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Cancer Letters 201 (2003) 41–49
CANCER Letters
www.elsevier.com/locate/canlet

Modified arabinoxylan rice bran (MGN-3/Biobran) sensitizes human T cell leukemia cells to death receptor (CD95)-induced apoptosis

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Received 10 February 2003; received in revised form 10 June 2003; accepted 23 June 2003

Abstract

MGN-3, an arabinoxylan extracted from rice bran that is treated enzymatically with an extract from Shiitaki mushrooms, is an effective biological response modifier that increases NK cell activity, and potentiates the activity of conventional chemotherapeutic agents. In this study, we investigated the effect of MGN-3 on death receptor-induced apoptosis in the human leukemic HUT 78 cell line. HUT 78 cells were pre-treated with MGN-3, and then were incubated with the agonistic antibody against death receptor (Fas, CD95). Apoptosis was determined by the propidium iodide technique using FACScan. Activation of caspase 3, caspase 8, and caspase 9 was determined by flow cytometry. Mitochondrial membrane potential was measured with $DIOC_6$ dye using FACScan. Expression of CD95 and Bcl-2 were measured by flow cytometry. In a dose-dependent manner, MGN-3 enhanced anti-CD95 antibody-induced apoptosis. Increased cell death was correlated with increased depolarization of mitochondrial membrane potential and increased activation of caspase 3, caspase 8, and caspase 9. MGN-3 treatment had no effect on the level of expression of CD95, but it caused down regulation of Bcl-2 expression. These results suggest that MGN-3 increases the susceptibility of cancer cells to undergo apoptosis mediated by death ligands, which may be relevant for anti-cancer activities.

Keywords: Apoptosis; MGN-3; Caspase; Mitochondria; HUT 78; Bcl-2

1. Introduction

MGN-3 is a denatured hemicellulose which is obtained by reacting rice bran hemicellulose with multiple carbohydrate hydrolyzing enzymes from the Shiitake mushrooms. The main chemical structure of MGN-3 is an arabinoxylan, with a xylose in its main chain and an arabinose polymer in its side chain [1]. We have previously reported that MGN-3 augments NK, T, and B cell functions both in vitro and in vivo [1–5]. We have recently demonstrated a direct effect of MGN-3 on tumor cell growth. MGN-3 arrested the growth of cutaneous squamous cell carcinoma (SCC13) cell line in conjunction with an increase in intracellular levels of IL-10 and IL-12 as compared to control untreated cells [6]. In addition, when MGN-3

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doi:10.1016/S0304-3835(03)00458-0

was administered in conjunction with conventional chemotherapeutic agents, it was highly effective in inducing remission of cancer in animal models [7].

Apoptosis (programmed cell death) is a physiologic form of cell death that plays an important role in normal development, tissue homeostasis, and pathological situations [8,9]. There are two major pathways of apoptosis: the death receptor pathway and a mitochondrial (intrinsic) pathway [10–12]. CD95 is a death receptor that belongs to the tumor necrosis factor receptor (TNF R)/nerve growth factor receptor (NGFR) gene superfamily [13,14]. Oligomerization of CD95 by CD95 ligand (CD95L) or agonistic anti-CD95 antibody activates the apoptotic pathway by recruiting adapter protein Fas associated death domain (FADD) [14,15]. FADD then recruits caspase 8 to the death receptor complex (DISC). Auto activation of caspase 8 at the DISC is followed by activation of effector caspases, including caspase 3 [16]. In certain cell types, termed type II, activated caspase 8 has been shown to cleave a Bcl-2 family member, Bid, resulting in the production of truncated Bid. This affects mitochondria and releases cytochrome C [17]. Cytochrome C couples with adaptor protein Apaf-1 to activate caspase 9. Caspase 9 then activates caspase 3. Caspase 3 acts on several substrates to produce the morphological and biochemical changes in apoptosis.

Death receptors are expressed in a variety of tumor cells, but a number of tumor cells are resistant to death ligands, despite expressing substantial amounts of CD95 on their surface. This suggests that the expression of death receptors is not sufficient to allow an apoptotic response [17,18]. Recent research has focused on identifying agents that increase the susceptibility of cancer cells to undergo apoptosis mediated by death receptors. This study was undertaken to determine whether MGN-3 can also increase the susceptibility of cancer cells to anti-CD95 antibody-induced apoptosis.

2. Materials and methods

2.1. MGN-3

MGN-3 is an arabinoxylan extracted from rice bran that is treated enzymatically with an extract from Shiitake mushrooms. It contains polysaccharides (β1,3-glucan and activated hemicellulose). MGN-3 was freshly prepared by dissolving in distilled H_2O at concentration of 30 gm/l. MGN-3 was provided by Daiwa Pharmaceuticals Co. Ltd, Tokyo, Japan.

2.1.1. Cell culture and test exposures

The human T cell leukemic HUT 78 cell line (ATCC, Manassas, VA) was maintained in a suspension culture at 37 °C 5% CO_2/95% air in RPMI-1640 (GIBCO/BRL, Grand Island, NY) supplemented with 10% FBS (GIBCO/BRL), 2 mM glutamine, 100 U penicillin and 100 μg/ml of streptomycin. For experimentation, cells in log phase growth were cultured with MGN-3 (100–1000 μg/ml) for 3–24 h and then were stimulated with agonistic anti-Fas (CD95) antibody for an additional 24 h. Following exposure to anti-CD95 antibody, cells were washed with PBS, and then were used for different assays described below.

2.1.2. Apoptosis

Cellular DNA content was measured following extraction of degraded DNA from apoptotic cells by propidium iodide (PI) staining, as described by Darzynkiewicz et al. [19]. Briefly, cells (1×10^6 ml^{-1}) were fixed in 70% ethanol, washed with PBS and were re-suspended in the DNA extraction buffer (0.2 M Na_2HPO_4 with 0.1 M citric acid pH 7.8). Following extraction, cells were washed and incubated in DNA staining solution (20 μg/ml PI in PBS containing 50 μg/ml RNase A). Cells were stained for 30 min at room temperature in the dark and were analyzed by FACScan (Becton Dickinson, San Jose, CA). After exclusion of necrotic debris, the sub G0/G1 peak was used to quantify apoptosis.

2.1.3. Intracellular activity of caspases 8, 9 and 3

The method is based on carboxyfluorescein labeled fluromethyl ketone (FMK)-peptide inhibitors of caspases. These inhibitors are cell permeable and non-toxic. Once inside the cells, these inhibitors bind covalently to the active caspase. Caspase positive (+) cells are distinguished from caspase negative (−) cells with aid of flow cytometry. Briefly, cells undergoing apoptosis were loaded with fluorescein

labeled FMK-peptide inhibitors (FAM-LETD-FMK for caspase 8, FAM-LEHD-FMK for caspase 9, and FAM-DEVD-FMK for caspase 3; Intergen Company, NY). After 1 h incubation, the cells were washed to remove unbound caspase, and cells that contained bound inhibitor were quantified using a FACScan flow cytometer.

2.2. Mitochondrial potential $\Delta\psi_m$

Variations of the mitochondrial transmembrane potential $\Delta\psi_m$ during apoptosis was studied using 3′3′-dihexyloxacarbocycnine dye [$DIOC_6$ (3)] (Molecular Probes, Eugene, OR). This cyanine dye accumulates in the mitochondrial matrix under the influence of the $\Delta\psi_m$. 5×10^5 cells/ml were incubated with 0.5 μM $DIOC_6$ (3) for 30 min at 37 °C. Cells were transferred on ice for FACS analysis. Forward and side scatters were used to gate and exclude cellular debris using a FACScan. Cells were excited at 488 nm and green fluorescence was collected on FL1 at 530 nm. Five thousand cells were analyzed. Data was acquired and analyzed using Cell Quest software (Becton Dickinson).

2.3. Expression of CD95 and Bcl-2

Cells were stained with FITC-labeled anti-human CD95 antibody or isotype control IgG (B.D. Biosciences) and the surface expression of CD95 was determined by flow cytometry. For detection of Bcl-2, cells were first fixed and permeabilized with ice-cold 70% methanol. They were then stained with FITC labeled anti-Bcl-2, or isotype control (Dako Corp, Carpintaria, CA). Cells were washed and analyzed by FACScan. The percentages of cells expressing CD95, Bcl-2, and mean fluorescence intensity (an indicator of density of the molecules/cell) were determined.

Statistical analysis. A two-tailed student's test was used to determine the significance of differences between experimental and control groups. Statistical analysis of histograms was performed by the Kolmogorov–Smirnov statistics. AD value of >0.2 is considered statistically significant.

3. Results

3.1. MGN-3 sensitizes HUT 78 cells to death receptor-mediated apoptosis

Leukemia cell line (HUT 78) was pre-treated with MGN-3 and then incubated with agonistic anti-CD95 antibody. Twenty-four hours post-incubation, apoptosis was determined by the PI technique, using a FACScan flow cytometer. Specific apoptosis was calculated as the percentage of experimental apoptosis-percentage of spontaneous apoptosis. The data in Fig. 1(a) shows a representative histogram plot and Fig. 1(b) shows data from three independent experiments. MGN-3 alone at concentrations of 100–1000 μg/ml had minimal effect on apoptosis (specific apoptosis 2.5–4.5%). Anti-CD95 antibody induced apoptosis in 29% of HUT 78 cells (specific apoptosis = 20%). However, when leukemic cells were pre-treated with MGN-3, followed by the anti-CD95 antibody, a significant increase in the number of apoptotic cells (specific apoptosis 35–42%) ($P < 0.01$) was noted. This represents a 200% increase as compared to anti-CD95 antibody alone. Lower concentration <100 μg/ml did not sensitize HUT 78 cells to anti-CD95 antibody-induced apoptosis.

3.2. MGN-3 increases activation of caspase 8, caspase 9, and caspase 3

Apoptosis via the death receptor (CD95) is mediated by activation of the caspase cascade. In order to determine the steps in CD95 mediated apoptosis that were affected by MGN-3, we examined the activation of proximal caspases (caspase 8, caspase 9) and the executioner caspase (caspase 3). HUT 78 T cells were exposed to MGN-3 for 24 h and were incubated with anti-CD95 antibody for 6 h. The proportion of cells with active caspase 8, caspase 9, and caspase 3 were determined with a Caspatag caspase detection kit using FACScan. Fig. 2 shows data from three experiments for activation of caspases. The data shows that anti-CD95 antibody induced the activation of caspase 8, caspase 9, and caspase 3 in HUT 78 cells. It also demonstrates that the proportion of cells with increased active caspase 8, caspase 9, and caspase 3 was higher in MGN-3 treated cells than in untreated control cells. Moreover,

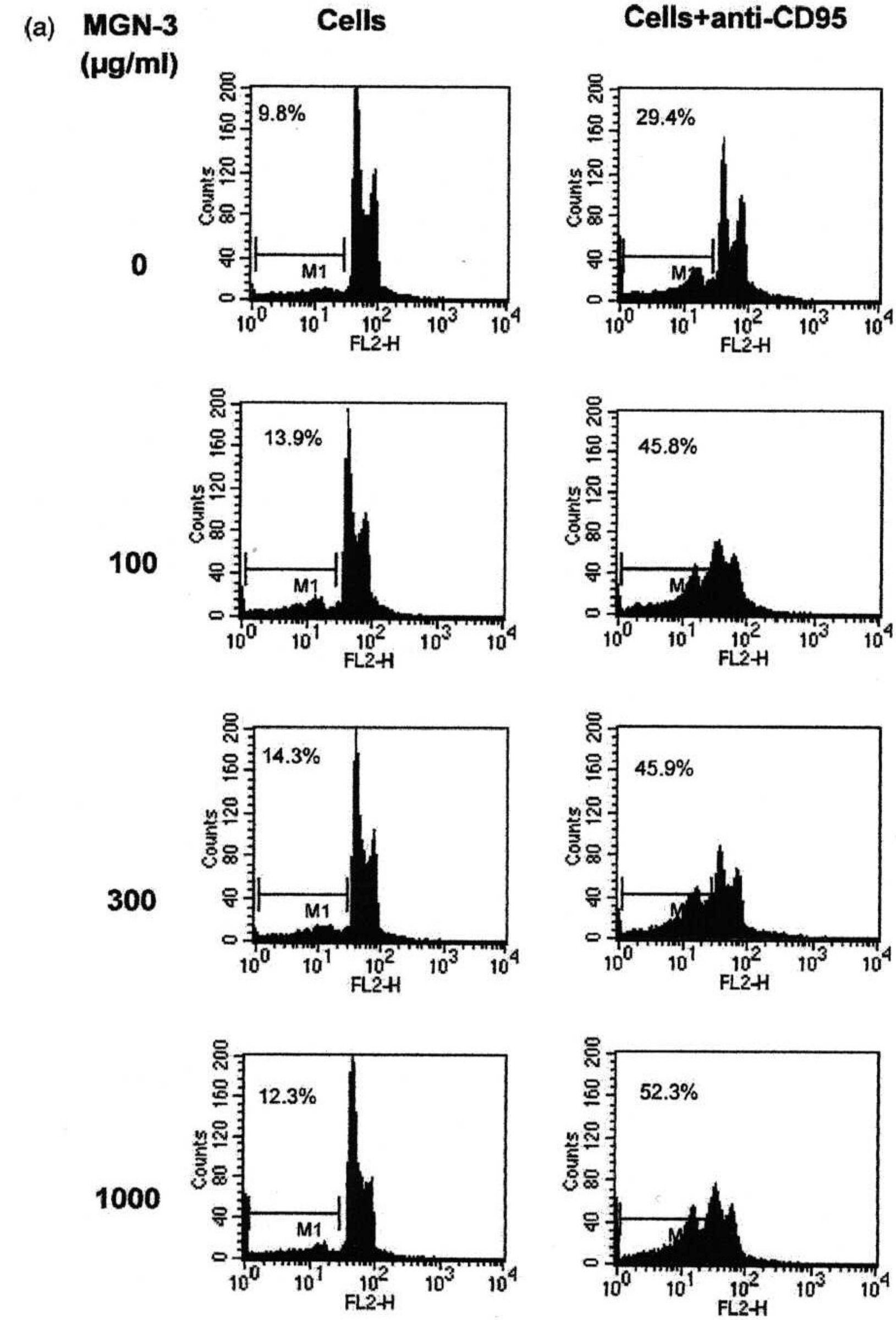

Fig. 1. Effect of MGN-3 on anti-CD95 antibody-induced apoptosis. Cells (HUT 78) were pre-treated with MGN-3 for 3 h and then were incubated with agonistic anti-CD95 antibody. Apoptotic cells were determined by PI technique using FACScan flow cytometer. (a) A representative histogram showing increased apoptosis and (b) represents the mean ± SD of three experiments. Combination = MGN-3 at 100, 300, 1000 µg/ml followed by anti-CD95 antibody. *Significant at $P < 0.01$.

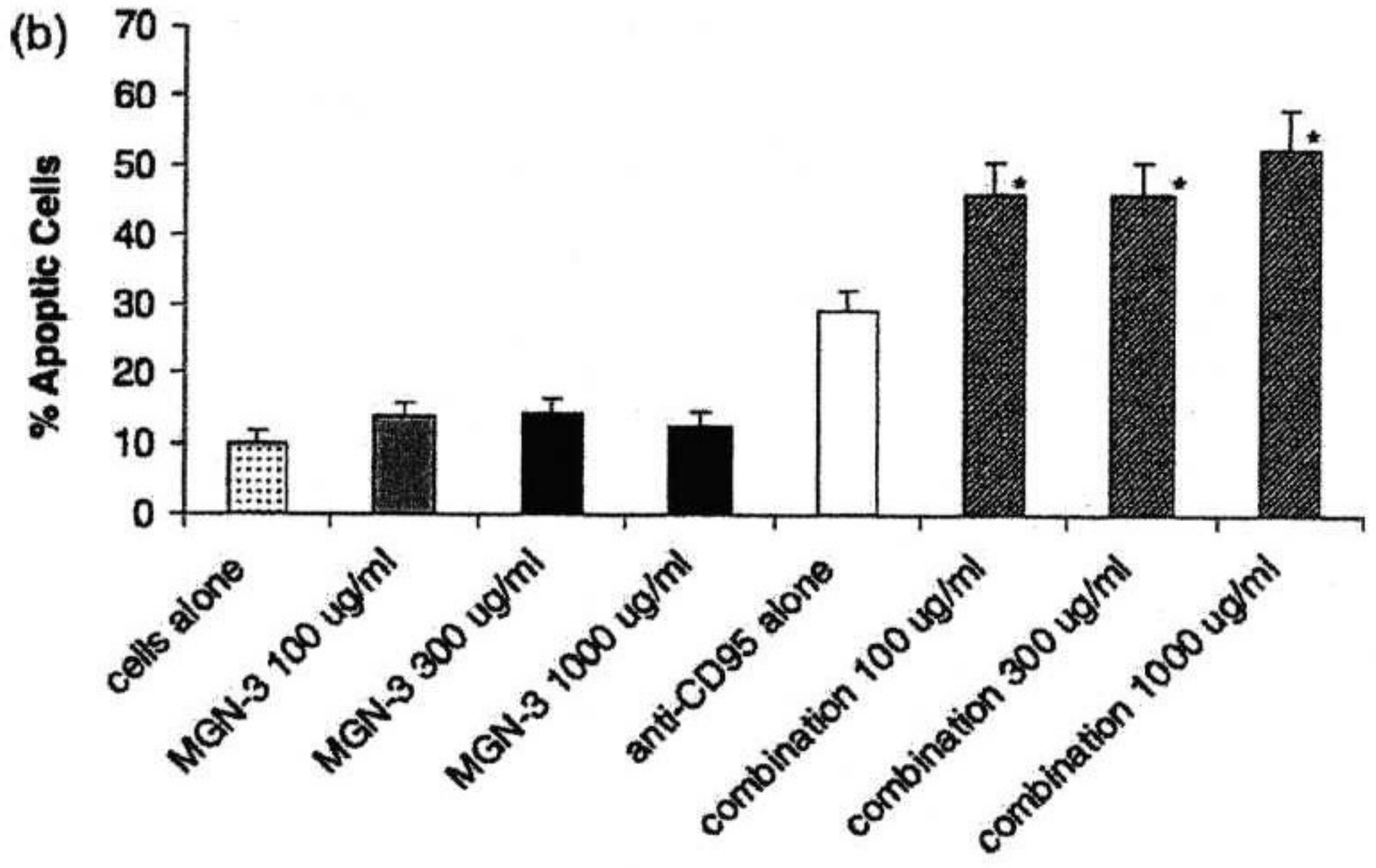

Fig. 1 (*continued*)

the mean fluorescence intensity of caspase 8 and caspase 3 was significantly higher in MGN-3 pre-treated cells followed by anti-CD95 antibody in comparison with untreated cells. This would suggest that pre-exposure to MGN-3 led to increased activation of proximal and executioner caspases.

3.3. MGN-3 does not alter the level of expression of the death receptor CD95

To determine the possibility that the increased susceptibility of MGN-3 treated cells is due to up regulation of a death receptor, CD95 expression on HUT 78 cells treated with and without MGN-3 was determined. The percentage of cells expressing CD95 and the density of CD95 on MGN-3 treated cells was similar to that of untreated cells.

3.4. MGN-3 disrupts mitochondrial membrane potentials

Stimulation of the CD95 receptor leads to the disruption of the mitochondrial membrane potential [20,21] and to the release of mitochondrial contents. This results in activation of caspase 9 and caspase 3. To further characterize the sensitizing action of MGN-3 on the death receptor pathway of apoptosis, we determined mitochondrial membrane potentials. HUT 78 cells treated with or without MGN-3 were exposed to anti-CD95 antibody, and mitochondrial potential was determined by flow cytometry using membrane potential sensitive $DIOC_6$ (3) dye. The data in Fig. 3 shows that a significantly higher proportion of MGN-3 pre-treated HUT 78 cells exhibit decreased membrane potential as compared to untreated cells. MGN-3 alone had no significant effect on mitochondrial membrane potential.

3.5. MGN-3 downregulates Bcl-2 expression

Bcl-2 is an anti-apoptotic molecule that is shown to protect the cells from apoptosis induced by diverse agents [22,23]. To further investigate the effect of MGN-3, we tested its effect on Bcl-2 expression. The data in Table 1 shows that MGN-3 caused a significant decrease in the level of expression of this anti-apoptotic protein.

4. Discussion

Susceptibility to apoptosis is a prerequisite for successful treatment of cancer cells by natural killer cells, cytotoxic T cells, chemotherapy, or radiation therapy. In this study, we have established the fact that MGN-3, a biological response modifier (BRM), sensitizes human leukemic HUT 78 cells to anti-CD95 antibody-induced apoptosis. This conclusion is based on the following evidence: MGN-3 on its own, at 100–1000 μg/ml, resulted in a 3–4% apoptotic cell

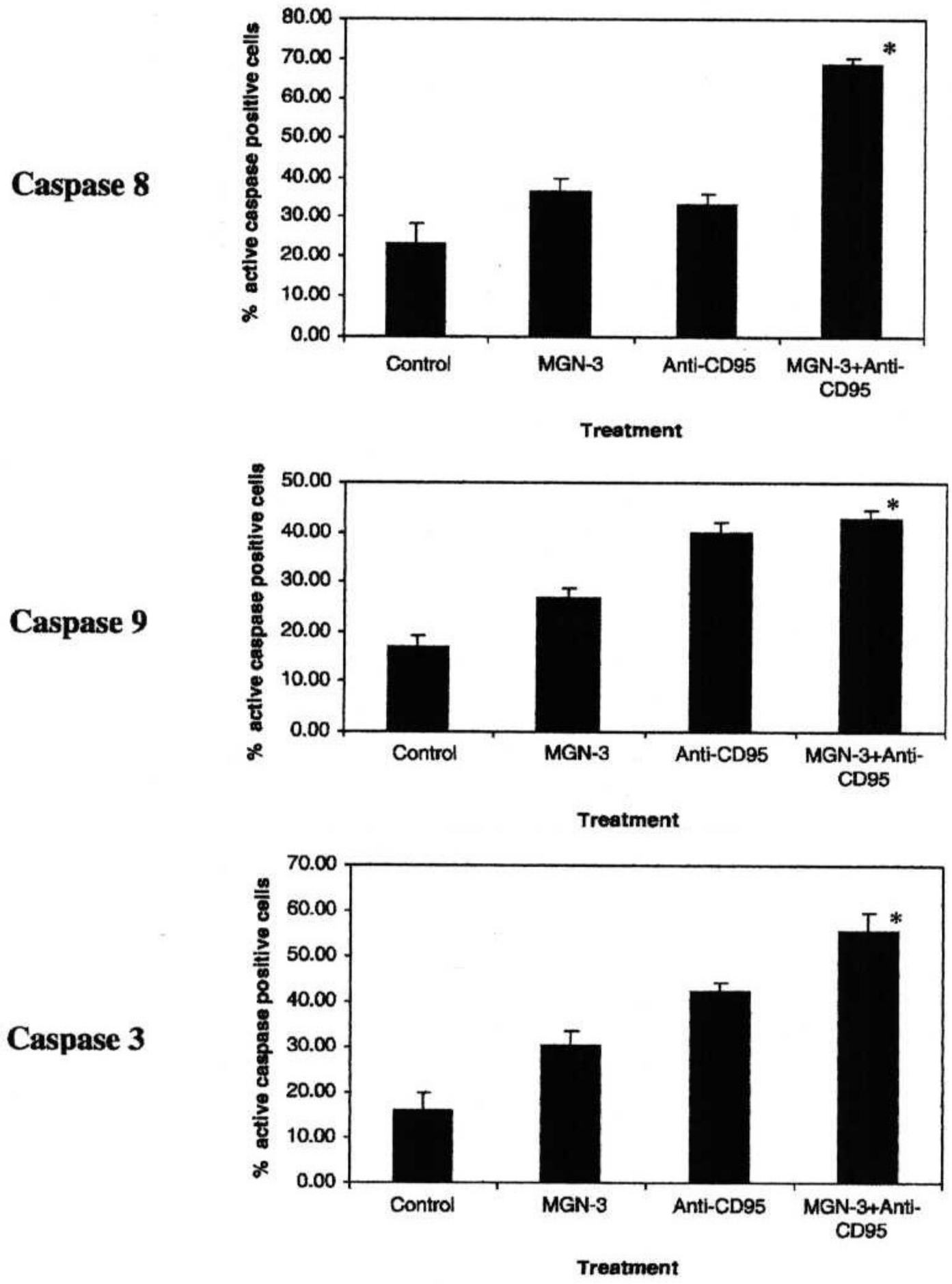

Fig. 2. Increased activation of caspases 3, 8, and 9. Cells were treated with MGN-3 and were incubated with anti-CD95 antibody, and intracellular active caspases 3, 8, and 9 were determined with caspatag caspases 3, 8, and 9 determination kit using FACScan. Data represent the mean ± SD of three experiments. *Significant at $P < 0.01$.

death, while CD95 stimulation caused a 20% apoptosis. When cells were treated with both MGN-3 followed by the anti-CD95 antibody, 35–42% apoptic cells were observed. This represents a 200% increase, as compared to using the anti-CD95 antibody alone. (Fig. 1(a) and (b)). This was correlated with the increased number of cells having active caspase 8, caspase 9, and caspase 3 in MGN-3 pre-treated cells that were subsequently exposed to anti-CD95 antibody, as compared to cells exposed to anti-CD95 antibody alone.

The recommended dose of MGN-3 for a human weighing 60 kg is 3 g per day by mouth [2]. Studies in experimental animals have shown that doubling of this dose is well tolerated without toxic manifestation in terms of body weight of mice (Personal Communications). Currently, there is not a significant body of research on the effect of natural BRMs against cancer cell apoptosis. Fan et al. [24] demonstrated that a lipoprotein fraction of rice bran induces apoptosis and growth inhibition in cultured human endometrial adenocarcinoma cells (Sawano). Miyoshi et al. [25]

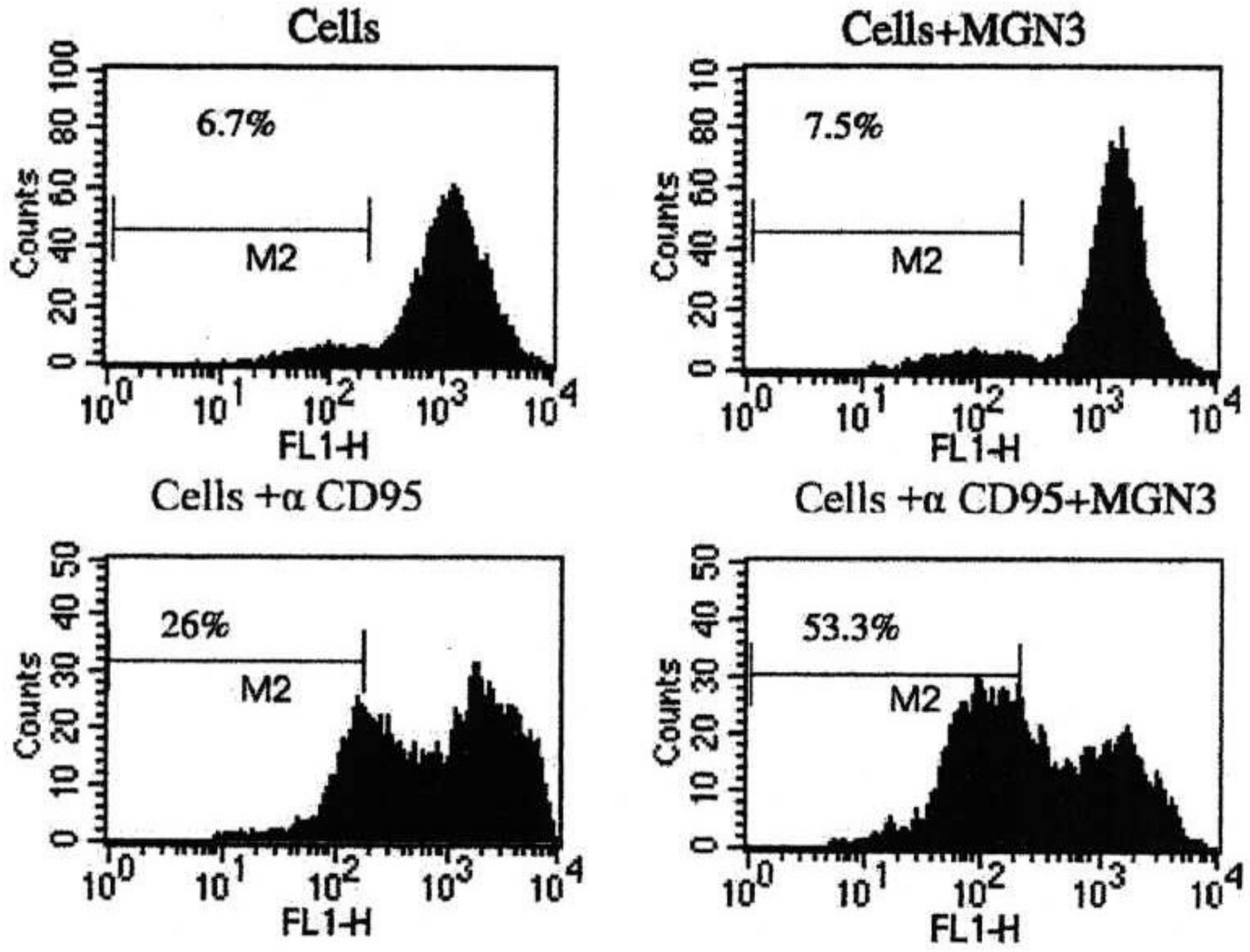

Fig. 3. Effect of MGN-3 on mitochondrial potential. HUT 78 cells were incubated with or without anti-CD95 antibody in the presence or absence of MGN-3 for 2 h. The cells were then stained with $DIOC_6$ and were subjected to flow cytometry analysis to determine the $\Delta\psi_m$. The numbers in the histogram represent the proportion of cells exhibiting decreased membrane potential.

reported that rice bran agglutinin (RBA) induces growth arrest and apoptosis in human monoblastic leukemia U937 cells. Han et al. [26] showed that mycelial extracts of Coprinus disseminatus (pers. Fr.) induce apoptosis in the human cervical carcinoma cells via activation of caspase 3. Sigounas et al. [27] studied the effect of *dl*-α-tocopherol (vitamin E) on different cancer cell lines and they found that *dl*-α-tocopherol induced apoptosis in erythroleukemia, prostate, and breast cancer cells with different degrees of sensitivity. In another study, apoptic activity of Chinese herbal preparation PC SPES was examined. Halicka et al. [28] found that treatment with PC SPES resulted in an increased percentage of cells with fractional DNA content in histiocytic lymphoma U937 and prostate adenocarcinoma PC 3 lines. They reported that apoptosis occurred through the down-regulation of the expression of Bcl-2.

To investigate the mechanism by which MGN-3 modulates CD95-mediated apoptosis, the effect of MGN-3 on known effector molecules in anti-CD95 antibody-induced apoptosis was examined. In this study we have shown that MGN-3 up regulates the activation of proximal caspase 8, and downstreams caspase 9 and executioner caspase 3. MGN-3 also caused a significant decrease in the level of expression of anti-apoptotic protein Bcl-2, which is shown to protect the cells from apoptosis induced by diverse agents [22,23]. In addition, MGN-3 treated cells, upon activation with anti-CD95 antibody, showed a significant decrease in mitochondrial membrane potential. This may play a critical role in the sensitization induced by MGN-3, since Bcl-2 exerts its antiapoptotic effect by maintaining the integrity of mitochondria and preventing the release of proapoptotic molecules from its intermembrane space [22,23].

The mechanism(s) by which MGN-3 increases the activation of caspases and downregulates Bcl-2 expression is not known. We have previously shown

Table 1
Effect of MGN-3 on the expression of Bcl-2

Cells treated with	Bcl-2 positive cells (%)	MFC
None	100	740
MGN-3	100	559*

HUT 78 cells (1 × 106 cells/ml) were cultured in the absence or presence of MGN-3 (1000 μg/ml) for 24 h. Expression of Bcl-2 was determined by staining the cells with anti-human Bcl-2 antibody and flow cytometry. MFC = mean fluorescence channel number.

* Significantly different from control untreated cells $D = 0.30$.

that MGN-3 increases the production of tumor necrosis factor α (TNF-α) and interferon-γ (IFN-γ) [5]. In tumor cells, both TNF-α and IFN-γ induce or modulate cell death via activation of caspases [10,11, 29,30]. It is possible that MGN-3 increases activation of caspases in HUT 78 cells via inducing the production of TNF-α and IFN-γ.

Defects in apoptosis pathways contribute to the resistance of tumor cells to chemotherapeutic agents, radiation, and to cellular immune responses (NK and cytotoxic T cell-mediated apoptosis). Currently, there is much interest in identifying natural products that can boost host cell-mediated immune responses against cancer, as well as agents that increase the susceptibility of cancer cells to undergo apoptosis. In this study, we have shown that MGN-3, a BRM extracted from rice bran, sensitizes the leukemia cells to apoptosis mediated by the anti-CD95 antibody. We are currently investigating the sensitizing activities of MGN-3 against a range of additional leukemic cell lines. The precise mechanism(s) of action of MGN-3 remains to be investigated. Nevertheless, our results suggest that MGN-3 could be used to improve the treatment of certain human leukemias.

Acknowledgements

We are greatly indebted to Dr. Magda Shaheen at the Research Center in Minority Institutions (RCMI) program, Drew University of Medicine and Science, for assistance in statistical analysis. This work was supported by a grant from Daiwa Pharmaceutical Company, Tokyo, Japan.

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