused a marked increase of reactive oxygen intermediates (ROI) generation measured by the 2′,7′-dichlorofluorescin method. However, oxidative damage judged by the production of lipid peroxidates and the nitroblue tetrazolium-reducing ability were enhanced more significantly by simultaneous treatment with vesnarinone and C2-ceramide than by vesnarinone alone. Moreover, vesnarinone inhibited catalase function both at the protein and activity level, and this inhibition was synergistically enhanced by C2-ceramide, and vesnarinone-induced oxidative damage and apoptosis were significantly suppressed by treatment of HL-60 cells with purified catalase. C2-ceramide enhanced vesnarinone-induced inhibition of the ROI-scavenging enzyme catalase at the levels of protein and activity in HL-60 cells; in contrast, however, vesnarinone did not induce ceramide generation, oxidative damage, or catalase depletion in HL-60/ves cells, where vesnarinone could not induce apoptosis. Taken together, the results suggest that vesnarinone induces myeloid cell apoptosis by increasing oxidative damage via ceramide-induced inhibition of catalase function.

Vesnarinone (3,4-dihydro-6-[4-(3,4-dimethoxy-benzoyl)-1-piperazinyl]-2(1*H*)quinolinone) was developed as an inotropic agent for treatment of congestive heart failure, but its clinical use is restricted because of the occurrence of agranulocytosis as a side effect, even though it significantly improves heart function (Feldman et al., 1993, 1996; Cohn et al., 1998). Apoptosis may be a cause of agranulocytosis: vesnarinone was reported to induce growth inhibition in many cell systems, such as salivary carcinoma cells, pancreatic cancer cells, squamous cells, hepatocellular carcinoma cells, and glioma cells (Nio et al., 1997; Yoneda et al., 1998; Kubo et al., 1999; Tanaka et al., 1999). Among hematopoietic cells, vesnarinone inhibited the colony formation of hematopoietic stem cells and the proliferation and differentiation of human myelocytic leukemia HL-60 cells by suppressing the secretion of growth factors from stroma cells (Fujiwara et al., 1997). Clinical studies of vesnarinone in patients with advanced cancer or cardiac disease found the tolerable side effects, including leukopenia, skin rash, increased liver enzymes and prolongation of QT interval, but still the incidence of neutropenia ranged from 0.2 to 2.5% (Feldman et al., 1993; Cohn et al., 1998).

The sphingolipid ceramide has recently emerged as a novel signal regulator of cell differentiation and apoptosis, although it was recognized merely as one of components of cell membrane (Hannun, 1996; Okazaki et al., 1998). It was previously reported that 1α,25-dihydroxyvitamin D3 increased ceramide generation through sphingomyelin hydrolysis to induce the differentiation of human myeloid leukemia HL-60 cells toward myeloid lineage, and this metabolic pathway was named the “sphingomyelin cycle” (Okazaki et al., 1989, 1990). In terms of the induction of apoptosis, many kinds of stress, including TNF-α, anti-Fas antibody, ultraviolet, irradiation and heat shock, have been recognized as

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ABBREVIATIONS: TNF-α, tumor necrosis factor-α; ROI, reactive oxygen intermediates; DCFH, 2′,7′-dichlorofluorescein; C2-ceramide, *N*-acetyl sphingosine; DAPI, 4′,6-Diamidine-2′-phenylindole dihydrochloride; DA, diacetate; CPA, *cis*-parinaric acid; PBS, phosphate-buffered saline; DAG, diacyl glycerol; NBT, nitroblue tetrazolium; TBS-T, Tris-buffered saline-Tween 20; ANOVA, analysis of variance.

inducers of ceramide generation (Okazaki et al., 1998). Antitumor agents, including daunorubicin, vincristine, and cytosine arabinoside, also increased ceramide generation to induce apoptosis, whereas drug-resistant cell lines showed the failure of ceramide generation by increasing glucosylceramide transferase activity or sphingomyelin synthase (Cabot et al., 1996; Kitano et al., 1998).

Reactive oxygen intermediates (ROI) were recognized as key mediators of ceramide-induced apoptosis, because many apoptosis-inducing stresses can increase intracellular oxidative damage in parallel with an increase of ceramide (Degli Esposti and McLennan, 1998; Takao et al., 2000). For example, when generation of superoxide and hydrogen peroxide (H_2O_2) was detected by the 2′,7′-dichlorofluorescein (DCFH) method anti-Fas antibody was reported to increase intracellular ROI through the ceramide-induced inhibition of electron transport in mitochondria (Quillet Mary et al., 1997). However, whether ceramide action and ceramide-induced oxidative damage are involved in vesnarinone-induced apoptosis remains unknown.

Therefore, we first examined the specificity of vesnarinone-induced toxicity in various hematopoietic cells, and then, to investigate the mechanism of vesnarinone caused agranulocytosis, we investigated the connection between oxidative damage and proapoptotic ceramide action in vesnarinone-induced myeloid cell apoptosis. We showed that vesnarinone induced myeloid HL-60 cell apoptosis via an increase of oxidative damage through ceramide-depleted catalase function and that the increase of ceramide, oxidative damage, and catalase depletion were not detected in vesnarinone resistant HL-60 cells (HL-60/ves). We also discuss the possible implications of antioxidative effects in clinically observed vesnarinone-induced agranulocytosis.

Experimental Procedures

Materials. C2-ceramide (*N*-acetyl sphingosine) was purchased from Matreya, Inc. (State College, PA). Glutathione (reduced form) was from Wako (Osaka, Japan). 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) was from Nacalai Tesque (Kyoto, Japan). 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and *cis*-parinaric acid (CPA) were from Pierce (Tokyo, Japan). Anti-catalase antibody was from Calbiochem (San Diego, CA). Vesnarinone was kindly provided by Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan). Other chemicals and reagents including purified catalase were obtained from Sigma (Tokyo, Japan).

Cell Culture. Human leukemia HL-60 cells were purchased from Riken Cell Bank (Dr. T. Ohno, Tsukuba, Ibaraki, Japan), and HL-60/ves cells were cloned from vincristine-resistant HL-60 cells kindly gifted by Dr Center, M.S. (Ma et al., 1992). Other cells were from the American Type Culture Collection (Manassas, VA). All cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum (Upstate Biotechnology, Inc., Lake Placid, NY) at 37°C in a 5% CO_2 incubator. The cells were washed three times in phosphate-buffered saline (PBS) and resuspended in RPMI 1640 medium containing 5 μg/ml transferrin, 5 μg/ml insulin, and 2% fetal bovine serum, and then used for the experiments. The viable cells, which excluded trypan blue dye, were counted under a microscope using a hemocytometer at the indicated times.

Detection of Apoptosis by Nuclear Condensation and Fragmentation. Apoptosis as judged by nuclear condensation and fragmentation was identified by staining the cells with DAPI. In brief, cells were washed and fixed with 1% glutaraldehyde for 30 min and then labeled with 2 μg/ml DAPI. After labeling, apoptotic cells were visualized under a fluorescent microscope (BX60-34FFB1; Olympus, Tokyo, Japan). Morphological changes of apoptotic cells were also confirmed with May-Giemsa staining. Cells with condensed or fragmented nuclei were scored as apoptotic cells. At least 200 cells were counted in each experiment, and the data are the means of at least three independent experiments.

Ceramide Quantitation. After extracting the lipids according to the Bligh and Dyer method, ceramide levels were enzymatically measured by using *Escherichia coli* diacylglycerol (DAG) kinase, which converts ceramide to ceramide-1 phosphate and corrected by phospholipid phosphate as described before (Okazaki et al., 1989). The lack of change of DAG kinase activity during the procedure was confirmed by experiments using 40 nmol of C2-ceramide in the reaction mixture as an internal standard, and the amount of phospholipid phosphate was shown to parallel the viable cell numbers as described in the text.

Detection of ROI Production by DCFH Method. Production of ROI was measured with DCFH-DA, which is cell permeable and is oxidized inside the cells to fluorescent 2′,7′-dichlorofluorescein in the presence of ROI. Cells treated with vesnarinone or untreated cells were washed with PBS twice, resuspended in 1 ml of PBS, and incubated with 5 μM DCFH-DA for 15 min at 37°C. Then the cells were washed in PBS, and fluorescence was determined using flow cytometry on a FACScan (Becton Dickinson) with an excitation wavelength of 488 nm and an emission wavelength of 530 nm (Bass et al., 1983).

Determination of Lipid Peroxidation. Lipid peroxidation was indicated by the decrease in CPA fluorescence measured using a fluorescence spectrophotometer F-3000 (Hitachi). In brief, cells, after coincubation with C2-ceramide and/or vesnarinone for the indicated times, were washed with PBS twice and resuspended in a 3-ml volume of RPMI-1640 medium, then were incubated with 5 μM CPA for 30 min. Loss of fluorescence was monitored for 1 min with excitation and emission wavelengths of 318 nm and 420 nm, respectively (Hedley and Chow, 1992).

Measurement of NBT-Reducing Ability. After treatment, the cells were resuspended in 0.1 ml of 400 ng/ml phorbol 12-myristate 13-acetate; Sigma, MO) and 0.1 ml of 1 mg/ml NBT (Nacalai Tesque, Inc., Kyoto, Japan) solution in PBS and incubated for 1 h at 37°C. After incubation, the cells were centrifuged and resuspended in 100 μl of PBS. They were then smeared on a glass slide and counterstained with 0.3% (w/v) safranin O (Nacalai Tesque, Inc.) in methanol. Positive cells reduce NBT, yielding intracellular deposits of black-blue formazan dye; this ability was determined by the microscopic examination of over 200 cells. The results were expressed as a percentage of the total cells that were positive for NBT reduction.

Assay for Internucleosomal DNA Fragmentation. DNA extraction was performed using the GENOME DNA isolation kit (Bio 101, Vista, CA) according to the manufacturer's protocol with slight modifications. Cells with or without treatment by various drugs were collected by centrifugation in a Eppendorf tube and washed once in PBS. They were resuspended in 185 μl of cell suspension solution. After the addition of 5 μl of RNase Mixx (Qbiogene, Illkrich, France) and 10 μl of cell lysis/denaturing solution, the cell lysate was incubated at 55°C for 15 min. Then 2.5 μl of Protease Mixx (Qbiogene) was added to the lysate, and the mixture was further incubated for 2 h. After the addition of 50 μl of Salt Out (Qbiogene, Illkrich, France) the mixture was cooled on ice for 20 min and centrifuged at 100,000*g* for 15 min at 4°C in a Hitachi Koki Himac CS 100 EX ultracentrifuge. Then 1 ml of 80% ethanol diluted with Tris/EDTA buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA) was added to the supernatant, cooled on ice for 20 min, and centrifuged at 12,000*g* for 15 min at 4°C in a microcentrifuge. The DNA pellet was dissolved in Tris/EDTA buffer, pH 8.0. The concentration of DNA was estimated by determining the absorbance at 260 nm. Electrophoresis was carried out in a 2% NuSieve-agarose (FMC Bioproducts, Rockland, ME) minigel in 1× Tris/acetate/EDTA buffer (40 mM Tris acetate, 1 mM

EDTA) at 100 V for 30 min. DNA was visualized under UV light after staining with ethidium bromide.

Western Blotting. The cells (5×10^6) were collected by centrifugation, washed in ice-cold PBS, and lysed in 400 μl of a lysis buffer (0.5% Triton X-100, 50 mM Tris/HCl, pH 7.6, 300 mM NaCl, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 4 mM EDTA, 1 mM sodium orthovanadate, 10 mM NaF, and 10 mM sodium pyrophosphate) at 4°C with sonication. The concentration of protein was measured using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). The samples were denatured by boiling in Laemmli sample buffer (25 mM Tris/HCl, pH 6.8, 0.5% SDS, 5% glycerol, 0.05% phenol red, and 1.5% 2-mercaptoethanol) for 5 min, subjected to SDS-polyacrylamide gel electrophoresis using a 10 or 15% running gel, and electroblotted to an Immobilon-P transfer membrane (Millipore Corp., Bedford, MA). Nonspecific binding was blocked by incubating the membrane with 10% Blockace (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) in TBS-T buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) overnight. Then, the membrane was washed in TBS-T buffer for 15 min three times and incubated with a 1:500 dilution of rabbit anti-human erythrocyte catalase antibody (Athens Research and Technology, Inc., Athens, Georgia) in TBS-T buffer for 1 h. The membrane was washed in TBS-T buffer for 15 min three times and incubated with a 1:1000 dilution of donkey anti-rabbit horseradish peroxidase-conjugate-d IgG (Amersham Biosciences, Piscataway, NJ) in TBS-T buffer for 30 min. After washing the membrane for three times for 5 min each in TBS-T buffer, the detection of catalase was performed using enhanced chemiluminescence Western blotting detection reagents (Amersham) according to the manufacturer's protocol.

Statistical Examination. Statistical significances in this work were calculated by ANOVA test.

Results

Effects of Vesnarinone on Cell Growth in Human Myeloid Precursor HL-60 Cells. Because vesnarinone was reported to cause agranulocytosis in a clinical trial, we examined whether myeloid precursor HL-60 cells were susceptible to its cytotoxicity. When myeloid HL-60 cells were treated with vesnarinone at the various concentrations up to 100 μg/ml, the viability was significantly decreased in a time- and dose-dependent manner (Fig. 1). We next examined whether vesnarinone-induced HL-60 cell death was caused by the induction of apoptosis. When the cells were treated with various concentrations of vesnarinone, the percentage of apoptotic cells judged by DAPI staining method was 35 ± 3, 12 ± 2, 3 ± 2, and 2 ±2% in the presence of 100, 50, 20, and 0 μg/ml of vesnarinone, respectively, 24 h after treatment (Fig. 1, inset). The finding that vesnarinone had marked cytotoxicity, as evidenced by induction of apoptosis in myeloid cells, suggested that analysis of the mechanism by which vesnarinone inhibited HL-60 cell growth should contribute to clarify the mechanism of agranulocytosis.

Increase of the Ceramide Content by Vesnarinone, and Synergistic Effects of C2-Ceramide on Vesnarinone-Induced Apoptosis in HL-60 Cells. As shown in Fig. 2, A and B, when the cells were treated with vesnarinone, ceramide generation was increased in a time- and dose-dependent manner. Ceramide content in the control cells was 7.1 ± 0.8 pmol/nmol of phospholipid. Vesnarinone increased ceramide generation within 12 h after treatment, and 50 μg/ml vesnarinone increased the ceramide content by 42% after 48 h of treatment. Because the cells treated with 50 μg/ml vesnarinone showed an inhibition of cell growth approximately 24 h after treatment, ceramide generation seemed to precede the execution of cell death. To determine the relation between vesnarinone-induced apoptosis and ceramide action, it was examined whether exogenous C2-ceramide influenced the effects of vesnarinone on induction of apoptosis. Simultaneous treatment of the cells with vesnarinone and various concentrations of C2-ceramide increased the proportion of apoptotic cells significantly compared with vesnarinone alone. As shown in Fig. 3A, the percentage of apoptotic cells induced by 50 μg/ml vesnarinone increased from 9 to 50% in the presence of 4 μM C2-ceramide, which alone induced only 15% of apoptotic cells, 12 h after treatment, demonstrating the synergistic effects of ceramide on vesnarinone-induced apoptosis. In addition, the synergistic effects of ceramide on vesnarinone-induced apoptosis were also confirmed by the increase of DNA fragmentation (Fig. 3B).

Effects of C2-Ceramide on Vesnarinone-Induced Oxidative Damage in HL-60 Cells. The generation of ROI, mainly consisting of H_2O_2, was measured by the DCFH method in the presence of either vesnarinone, C2-ceramide, or both. Vesnarinone (50 μg/ml) alone did not show a significant increase of fluorescence levels (Fig. 4A, b), whereas 3 μM C2-ceramide alone slightly increased the peak level of fluorescence compared with no treatment (Fig. 4A, a). Simultaneous treatment with C2-ceramide and vesnarinone caused a significant but faint increase of the fluorescence level compared with treatment with vesnarinone alone (Fig.

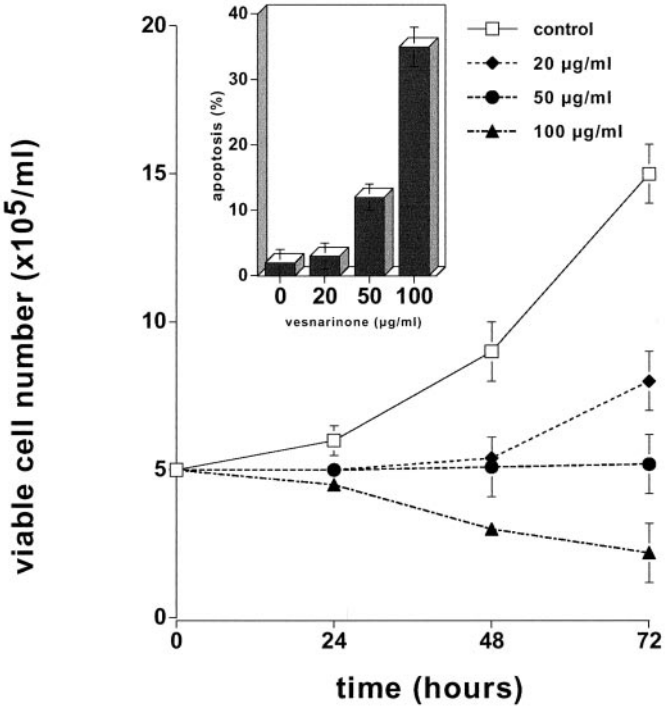

Fig. 1. Effects of vesnarinone on cell growth and apoptosis in human myeloid leukemia HL-60 cells. Human myeloid leukemia HL-60 cells at an initial concentration of 2.5×10^5/ml were treated with the indicated concentrations of vesnarinone for up to 72 h and harvested at the indicated times. The data in inset show the percentage of apoptotic cells after treatment with various concentrations of vesnarinone for 24 h compared with no treatment. The viable cell numbers were counted with the 0.025% trypan blue exclusion method by using a hemocytometer, and apoptosis was assessed by DAPI staining method under a microscope as described under *Experimental Procedures*. The results were obtained from three different experiments. The bars indicate 1 S.D.

4A, c). In contrast, even under conditions in which 100 μM H_2O_2 dramatically increased the ROI level (Fig. 4B, b), 50 μg/ml vesnarinone could still enhance ROI levels (Fig. 4B, c), suggesting that the increase of ROI accumulation was caused by the inhibition of ROI-scavenging system rather than direct enhancement of ROI generation by vesnarinone. Indeed, when we examined the extent of oxidative damage by assessing NBT-reducing ability and the extent of oxidized CPA, vesnarinone significantly increased the levels of NBT-positive cells and lipid peroxidation (Fig. 4, C and D). Moreover, simultaneous treatment with C2-ceramide showed a marked increase of vesnarinone-induced NBT-reducing ability and lipid peroxidation compared with vesnarinone or C2-ceramide alone (Fig. 4, C and D).

Effects of C2-Ceramide on Vesnarinone-Inhibited Catalase Function. As shown in Fig. 5A, the amount of catalase protein was slightly decreased 24 h after treatment with either vesnarinone or C2-ceramide alone, and catalase protein almost completely disappeared after simultaneous treatment with vesnarinone and C2 ceramide. The level of catalase activity was also decreased significantly after 24 h of simultaneous treatment with vesnarinone and C2-ceramide compared with vesnarinone alone (Fig. 5B). These results suggest that the mechanism by which vesnarinone inhibits catalase activity by decreasing its protein levels involves ceramide signaling.

Effects of Exogenous Catalase on Vesnarinone-Induced Oxidative Damage and Apoptosis. We next examined the role of exogenous catalase in vesnarinone-induced lipid peroxidation and apoptosis in HL-60 cells. Treatment with purified catalase blocked vesnarinone-induced lipid peroxidation and apoptosis (Fig. 6, A and B). Catalase (400 U/ml) decreased lipid peroxidation and apoptosis caused by 50 μg/ml vesnarinone from 136 to 112% and from 29 to 7%, respectively. These results suggest that oxidative damage regulated via catalase function is closely involved in vesnarinone-induced HL-60 cell apoptosis.

Lack of Increase of Ceramide, Lipid Peroxidation, and Apoptosis, and Lack of Depletion of Catalase at the Protein and Activity Levels by Vesnarinone in HL-60/ves Cells. To investigate whether ceramide action is re-

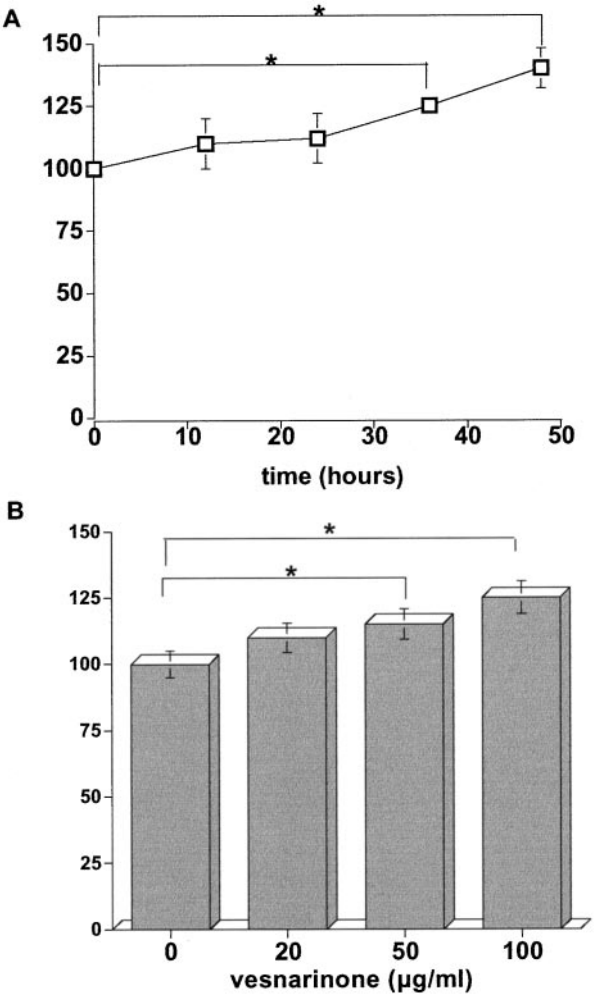

Fig. 2. Increase of ceramide content in a time- and dose-dependent manner by vesnarinone in HL-60 cells. The cells were treated with 50 μg/ml vesnarinone for the indicated times (0, 12, 24, 36, or 48 h) (A) or with the indicated concentrations (0, 20, 50, or 100 μg/ml) for 24 h (B). After harvesting the cells, the lipids were extracted by the Brigh and Dyer method and ceramide contents were measured by the DAG kinase method as described under *Experimental Procedures*. The results were obtained from more than three different experiments. The bars indicate 1 S.D. The vertical axis represents ceramide content as a percentage of control. The significance of differences of ceramide levels was determined by ANOVA. *, $p < 0.01$.

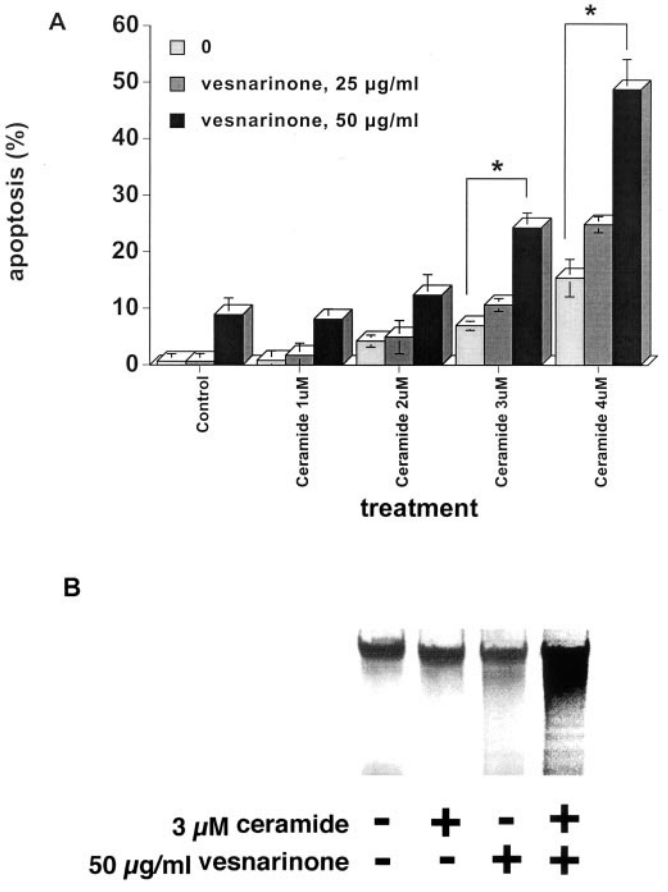

Fig. 3. Synergistic effects of C2-ceramide on vesnarinone-induced apoptosis in HL-60 cells. The cells were treated simultaneously with various concentrations of C2-ceramide (0, 1, 2, 3, or 4 μM) and vesnarinone (0, 25, or 50 μg/ml) for 24 h. Apoptotic cells showing nuclear condensation and fragmentation were counted by using the DAPI method (A) and DNA agarose gel electrophoresis (B) as described under *Experimental Procedures*. The results were obtained from three different experiments. The bars indicate 1 S.D. The significance of differences of apoptosis levels between the treatments was determined by ANOVA. *, $p < 0.01$ (A).

quired for vesnarinone-induced apoptosis and oxidative damage, we examined the effects of vesnarinone on induction of apoptosis, ceramide increase, and lipid peroxidation in HL-60/ves cells, which show marked resistance to vesnarinone-induced apoptosis (Fig. 7A). As shown in Fig. 7, B to E, vesnarinone induced apoptosis, ceramide generation, and oxidative damage, as judged by the extent of lipid peroxidation in a dose-dependent manner in HL-60 cells, whereas no increase of apoptosis, ceramide generation, and lipid peroxidation and no inhibition of catalase function were detected 3 days after treatment with vesnarinone at up to 100 μg/ml in HL-60/ves cells. These results clearly suggest that the ceramide-induced depletion of catalase at the levels of activity and protein and the subsequent increase of oxidative damage were required for vesnarinone-induced apoptosis in HL 60 cells.

Discussion

Vesnarinone was developed as an effective therapeutic agent exerting an inotropical effect for congestive heart failure, but it was found to induce neutropenia including severe agranulocytosis at the rate of 0.3 to 2.5% (Feldman et al., 1993; Cohn et al., 1998). Here, first of all, we examined

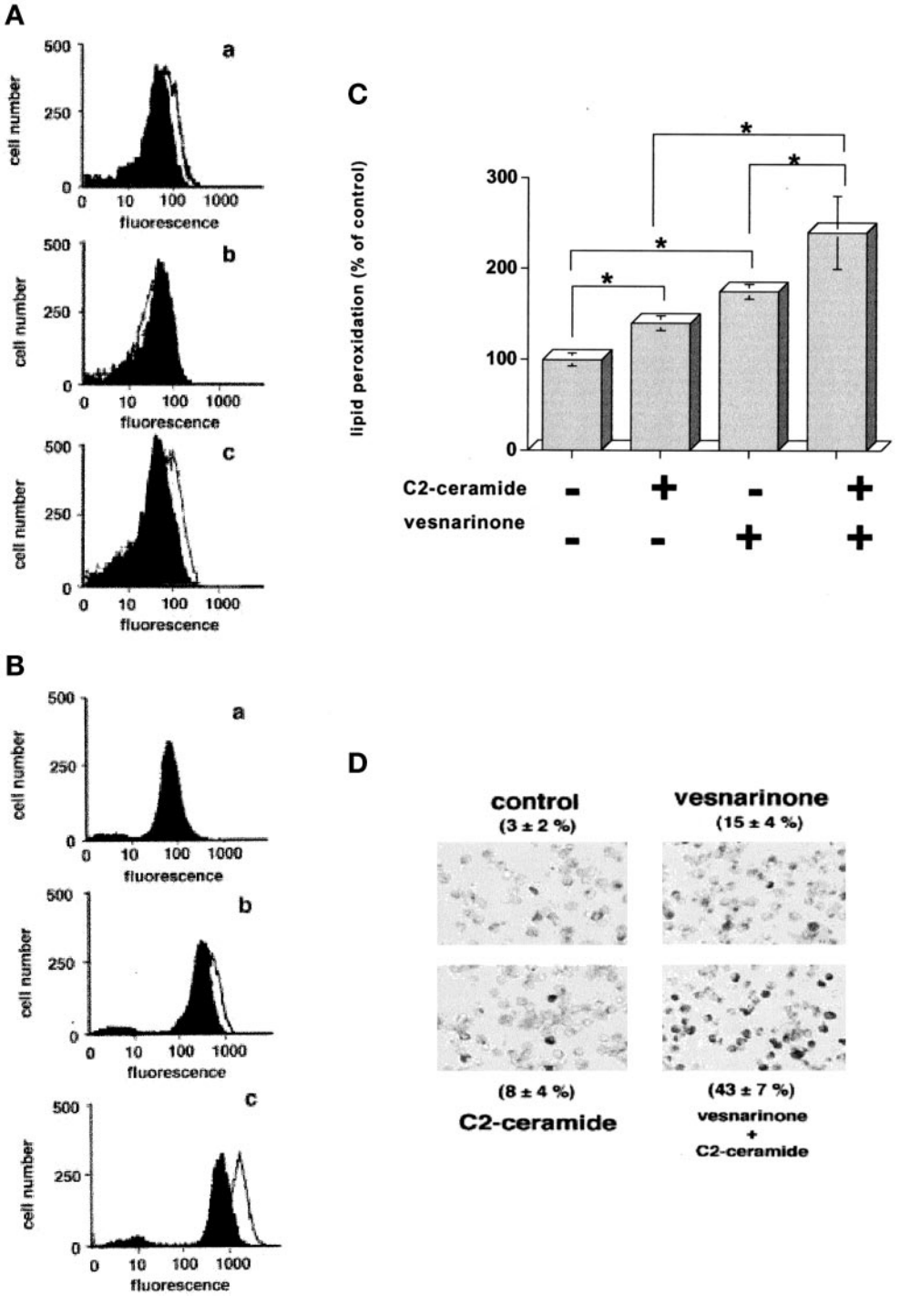

Fig. 4. Effects of vesnarinone and C2-ceramide on the generation of ROIand lipid peroxidation in HL-60 cells. A, the cells were treated with (open area) or without (filled area) 3 μM C2 ceramide (a), 50 μg/ml vesnarinone (b), or both (c) for 24 h, harvested and treated with DCFH for 30 min. Then, the fluorescence of the cells was measured using a FACScan as described under *Experimental Procedures*. The results are the representative of at least three different experiments. B, the cells were treated without (a) or with 100 μM H_2O_2 (b) and 1 mM H_2O_2 (c) for 10 min in the presence (open area) or absence (closed area) of 50 μg/ml vesnarinone. After the incubation with DCFH for 30 min, the fluorescence was measured by FACScan as described under *Experimental Procedures*. The results were the representative for at least three different experiments. C, the cells were treated with or without 3 μM C2-ceramide, 50 μg/ml vesnarinone, or both for 24 h, and harvested. Then, lipid peroxidation was determined by measuring the extent of oxidation of *cis*-parinaric acid as described under *Experimental Procedures*. D, the cells were treated with or without 3 μM C2-ceramide, 50 μg/ml vesnarinone, or both for 24 h, harvested and then NBT-reducing ability was determined as described under *Experimental Procedures*. The number in the parenthesis was the percentage of NBT-positive cells. The results are the representative of at least three different experiments. The bars indicate 1 S.D. The significance of differences of lipid peroxidation production between the treatments was determined by ANOVA test. $*$, $p < 0.01$ (C).

whether vesnarinone caused growth inhibition due to apoptosis specifically in myeloid-lineage HL-60 cells as shown in Fig. 1. In addition, it was shown that vesnarinone inhibited CD4-cross-linking–induced lymphocytic apoptosis by blocking the secretion of TNF-α and interferon-γ in Jurkat T cells (Oyaizu et al., 1996; Manna and Aggarwal, 2000) and may be further confirmed clinically by the finding that pancytopenia was rare compared with neutropenia as a hematological side effect of vesnarinone (Feldman et al., 1996).

In general, agranulocytosis is thought to be caused by direct drug-induced toxicity or immune-mediated reaction against hematopoietic stem cells (Meyer-Gessner et al., 1994; Ruvidic, 1996). Vesnarinone was reported to suppress the colony formation of blasts obtained from leukemia patients and the proliferation of several leukemia cell lines (Fujiwara et al., 1997). It was also reported that vesnarinone inhibited HL-60 cell growth indirectly by the decrease of cytokine secretion from the stroma cells, which support hematopoietic cell growth in the bone marrow (Aizawa et al., 1997; Nabeshima et al., 1997). In terms of the biochemical effects of vesnarinone, the following findings have been reported so far: (1) inhibition of phosphodiesterase and a subsequent increase of intracellular cAMP content (Sasayama and Matsumori, 1996; Jiang et al., 1999), (2) increase of calcium in whole cells (Sato et al., 1996), (3) modulation of potassium and sodium channels (Cavusoglu et al., 1995) and (4) activation of tyrosine phosphorylation of cell adhesion-related molecules (Sato et al., 1995). In addition, vesnarinone inhibited the secretion of lipopolysaccharide-induced TNF-α, interleukin 6, and granulocyte/monocyte colony stimulating factor (Shioi et al., 1994; Uetrecht et al., 1994; Matsumori and Sasayama, 1995) and induced cell cycle arrest in G_1 phase by inhibiting G_1 cyclin (Yokozaki et al., 1999) and cyclin E/Cdk2 kinase activity (Yoneda et al., 1998). However, the precise relation of these findings to vesnarinone-induced apoptosis remains to be determined.

It has been demonstrated that the generation of ROI is closely related to proapoptotic signaling in many cell types (Wong et al., 1989; Schulze-Osthoff et al., 1993). As shown in Fig. 4A, a significant increase of ROI generation by the simultaneous treatment of vesnarinone with C2-ceramide was detected. Similarly, the extent of NBT-reducing ability

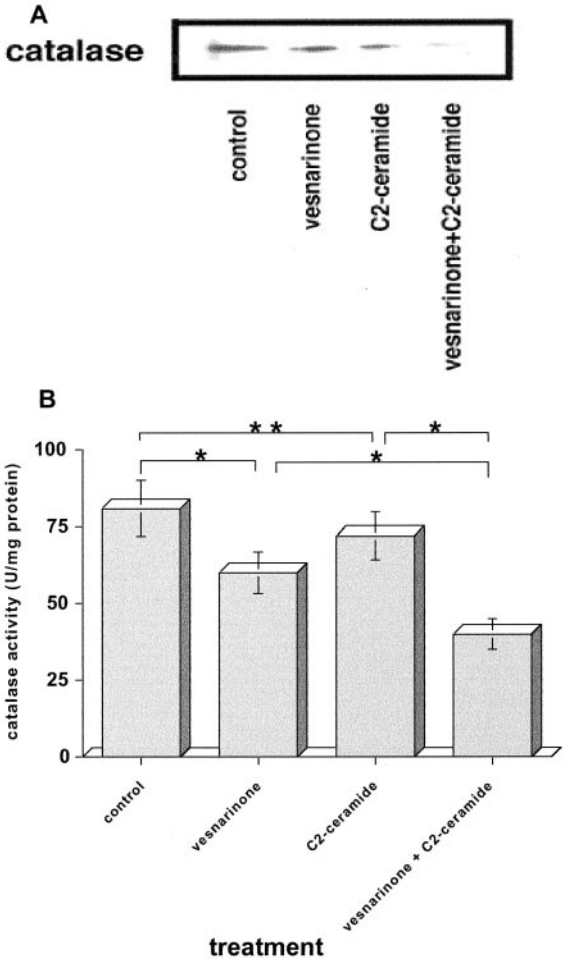

Fig. 5. Enhancing effects of C2-ceramide on vesnarinone-inhibited catalase function in HL-60 cells. The cells were treated with or without 3 μM C2-ceramide, 50 μg/ml vesnarinone, or both for 24 h and harvested. Catalase levels were detected by Western blotting analysis using anti-catalase antibody (A). The results are the representative for at least three different experiments. The activity of catalase was measured by the decrease of H_2O_2-induced oxidative status (B) as described under *Experimental Procedures*. The results were obtained from three different experiments. The bars indicate 1 S.D. The significance of differences of catalase activity level between the treatments was determined by ANOVA. *, $p < 0.01$. **, $p < 0.05$ (B).

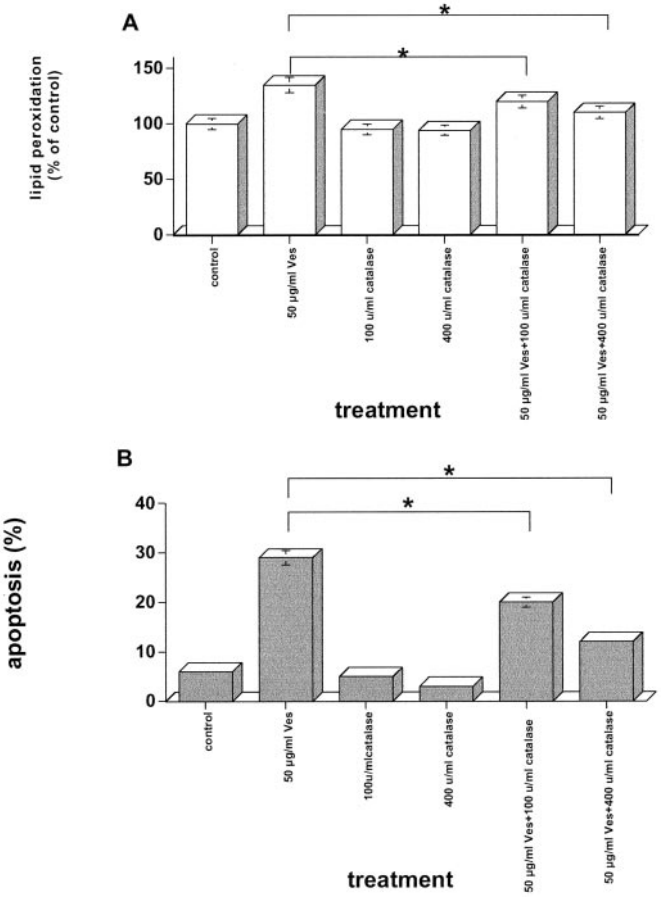

Fig. 6. Effects of exogenous catalase on vesnarinone-induced lipid peroxidation and apoptosis in HL-60 cells. The cells were treated with purified catalase at the concentration of 100 or 400 U/ml in the presence or absence of 50 μg/ml vesnarinone. Then, lipid peroxidation (A) and apoptosis (B) were examined as described under *Experimental Procedures*. The results were obtained from three different experiments. The bars indicate 1 S.D. The significance of differences of lipid peroxidation production (A) and apoptosis level (B) between the treatments was determined by ANOVA. *, $p < 0.01$.

and lipid peroxidation was significantly enhanced by simultaneous treatment with vesnarinone and C2-ceramide. In addition, even after the addition of an excess of H2O2, treatment with vesnarinone still increased the ROI level (Fig. 4B, c), and the extent of increase of ROI was much more than that caused by vesnarinone alone (Fig. 4A, a). Therefore, it is likely that vesnarinone increased oxidative damage, probably by inhibiting the ROI-scavenging system rather than by directly intensifying the generation of ROI, even though ROI measurement by DCFH method was reported to be careful to assess oxidative damage due to its modification by the intracellular enzymes such as peroxidase and esterase (Rota et al., 1999a,b). Indeed, as shown in Fig. 5, the protein and activity levels of catalase, which is recognized as one of the key enzymes for scavenging ROI (Mates, 2000), was depleted by vesnarinone in HL-60 cells, and this depletion was significantly enhanced in the presence of C2-ceramide. In contrast, the depletion of catalase and oxidative damage did not occur even at a higher concentration (100 μg/ml) of vesnarinone in HL-60/ves cells, which are resistant to vesnarinone-induced apoptosis (Fig. 7), suggesting that vesnarinone-increased oxidative damage is closely related to ceramide-depleted catalase function.

As far as we know, this is the first report to show the molecular mechanism by which vesnarinone induces apoptosis in myeloid HL-60 cells. We here showed that oxidative damage through ceramide-related signals was required for vesnarinone-induced apoptosis. This notion was supported by the findings that (1) vesnarinone increased the intracellular ceramide content in myeloid HL-60 cells in parallel with apoptosis induction (Figs. 1 and 2), (2) vesnarinone-induced oxidative damage caused by depletion of catalase function and apoptosis were synergistically enhanced by exogenous ceramide but blocked by treatment with the purified catalase (Figs. 3 to 5), and (3) ceramide generation and oxidative damage caused by depletion of catalase function were not induced by vesnarinone in apoptosis-resistant HL-60/ves cells (Fig. 7).

In summary, we showed that vesnarinone induced myeloid cell apoptosis more effectively than that of other lineages of cells by increasing oxidative damage through ceramide-depleted catalase function. This conclusion may lead us two possible clinical approaches; one is preventing vesnarinone-caused agranulocytosis by reducing oxidative damage with ROI scavengers or antioxidants in the patients with heart failure and another is searching for more effective chemotherapy of acute leukemia by improving the ability of vesnarinone to cause apoptosis, because the recent phase I study of vesnarinone in combination with gemcitabine seemed to be effective in patients with advanced solid tumors (Patnaik et al., 2000).

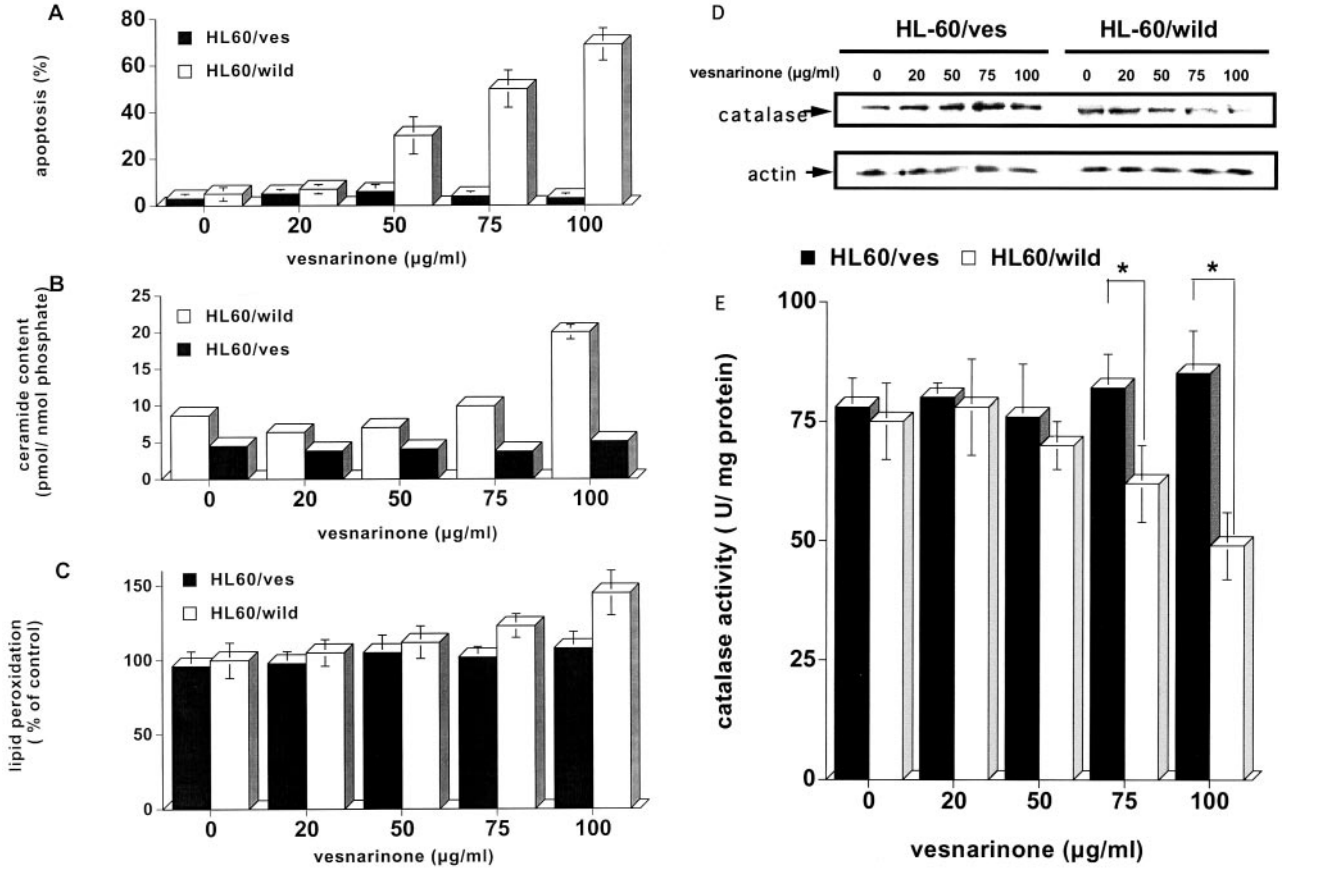

Fig. 7. No increase of ceramide generation, lipid peroxidation, and apoptosis, and no depletion of catalase protein and activity by vesnarinone in HL-60 or HL-60/ves cells. The cells were treated with various concentrations of vesnarinone (0, 20, 50, 75, or 100 μg/ml) for 72 h, and harvested. Induction of apoptosis (A), ceramide generation (B), lipid peroxidation (C), and catalase function (D, protein levels; E, activity levels) were examined as described under *Experimental Procedures*. The results were obtained from three different experiments or are the representative of at least three different experiments. The bars indicate 1 S.D. The significance of differences of catalase activity (E) between HL-60 and HL-60/ves cells was determined by ANOVA. *, $p < 0.01$.

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