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# Involvement of $Ca^{2+}$ Ion and Reactive Oxygen Species as a Mediator in Pradimicin-induced Apoptosis

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Pradimicin (PRM) induces apoptosis in mammalian cells which had been incubated with 1-deoxymannojirimycin (DMJ). Flow cytometric analysis revealed that PRM preferentially induced apoptosis to the cells of the G1 phase. Two possible mediators in this apoptotic cascade were identified. Exposure of DMJ-treated cells to PRM resulted in a rapid ($\sim$5 seconds) and slow ($\sim$30 minutes) elevation of the intracellular calcium level. Reactive oxygen species (ROS) were proved to be involved in this system by the fact that the apoptosis was completely inhibited by treating the cells with a ROS scavenger, *N*-acetylcysteine in prior to the PRM stimulation.

Apoptosis is an active process by which a multicellular organism eliminates redundant, defective, or destructive cells to control development, proliferation, immunological tolerance, and diseases. Many key different molecules such as reactive oxygen species, calcium ion, proteases, and kinases participate in apoptosis and there seems to be several pathways depending on the death signal. In the preceding paper, we reported that pradimicin specifically induces apoptosis in mammalian cells which carry high-mannose type oligosaccharides at the cell surface.[1] There are not many studies which have identified the involvement of cell surface glycans in apoptosis.[2~4] BAUM *et al.* reported that galectin-1 induced apoptosis of T cells by binding to *N*-linked oligosaccharides and the apoptosis was avoided when the formation of complex type glycoproteins had been blocked through the inhibition of mannosidase II by swainsonine.[4] Despite the findings of lectin-induced apoptosis, no such small molecules as PRM has ever been identified to induce apoptosis which is mediated by cell surface glycans. Though the initial object of our works on PRM was to elucidate its antifungal mechanism, we further investigated the PRM-induced apoptosis of mammalian cells owing to its unusual property of calcium-dependent binding to high-mannose type oligosaccharides. We herein report the relationship of the cell cycle and apoptosis and the involvement of intracellular $Ca^{2+}$ and reactive oxygen species (ROS) in PRM-induced apoptosis.

## Materials and Methods

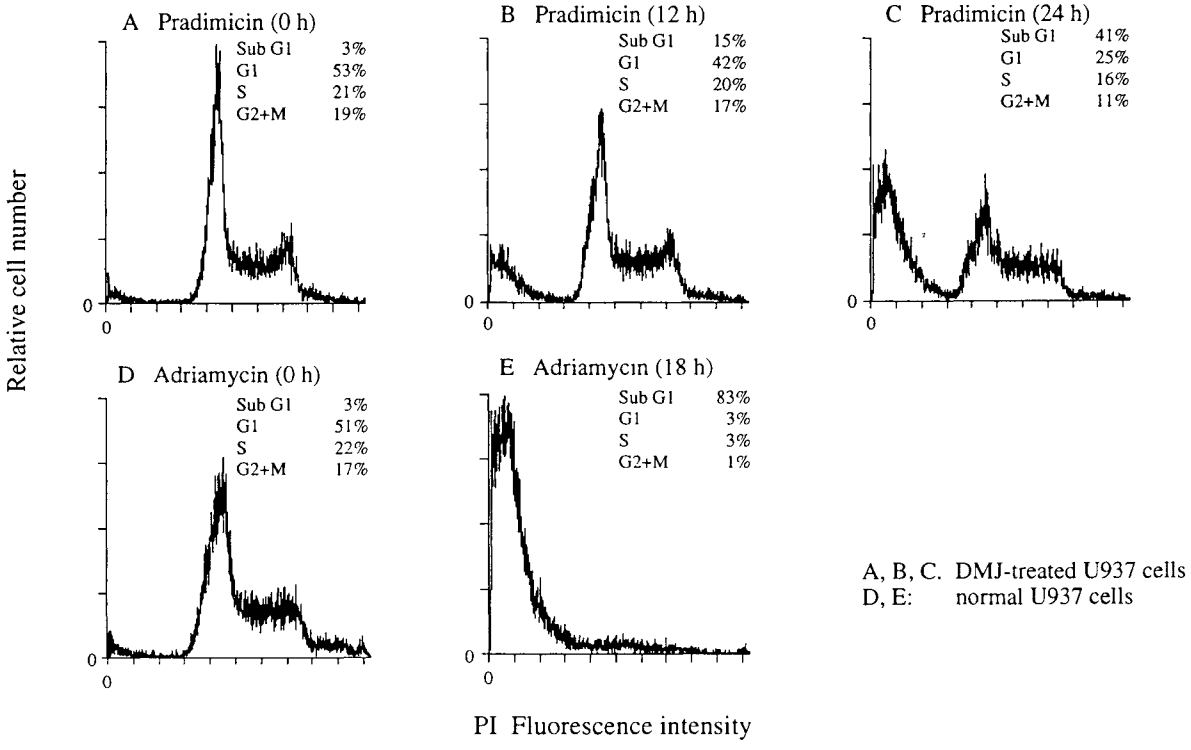
### Materials

Fura2-AM and BAPTA-AM were purchased from Molecular Probes, Inc. (Eugene, OR, USA) and dissolved in DMSO to give 10 mg/ml stock solution. *N*-Acetylcysteine was from Sigma Chemical Co. (St.Louis, MO, USA) and dissolved in RPMI 1640 medium (Nissui, Tokyo, Japan) to provide 1 M stock solution and the pH was adjusted to 7.1$\sim$7.3 by the addition of $NaHCO_3$. Z-Asp-$CH_2$-DCP, an ICE protease inhibitor, was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and diluted with DMSO to give 10 mg/ml stock solution. Cell Tak was from Cosmobio Co. (Tokyo, Japan). All stock solutions were kept at $-20$°C.

### Flow Cytometric Analysis

U937 cells ($2 \times 10^6$) were washed with PBS ($-$) three times. Then, the cells were incubated in 70% EtOH

Fig. 1. Detection of DNA fragmentation by flow cytometric analysis.




(200 $\mu$l) at $-20$°C for 4 hours, washed with PBS (−), incubated in phosphate-citrate buffer (4% 0.1 M citric acid in 0.2 M $Na_2HPO_4$) for 30 minutes at room temperature, suspended in PBS (−) (200 $\mu$l), and incubated with RNaseA (10 mg/ml, 10 $\mu$l) for 20 minutes at 37°C. The cells were resuspended in PBS (−) (1 ml) and stained with propidium iodide (1 mg/ml, 50 $\mu$l) at 4°C for 30 minutes under dark. Fluorescence intensity was quantified using a flow cytometer (Coulter Epics Elite).

Intracellular $Ca^{2+}$ Ion Concentration Measurement

U937 cells ($1 \times 10^6$/ml) loaded with 2 $\mu$M Fura2-AM for 20 minutes at 37°C were washed with PBS (+) three times, suspended in phenol red free RPMI 1640 medium (1 ml), and fixed on a culture dish coated with Cell Tak. Fluorescence was detected by a confocal laser microscope (Diaphoto TMD380, Nikon) and the intracellular $Ca^{2+}$ concentration was calculated utilizing an image analyzing system (ARGUS-50, Hamamatsu Photonics).

*N*-Acetylcysteine Treatment

DMJ-treated cells ($5 \times 10^4$/ml) were cultured with *N*-acetylcysteine (0 ~ 10 mM) for 3 hours at 37°C, washed with PBS (−) three times, and resuspended in RPMI 1640 medium ($2.5 \times 10^5$/ml). Then, the cells were treated with 50 $\mu$g/ml PRM in the presence of 1 mM $CaCl_2$ for 24 hours at 37°C and the cell viability was determined by MTS assay.

Z-Asp-$CH_2$-DCP Treatment

DMJ-treated cells ($2.5 \times 10^5$/ml) were incubated with 50 $\mu$g/ml PRM in RPMI 1640 medium containing 1 mM $CaCl_2$ in the presence of Z-Asp-$CH_2$-DCP (0 ~ 300 $\mu$g/ml) at 37°C. After 24 hours, the cell viability was determined by MTS assay.

## Results

PRM-induced Apoptosis and Cell Cycle

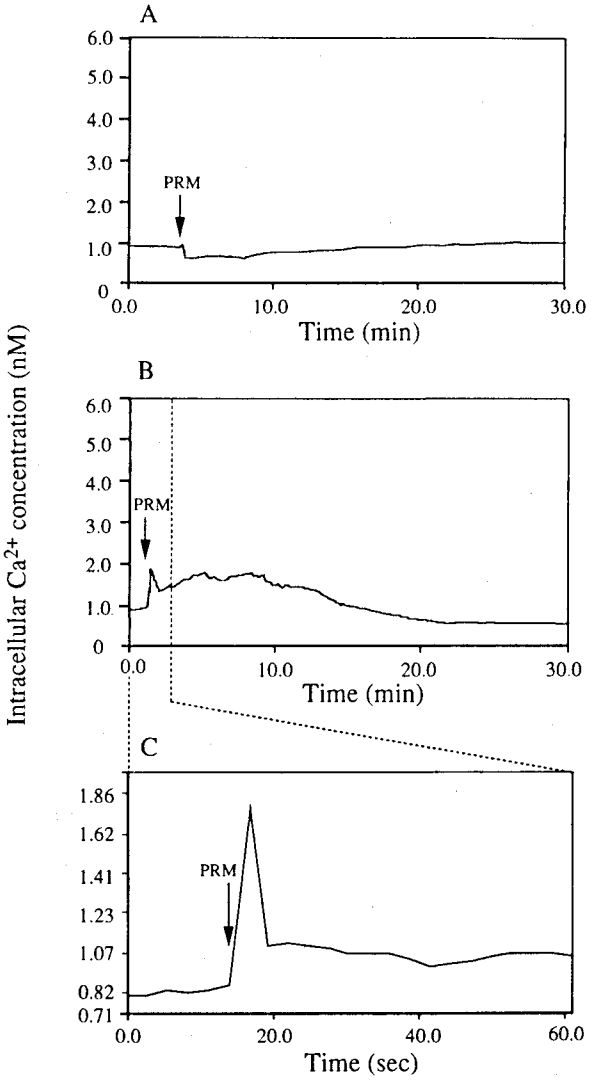
Flow cytometric analysis was performed to investigate the relationship between apoptosis and the cell cycle (Fig. 1). The cell cycle distribution of DMJ-treated cells showed a similar profile to that of normal cells, indicating that inhibition of α-mannosidase I by DMJ did not

affect the cell cycle (Fig. 1A, 1D). When the normal U937 cells were treated with adriamycin, a known apoptosis inducer, DNA fragmentation took place in most of the cells after 18 hours (Fig. 1D, 1E). PRM induced apoptotic DNA fragmentation in 15% and 41% of DMJ-treated cells after 12 and 24 hours respectively (Fig. 1B, 1C). This is in good accordance with the result that about 40% of the cells were determined to be dead by MTS assay after 24 hours under the same condition. Remarkably, DMJ-treated cells of the G1 phase were significantly decreased from 53% to 25% by PRM treatment. This result may indicates that the cells of the G1 phase is most sensitive to PRM.

## The Intracellular $Ca^{2+}$ Level in PRM-induced Apoptosis

Considering the property of PRM which binds to D-mannosides in calcium-dependent manner, we studied the intracellular $Ca^{2+}$ response to the PRM stimulation on U937 cells (Fig. 2). The time course of the intracellular $Ca^{2+}$ concentration of Fura2-AM-preloaded cells was monitored at 10 seconds interval after the exposure to PRM. When 50 $\mu$g/ml PRM was added to normal U937 cells, the $Ca^{2+}$ level was not changed (Fig. 2A). However, it was elevated instantly after the addition of 50 $\mu$g/ml PRM to the cells which had been incubated with 200 $\mu$g/ml DMJ for 48 hours (Fig. 2B). In order to analyze the profile in detail, the scanning was conducted at 2.2 seconds interval under the same condition. The $Ca^{2+}$ concentration was elevated within a second in response to the PRM stimulation, dropped to the normal level, and a subsequent gradual increase and declining followed in 20 minutes (Fig. 2B, 2C), indicating that there are two phases in the elevation of the intracellular $Ca^{2+}$ level. On the other hand, 10 $\mu$g/ml PRM, at which concentration significant apoptosis was not detected in DMJ-treated cells, did not affect the $Ca^{2+}$ concentration at all (data not shown). These findings suggested the possible involvement of $Ca^{2+}$ ions in signaling of PRM-induced apoptosis. The intracellular $Ca^{2+}$ concentration was not increased when DMJ-treated cells were loaded with BAPTA-AM, a cell permeant $Ca^{2+}$ chelator, before stimulating with 50 $\mu$g/ml PRM (data not shown). The suppression of the apoptosis by BAPTA-AM could not be proved due to its cytotoxity. In addition, the extracellular $Ca^{2+}$ influx could not be confirmed technically because the removal of the extracellular $Ca^{2+}$ by use of a chelator such as EGTA prevented PRM from binding to mannose-rich oligosaccharides.

Fig. 2. Time course of intracellular $Ca^{2+}$ level after PRM stimulation.




A: normal U937 cells (scanning interval = 10 sec)
B: DMJ-treated cells (scanning interval = 10 sec)
C: DMJ-treated cells (scanning interval = 2.2 sec)

## Reactive Oxygen Species in PRM-induced Apoptosis

Reactive oxygen species (ROS) have been suggested to be widely involved in apoptosis. Various death factors and stresses such as anti-Fas antigen, TNF-$\alpha$, antitumor agents, and irradiation activate the apoptotic cascade *via* ROS generation. In many systems, ROS-mediated apoptosis was suppressed by quenching ROS with anti-oxidants such as *N*-acetylcysteine (NAC). To test the involvement of ROS, we examined the effect of NAC on

Fig. 3. Effect of *N*-acetylcysteine on PRM-induced apoptosis.

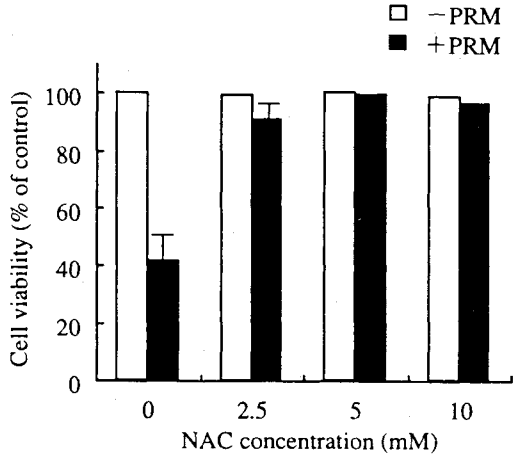
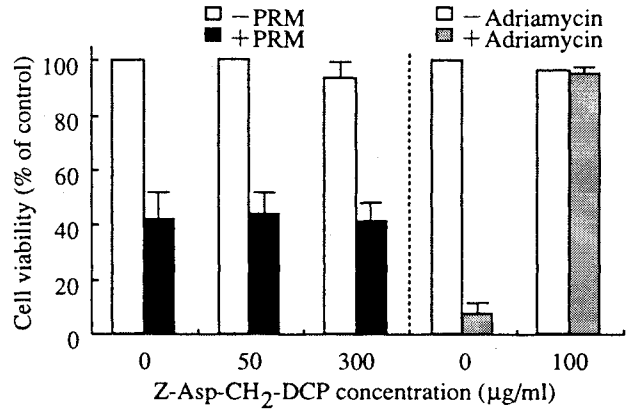



Fig. 4. Effect of Z-Asp-$CH_2$-DCB on PRM-induced apoptosis.




PRM-induced apoptosis (Fig. 3). DMJ-treated cells were incubated with NAC before the treatment with PRM. It was found that while the viability of the cells not treated with NAC was 41%, that of the cells treated with 2.5 mM NAC was 90%, and the apoptosis was completely inhibited by 5 mM NAC pretreatment. This result indicates that ROS is one of the mediators in PRM-induced apoptosis. The time lag between the apoptotic stimulus and ROS production varies from a few minutes to a couple of hours depending on the experimental system.[5,6] In our system, the intracellular $Ca^{2+}$ level was elevated within a second after the PRM stimulation and apoptotic morphology was detected at least 12 hours after the stimulation, suggesting that ROS generation took place between the two events.

### Involvement of ICE-protease

Caspases, a family of cysteine proteases, are supposed to play a central role in various types of apoptosis.[7] Interleukin-1$\beta$ converting enzyme (ICE or caspase-1) was found as a mammalian homologue of CED-3, a positive regulator of apoptosis in the nematode *Caenorhabditis elegans*. It was reported that apoptosis of U937 cells caused by agents such as adriamycin and TNF-$\alpha$ was completely inhibited by ICE protease inhibitor, Z-Asp-$CH_2$-DCB.[8] DMJ-treated cells were incubated with 50 $\mu$g/ml PRM in the presence of 50 ~ 300 $\mu$g/ml Z-Asp-$CH_2$-DCP. While the adriamycin-induced apoptosis of U937 cells was suppressed by the coincubation with 100 $\mu$g/ml Z-Asp-$CH_2$-DCB, PRM-induced apoptosis was not (Fig. 4). Thus, it is evident that PRM induces apoptosis of U937 cells in a different manner from antitumor agents like adriamycin without activating ICE or ICE-like proteases.

## Discussion

In this study, we have provided the evidences of possible involvement of $Ca^{2+}$ and ROS as a second messenger in PRM-induced apoptosis. The intracellular $Ca^{2+}$ concentration was instantly elevated by PRM stimulation. It is, however, uncertain whether the extracellular $Ca^{2+}$ influx or the release from $Ca^{2+}$ stores was the major cause of the elevation. The electron microscopic analysis of PRM-treated *Candida albicans* showed cell surface deformation accompanied with cell membrane and wall damage, implying the potent interaction of PRM with cell surface components.[9] A model was proposed on the basis of spectroscopic analysis and molecular modeling in which PRM forms an oligomeric calcium channel in the presence of D-mannosides.[10] Calcium ionophores like ionomycin are known to induce the extracellular $Ca^{2+}$ influx and activate $Ca^{2+}$-dependent protein phosphatase, calcineulin, resulting in apoptosis.[11] It is reported that the extracellular $Ca^{2+}$ influx regulates ROS production and conversely ROS is effective on intracellular $Ca^{2+}$ concentration.[6] The critical role of ROS in apoptotic signaling, however, seems to be the modulation of the activity of pro-

apoptotic proteins *via* regulating the oxidative state of cysteine residues which affect the active site and/or conformation of the proteins. Recent study revealed that apoptosis signal-regulating kinase 1 (ASK1), an upstream kinase in stress-activated protein kinase (SAPK) cascade, was activated *via* ROS-mediated dimerization of ASK1.[12] In parallel to this study, we have isolated PRM-resistant mutants of the yeast, *Saccaromyces cerevisiae*. The complement gene for the mutation was recently identified to code YPD1 (N. NOMURA *et al.*, unpublished results), a membrane protein that regulates osmorality in upstream of HOG1-MAP kinase cascade.[13] Taken together with these results, further studies of the mechanism of PRM-induced apoptosis and the identification of the target(s) in mammalian cells may offer an integrated view on the intrinsic biochemical property of PRM.

Acknowledgments

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