Flow Cytometric Estimation of Cytotoxic Activity of Rhodexin A Isolated from *Rhodea japonica* in Human Leukemia K562 Cells

Chisato UMEBASHI, a Natsuko YAMAMOTO, a Hiromi NAKAO, a Yukiko TOI, a
Lumi CHIKAHISA-MURAMATSU, b Kaori KANEMARU, a Toshiya MASUDA, a and Yasuo OYAMA* a

*Laboratories of Cell Signaling and Bioorganic Chemistry, Faculty of Integrated Arts and Sciences, The University of Tokushima; Tokushima 770–8302, Japan; and b Taiho Pharmaceutical Co. Ltd.; Hanno 357–8527, Japan.
Received November 5, 2002; accepted January 17, 2003

We have examined the cytotoxic effect of rhodexin A isolated from the extract of *Rhodea japonica* on human leukemia K562 cells using a flow cytometer and compared it with that of ouabain. Rhodexin A at 30 nM started to attenuate growth without affecting viability and further increases in the concentration of rhodexin A (100 nM or more) completely inhibited growth with decreasing viability. Rhodexin A at 30–100 nM increased the G1/M population, but decreased the G2/M population, suggesting cell cycle arrest in the G2/M phase. Rhodexin A at 100 nM increased the number of cells with hypodiploid DNA, indicating that rhodexin A induced apoptosis. The potency of rhodexin A to inhibit growth was greater than that of ouabain. The results indicate that rhodexin A exerts a potent inhibitory action on the growth of human leukemia K562 cells by inducing cell cycle arrest and apoptosis. Rhodexin A may also be a candidate for cancer treatment because there have been clinical reports of tumor regression in patients taking cardiac glycosides.

**Key words** cytotoxicity; human leukemia K562 cell; rhodexin A; *Rhodea japonica*

It is known that several types of wild plants can be poisons to man and some such as morphine, codeine, quinine, atropine, reserpine, vincristine and cardiac glycosides are used in medicine. 3,10 Of these medicines, cardiac glycosides are a class of natural plant products used to treat patients with congestive heart failure and/or cardiac arrhythmia. In spite of the name, some cardiac glycosides have been shown to induce an inhibitory action on human tumor cell lines (glioblastoma, prostate adenocarcinoma, and lymphoma), 3—5 and there are clinical reports of tumor regression in patients taking other cardiac glycosides. 5 Therefore, there is growing interest in evaluating cardiac glycosides as anticancer drugs since some of the more than 200 naturally occurring cardiac glycosides may be useful in cancer treatment.

*Rhodea japonica* is commonly cultivated for traditional gardening and aesthetic flower arrangement in Japan. Its leaves and roots also contain cardiac glycosides such as rhodexins. 3 Of the rhodexins, it was reported that rhodexin A exerted a digitalis-like action in the cat heart. 3 An alcoholic extract of the above ground parts of *Cryptostegia grandiflora* showed inhibitory activity against human tumor KB cells and one of its active ingredients seemed to be rhodexin B. 3 Therefore, we have evaluated the cytotoxic actions of rhodexin A on human leukemia K562 cells using a flow cytometric technique 10 in order to determine if rhodexin A may be a potential anticancer agent.

**MATeRIALS AND METHODS**

**Rhodexin A** Fresh leaves of *Rhodea japonica* (7.2 kg), which was collected in Aoi, Tokushima, Japan, were extracted with methanol (36 l) for 23 d at room temperature. After filtration of the extract, the filtrate was concentrated to ca. 3.5 l in vacuo at 40 °C. The residue was extracted with hexane (2.1) and ethyl acetate (3 l), successively. The ethyl acetate soluble part was evaporated to give a green paste (48 g). Ten grams of the paste was then subjected to column chromatography on Diaion HP-20TM (500 ml) (Mitsubishi Chemicals, Tokyo), which was eluted with stepwise increases of methanol in water (10%, 20%, 30%, 40%, and 60%, in a volume of 1 l). The eluate obtained with the 60% methanol was collected and evaporated to give a pale yellow solid (1.4 g). Careful recrystallization of the solid with ethanol afforded pure rhodexin A (210 mg) as colorless cubes (mp 246—266 °C). Structural identification of the isolated rhodexin A was concluded by means of 1H- and 13C-NMR and FAB-MS.

**Cell Preparation and Analysis on Cytotoxicity** Human leukemia K562 cells were cultured in the 24 wells of Falcon tissue culture plates placed in a CO2 incubator (Sanyo, Tokyo) at a temperature of 37 °C. Each well contained 2 ml of RPMI 1640 medium with glutamine (300 mg/l) and 10% fetal bovine serum (Dainippon Pharmaceutical Co. Ltd., Osaka).

Cytograms (forward scatter intensity versus side scatter intensity) were obtained from K562 cells by a flow cytometer (Cyt-ACE150, JASCO, Tokyo). Cell growth and viability were estimated using propidium iodide (Molecular Probes Inc., Eugene, U.S.A.). Since propidium iodide, a dye highly impermeant to intact plasma membranes, is unable to stain living cells, K562 cells exerting propidium fluorescence are dead or have compromised membranes. Propidium fluorescence was measured 2 min after adding propidium iodide to medium to achieve a final concentration of 5 μM. The excitation wavelength for propidium was 488 nm. Emission was monitored at wavelength of 600±20 nm. Cell viability in the present study indicates the percentage population of cells which were not stained with propidium to the total number of cells. The number of cells counted during a programmed time of 30 s by a flow cytometer was correlated to those estimated by an erythrocytometer with a coefficient of 0.98. 10 Thus, a flow cytometer with propidium iodide simultaneously estimated the effects of the drugs on the growth and viability of cells in the medium. 10

A conventional technique such as MTT assay detects living cells but not dead cells since it is based on the ability of...
mitochondria in living cells to convert tetrazolium into formazan by dehydrogenase. The medium contains not only living cells but also dead cells. However, it would be necessary to simultaneously estimate respective populations of living and dead cells if the effect of a toxic substance is examined on growing cells. There is a benefit to using flow-cytometric measurements of cell growth and viability with propidium iodide. The number of cells measured by a flow cytometer includes those of living and dead cells that are distinguishable by propidium fluorescence. Such a measurement allows us to simultaneously estimate cell growth and viability.

To reveal the distribution of DNA content in K562 cells, DNA was stained with propidium iodide. DNA staining solution contained 0.1% Triton X-100 (Sigma Chemical), 3 mM EDTA (Katayama Chemical), 0.05 mg/ml RNase A (Sigma Chemical) and 100 μM propidium iodide in phosphate buffer solution. The cells were resuspended in DNA staining solution for at least 1 h at room temperature. Propidium fluorescence was measured from 2000 cells by a flow cytometer. The excitation wavelength for propidium was 488 nm and the emission was detected at 600±20 nm.

**Data Presentation and Statistics** Numerical values of experimental data are presented as the mean±standard deviation (S.D.) in this study. Statistical analysis was performed by Student's paired t test and/or an overall test of significance using an F-ratio derived from one-way analysis of variance. A p value of <0.05 was considered significant.

**RESULTS AND DISCUSSION**

**Effect of Rhodexin A**

K562 cells became confluent for up to 72 h under the control condition. After 72 h of incubation of K562 cells in the absence and presence of rhodexin A, the number of cells counted during a programmed time (30 s) by a flow cytometer under the control condition was 4576 whereas there were 760 and 336 in the presence of 30 nM and 100 nM rhodexin A (Fig. 1B), respectively. The results indicate that the incubation of K562 cells with 30 and 100 nM rhodexin A for 72 h inhibited cell growth. The number of cells measured by a flow cytometer under the control condition was 4576 whereas there were 760 and 336 in the presence of 30 nM and 100 nM rhodexin A (Fig. 1B), respectively. The results indicate that the incubation of K562 cells with 30 and 100 nM rhodexin A for 72 h inhibited cell growth. The number of cells measured by a flow cytometer under the control condition was 4576 whereas there were 760 and 336 in the presence of 30 nM and 100 nM rhodexin A (Fig. 1B), respectively. The results indicate that the incubation of K562 cells with 30 and 100 nM rhodexin A for 72 h inhibited cell growth. The number of cells measured by a flow cytometer under the control condition was 4576 whereas there were 760 and 336 in the presence of 30 nM and 100 nM rhodexin A (Fig. 1B), respectively. The results indicate that the incubation of K562 cells with 30 and 100 nM rhodexin A for 72 h inhibited cell growth.
number of cells consisted of those of living and dead cells. As shown in Fig. 1C, the percentage of cells stained with propidium, which were dead cells, was 8.9% under the control condition. The 72 h incubation with rhodexin A at 100 nM, but not at 30 nM, increased the percentage of dead cells to 36.3%. The results show that rhodexin A at 30 nM inhibited cell growth without affecting cell viability.

It has been reported that rhodexin A exerts a ouabain-like action. Therefore, the effect of rhodexin A on K562 cells was compared with that of ouabain. As shown in Fig. 2A, 30 and 100 nM rhodexin A and 100 nM ouabain significantly inhibited the cell growth during a 72 h incubation. The dose–response relations for the inhibitory actions of rhodexin A and ouabain after 72 h incubation are shown in Fig. 2B. The effects of 10 nM rhodexin A and 30 nM ouabain were not significant, indicating their steeper dose–response relations. With regards to the effects on the cell viability (Fig. 3A), rhodexin A at 100 nM significantly reduced the cell viability after 48 h of incubation while 30 nM rhodexin A did not. Ouabain at 100 nM also reduced cell viability after 72 h of incubation. The dose–response relations for the effects of rhodexin A and ouabain on the viability are shown in Fig. 3B. The results indicate that the cytotoxic action of rhodexin A is greater than that of ouabain.

Possible Mechanism To determine the mechanism of the inhibitory action of rhodexin A on the growth of K562 cells, the time-dependent effect of rhodexin A on their cell cycle was examined. Rhodexin A at 30 nM time-dependently decreased the peak amplitude of the G0/G1 phase, but increased that of the G2/M phase (Fig. 4A). Ouabain at 100 nM mimicked the actions of 30 nM rhodexin A. These effects became more profound when the concentration of rhodexin A was increased to 100 nM. The results suggest that rhodexin A may delay the progression of the G2/M phase, resulting in the relative accumulation of cells in the G2/M phase. The cell cycle of K562 cells in the presence of 100 nM rhodexin A clearly shows cells with hypodiploid DNA. As shown in Fig. 4B, 30 nM and 100 nM rhodexin A and 100 nM ouabain increased the percentage of cells with hypodiploid DNA in a time-dependent manner.

Rhodexin A at 20—30 nM significantly suppressed the growth of K562 cells (Figs. 1, 2) without a significant decrease in viability (Figs. 1, 3). However, 48—72 h of incubation with 100 nM rhodexin A significantly decreased cell viability (Fig. 3). The cell cycle study shown in Fig. 4A revealed that rhodexin A at 30—100 nM decreased the population in the G0/G1 phase and increased that in the G2/M phase, suggesting a blockade of mitosis by rhodexin A. Further-
more, rhodexin A at 100 nM greatly increased the population of cells with hypodiploid DNA (Fig. 4A). As shown in Fig. 4B, rhodexin A time- and dose-dependently increased the population of cells with hypodiploid DNA, suggesting apoptosis induced by rhodexin A. The effects of cardiac glycosides such as ouabain and digitonin on tumor cells have been reported to be linked to their abilities to induce sustained Ca$^{2+}$ increases in the cells. Therefore, rhodexin A may increase the Ca$^{2+}$ concentration in K562 cells. The effect of rhodexin A on the cell cycle of K562 cells (Fig. 4A) is similar to that of triphenyltin which induces sustained Ca$^{2+}$ increases in K562 cells. Thus, it may be concluded that the inhibitory action of rhodexin A on the growth of K562 cells is due to a blockade of mitosis and induction of apoptosis and that rhodexin A is also a potential treatment for cancer.

Acknowledgment We would like to thank the Nobeno Branch, JA Anan, Aioi, Tokushima, Japan for supplying the plant materials.

REFERENCES