Short Communication

Pradimicin, a Mannose-binding Antibiotic, Induced Carbohydrate-mediated Apoptosis in U937 Cells

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Pradimicin (PRM), a mannose-binding antifungal antibiotic, recognizes a α-mannoside in the presence of calcium. We demonstrated that BMY-28864, a semi-synthetic analog of PRM, induced apoptosis in U937 cells which had been incubated with 1-deoxy mannnojirimycin (DMJ). Characteristic morphological changes such as formation of apoptotic bodies and DNA fragmentation were observed in apoptotic cells.

Key words: apoptosis; pradimicin; 1-deoxymannojirimycin

Pradimicins (PRMs)\(^1\) and benamomycins,\(^2\) isolated independently from rare actinomycetes in 1988, belong to a family of benz[a]naphtacenequinone antibiotics.\(^3\) PRMs show a potent and highly selective antibiotic activity against fungi including Candida, Aspergillus, and Cryptococcus.\(^3\) Studies on the mode of action of PRM found that the specific binding of PRM to cell surface mannan in the presence of calcium induced a rapid leakage of potassium ions and morphological changes in cellular and nuclear membranes in Candida albicans, leading to cell death.\(^4\) - \(^7\) Remarkably, PRM inhibited the infection by human immunodeficiency virus (HIV) of T cells by binding to high mannose type oligosaccharides on gp120, which initiates the fusion of the virus to the T cell.\(^8\) Although PRM shows no cytotoxicity to mammalian cells expressing complex type oligosaccharides with sialic acids at their termini dominantly, incubation with 1-deoxy mannnojirimycin (DMJ), an α-mannosidase inhibitor, rendered rat basophil leukemia RBL-1 cells susceptible to PRM (T. Ueki and T. Oki, unpublished preliminary results). Based on these findings, we speculated that the interaction of PRM with mannosereich carbohydrates was involved in its fungicidal action and the molecular target was a glycoprotein on the cell surface, presumably one involved in a signal transduction pathway. In our preliminary study using yeasts, the specific binding site was not detectable due to the non-specific binding to the abundant mannan in the yeast cell surface. To minimize the non-specific binding, we chose a human myeloid leukemia U937 cell line for further study, hypothesizing that mammalian cells expressing high mannose type oligosaccharides react to PRM in the same manner as yeast cells.

Initially, we analyzed the time-dependence of the level of high mannose type oligosaccharide expression induced by the treatment with DMJ (Sigma Chemical Co., MO, U.S.A.). The extent of high mannose type oligosaccharide expression on the cell surface relative to that of control cells was estimated by measuring the fluorescent intensity of bound FITC-concanavalin A (FITC-Con A, Seikagaku Co., Tokyo), an FITC-labelled mannose-binding lectin. U937 cells were cultured in RPMI 1640 (Nissui Pharmaceutical Co., Tokyo) with 10% fetal bovine serum (JRH Biosciences Co., Tokyo) and 100 μg/ml kanamycin in the presence of 200 μg/ml DMJ at 37°C in a 5% CO₂ incubator. After various lengths of time, the cells were treated with FITC-Con A (50 μg/ml) in phosphate buffered saline containing 1 mM MgCl₂ and 1 mM CaCl₂ (PBS) at 37°C for 15 min, washed with PBS, and suspended in PBS. The fluorescent intensity of FITC was measured at 522 nm and the amount

\[ \text{Fig. 1. Expression of High Mannose Type Oligosaccharides Induced by DMJ.} \]

\[ \text{The experimental details are described in the text.} \]

\[ \text{Fig. 2. Dose-dependence of PRM on the Cell Death.} \]

\[ \text{The experimental details are described in the text.} \]

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of the adsorbed FITC-Con A was calculated. The amount of adsorbed FITC-Con A intensively increased after 3 hours of incubation, followed by a slight increase after 18 hours (Fig. 1). The result indicates that DMJ induced the elevation of the level of high mannose type oligosaccharide expression.

The effects of PRM on DMJ-treated cells were examined dose-dependently (Fig. 2). After 48 hours of incubation with DMJ (200 μg/ml), U937 cells were incubated with BMY-28864,9 a semi-synthetic analog of PRM FA110 prepared using a PRM-producing microorganism that was kindly provided by Bristol-Myers Squibb Research Institute (Wallingford, CT, U.S.A.), in the range from 5 to 100 μg/ml at 37°C for 24 hours. Cell viability was assayed using the MTS assay (Cell Titer 96 Aqueous, Promega Co., WI, U.S.A.) in 96-well microplates according to the supplier's protocol. The absorbance of the wells at 570 nm was read using an ELISA Reader. PRM induced cell death dose-dependently while control cells were not susceptible to PRM.

The PRM-induced cell death was investigated time-dependently (Fig. 3). After 48 hours of incubation with DMJ (200 μg/ml), cells were treated with 50 μg/ml PRM in the absence of DMJ. The percentage of the living cells decreased by 53% after 24 hours, but increased by 79% after 72 hours. This recovery of the cell growth can be explained by the regeneration of normal oligosaccharides on the cell surface in the absence of DMJ. To test this hypothesis, we incubated the cells with PRM in the continuous presence of DMJ (200 μg/ml). In this case, the regeneration of normal oligosaccharides was continuously inhibited and the viability was 26% after 72 hours.

DMJ dose-dependently affected the susceptibility of the cells to PRM (Fig. 4). After 48 hours of incubation with various concentrations of DMJ, U937 cells were treated with 50 μg/ml PRM for 24 hours and the cell viability was assayed using the MTS assay. Our results suggest that

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Fig. 3. Course of the Effects of PRM on Death of U937 Cells. U937 cells were incubated with DMJ (200 μg/ml) for 48 h. (1) After removal of DMJ, the cells were treated in the absence of PRM (●, control) or with 50 μg/ml PRM (▲); (2) the cells were continuously incubated in the presence of DMJ (200 μg/ml) with 50 μg/ml PRM (■) or without PRM (×). Cell viability was measured by trypan blue dye exclusion.

Fig. 4. Dose-dependence of DMJ on PRM-induced Cell Death. The experimental details are described in the text.

Fig. 5. Fluorescence Micrographs of Hoechst 33258 Stained Cells. Cells were fixed with 1% glutaraldehyde for 30 min and treated with 0.2 mg Hoechst 33258 in PBS for 3 min. After washing with PBS, preparations were analyzed with an excitation wavelength of 345 nm. (A) After 48 h of incubation with DMJ (200 μg/ml), U937 cells were treated with PRM (50 μg/ml) for 24 h; (B) normal U937 cells.
PRM-induced cell death relates closely to the expression of high mannose type oligosaccharides.

We undertook several experimental approaches to detect PRM-induced apoptosis. The dead cells showed characteristic morphological changes in apoptotic cells under a microscope. The entire cell and the nuclei bunched into small spherical structures called apoptotic bodies. The condensation and fragmentation of nucleosomal DNA were also observed in the nuclei of Hoechst 33258 (Molecular Probes Inc., OR, U.S.A.) stained cells under a fluorescence microscope (Fig. 5). After 48 hours of incubation with PRM, the DNA fragmentation was detected only in DMJ-treated cells (Fig. 6, lane 5). The ladder pattern was similar to that of the DNA of U937 cells treated with cycloheximide (100 µg/ml), a reference apoptosis inducer. It is widely suggested that reactive oxygen species (ROS) serve as an intracellular signal of the apoptotic cascade and ROS scavenger like N-acetylcysteine (NAC) inhibits the apoptotic signal transduction.11,12 To test the involvement of ROS in this process, DMJ-treated cells were incubated with 20 mM NAC for 30 min before the treatment with PRM (50 µg/ml, 24 h). NAC completely suppressed the cytoidal action of PRM, morphological change, and DNA fragmentation in the DMJ-treated cells. In light of the chemical inactivity of NAC to PRM, the result indicates that NAC reduced the ROS in the downstream of the apoptotic signal triggering by PRM. Since some lectins are capable of inducing apoptosis by binding to cell surface carbohydrates,13-15 we examined the effects of concanavalin A (Con A, Seikagaku Corp., Tokyo), a mannose-binding lectin, on DMJ-treated U937 cells. After 24 hours of incubation with Con-A (50 µg/ml), no significant morphological changes or DNA fragmentation was observed. In this study, we presented evidence suggesting that PRM can induce apoptosis in U937 cells through the interaction with high mannose type oligosaccharides at the cell surface. Further experiments aim at the identification of the receptor of PRM and the analysis of the mechanism of apoptosis induction.

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References

Fig. 6. Detection of DNA Laddering on Agarose Gel Electrophoresis. Cells were suspended in lysis solution (10 mM Tris, pH 7.5, 0.1% NaCl, 1 mM EDTA, 3% SDS, and 0.1 mg/ml Proteinase K). After incubation at 60°C for 4h, the cellular DNA was extracted with phenol/chloroform and precipitated with ethanol according to the standard protocol. The DNA precipitates were incubated with 20 µg/ml RNase A in TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA) at 37°C for 30 min. The DNA was separated in 2% agarose gels and detected by UV illumination after ethidium bromide staining. Lane 1, HincII-digested 6X 174 DNA; lane 2, U937 cells; lane 3, U937 cells incubated with DMJ (200 µg/ml) for 48 h; lane 4, normal U937 cells were treated with PRM (50 µg/ml) for 24 h; lane 5, After 48 h of incubation with DMJ (200 µg/ml), U937 cells were treated with PRM (50 µg/ml) for 24 h; lane 6, After 48 h of incubation with DMJ (200 µg/ml), U937 cells were treated with Con A (50 µg/ml) for 24 h; lane 7, U937 cells were treated with cycloheximide (100 µg/ml) for 24 h; lane 8, HincII-digested λ DNA.