

Synergistic Role of Arabinoxylan Rice Bran (MGN-3/Biobran) in *S. cerevisiae*-induced Apoptosis of Monolayer Breast Cancer MCF-7 Cells

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Abstract. We have recently demonstrated that breast cancer cell (BCC) lines undergo apoptosis following phagocytosis of *S. cerevisiae*. Arabinoxylan rice bran extract (MGN-3/Biobran) has been shown to enhance this effect. Since previous data were obtained using cells in suspension, the present study was undertaken to examine monolayer BCC that more closely model cancer cell growth. Monolayers of both breast cancer (MCF-7) and non-tumorigenic breast epithelial (MCF-10A) cells grown on glass coverslips were cultured with heat-killed *S. cerevisiae* at a ratio of 1:10, respectively. MCF-7 cells phagocytized yeast in a time-dependent manner, 6.9% to 14.3% from 1 h to 4 h, respectively, with a 2-fold increase in the presence of MGN-3. On the other hand, there was virtually no phagocytosis of yeast by MCF-10A cells. Similarly, yeast-induced apoptosis of MCF-7 cells occurred in a time-dependent manner, from 11.5% after 1 h to 21.7% after 4 h, and was enhanced in the presence of MGN-3. These data may have implications in the treatment of breast cancer.

Apoptosis involves a cascade of biochemical events that is tightly regulated. Some of the elements in this cascade include the activation of specific cysteine proteases, called caspases, mitochondrial release of death factors and, finally, DNA fragmentation (1). Many anticancer drugs function by inducing apoptosis (2-8). The specific intercellular damage induced by many therapeutic agents has been characterized and shown to involve the Fas/FasL system (5), mitochondria (6) and DNA damage (7, 8). With respect to the killing of

cancer cells by immune cells, earlier studies showed that the effector lymphocytes, such as NK and T cells, use two pathways to kill tumor cell targets. The first pathway utilizes ligation of FasL to its Fas receptor inducing apoptosis (9-13). The second pathway utilizes the pore-forming protein perforin and the serine protease granzyme B in the induction of target cell lysis (14, 15). On the other hand, tumor cells develop multiple strategies to escape from the apoptosis induced by either the host defense system or chemotherapeutic agents (16). These strategies include neutralizing Fas-FasL (17-19), down-regulation of death receptor-mediated apoptosis, alterations in the mitochondrial pathway of apoptosis and up-regulation of anti-apoptotic proteins (e.g. FLIP, IAPs) and of multidrug resistance proteins (20-22). Tumor cells also can inactivate CTL through the induction of apoptosis and the secretion of immunosuppressive factors (23, 24). In addition, tumor cells of T and B cell origin exhibit phagocytic activity against lymphocytes *in vitro* (25) and against autologous lymphocytes in cancer patients *in vivo* (26, 27).

It is, therefore, of particular interest to find agents that induce apoptosis of cancer cells with minimal side-effects. We have recently provided the first evidence that heat-killed non-pathogenic *S. cerevisiae*, baker's and brewer's yeast, induces apoptosis of breast cancer cells (BCCs) (28, 29), tongue squamous cell carcinomas and colon adenocarcinomas (30). Cancer cells in these studies were grown in suspension. Since BCCs grow in a monolayer, we thought it of particular interest to examine: i) whether BCCs also take up the yeast when growing in monolayer; ii) whether rice bran extracts (MGN-3/Biobran) can synergize the apoptotic effect of yeast and iii) whether yeast-induced apoptosis is selective for cancer cells. Therefore, the yeast effects against MCF-10A breast epithelial cells were examined. In this study, it was found that monolayer MCF-7 cells undergo apoptosis following attachment/ingestion of yeast and that this process is enhanced in the presence of MGN-3 and may suggest cancer cell specificity.

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Materials and Methods

Cell lines. The human breast cancer MCF-7 cell line and the MCF-10A breast epithelial cell line, purchased from the American Tissue and Culture Collection (ATCC), Manassas, VA, USA, were used. Cells were maintained in our laboratory in complete medium (CM) consisting of RPMI-1640, supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and 100 µg/ml of streptomycin and penicillin.

Preparation of *S. cerevisiae*. Commercially available baker's and brewer's yeast, *S. cerevisiae*, was used. The yeast was heat-killed by incubation for 1 h at 90°C, washed once and re-suspended in phosphate-buffered saline (PBS) prior to application. Quantification was carried out using a hemocytometer, and cell suspensions were adjusted to 1x10⁷ cells/ml.

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 it in distilled H₂O at a concentration of 100 mg/mL. MGN-3 was provided by Daiwa Pharmaceuticals Co. Ltd., Tokyo, Japan.

Growth of monolayer MCF-7 and MCF-10A cells in 8-well plates. A model assay system was developed to examine apoptosis of monolayer MCF-7 and MCF-10A cells following culture with yeast. The system allows the phagocytosis of yeast and apoptosis by adherent and non-adherent MCF-7 and MCF-10A cells to be monitored. For this purpose, MCF-7 and MCF-10A cells were allowed to grow in 8-well plates (26X33 mm each) (LUX Scientific Corp., Thousand Oaks, CA, USA). A cover glass was placed at the bottom of each well. 1x10⁵ MCF-7 cells/mL were pipetted into each well and allowed to adhere. After 2 h, the cells were washed once with 1 mL CM. Yeast (1x10⁶ cells in 5 µl), in the presence or absence of MGN-3 (100 µg/ml), were added to each well and incubated at 37°C and 5% CO₂. At time-periods between 1-4 h, both non-adherent and adherent cells were examined as follows:

Non-adherent cells: The medium containing non-adherent tumor cells (1 ml) was transferred to glass tubes. From the cell suspension, 200 µl were used to make cytospin preparations (Shandon Southern Instruments, Sewickly, PA, USA). Preparations were fixed in 100% methanol, air-dried, stained with 4% Giemsa for 15 min (Sigma-Aldrich Corp., St. Louis, MO, USA) and examined using oil immersion and a light microscope fitted with a 100x objective (Nikon, Tokyo, Japan). The number of apoptotic cells in the 200 µl were counted and multiplied by 5 to give the total number of apoptotic non-adherent cells in the cell suspension (Z). The percentage of apoptotic cells = $Z / \text{total number of cells} [100,000] \times 100$.

Adherent cells: Cover glasses containing adherent cells were carefully removed, air-dried, mounted on slides and treated as outlined for non-adherent cells. The data collected were analyzed for percent attachment, phagocytosis and apoptosis.

Assessment of attachment and phagocytic assay. The adherent monolayer MCF-7 and MCF-10A cells cultured with yeast in the presence or absence of MGN-3 were used to examine the percent of attachment. The assessment of attachment of yeast by tumor cells was calculated as the percentage of 200 tumor cells that attached to one or more yeast. With respect to phagocytosis, a previously

reported assay was employed with slight modifications (31, 32). In brief, the adherent monolayer MCF-7 and MCF-10A cells cultured with yeast in the presence or absence of MGN-3 were used to examine the percent of phagocytosis as in (B). Similarly, the assessment of uptake of yeast by tumor cells was calculated as the percentage of 200 tumor cells that ingested one or more yeast cell.

Confirmatory experiments of phagocytosis. Three different experiments were carried out in order to determine whether observations reflected actual phagocytosis of yeast by MCF-7 cells or simple adherence of yeast to cancer cells. The experiments are as follows:

Fluorescence microscopy: Phagocytosis was examined using yeast labelled with propidium iodide (PI). Fluorescent yeast were added to monolayer MCF-7 cells in 8-well plates at a ratio of 10:1. After 2 h, the number of adherent cancer cells that attached/phagocytized fluorescent yeast was counted using a fluorescence microscope fitted with a 100x objective (Nikon).

Effect of low temperature: A set of experiments were carried out to examine the effect of cooling on the phagocytic activity of MCF-7 cells. For this purpose, monolayer MCF-7 cells were cultured with yeast at a ratio of 1:10 at 4°C for 2 h. The percent of attachment and phagocytosis were subsequently examined by cytospin preparations. The results were compared with cells cultured with yeast at 37°C.

Treatment with cytochalasin B: Monolayer MCF-7 cells (2x10⁵ cells/ml) were incubated with cytochalasin B, (10 µg/ml) for 1 h at 37°C. The cells were washed twice with PBS and incubated with yeast at a MCF-7 cell to yeast ratio of 1:10. After 2 h, the percent of attachment and phagocytosis were determined by cytospin preparations.

Assessment of tumor cell survival and apoptosis. Monolayer cells were cultured with yeast at a ratio of 1:10. To determine the effects of yeast and MGN-3 treatments on cell survival, apoptotic adherent and non-adherent cells were identified at different intervals, and cell apoptosis was evaluated by 4 different methods:

Annexin V staining: MCF-7 cells were incubated with or without yeast (1:10 ratio) for 2 h at 37°C. Cells that detached from the monolayer were collected, washed with PBS and suspended in 100 µl of Annexin V binding buffer (CalTag Laboratories, Burlingame, CA, USA); 5 µl of FITC-labelled Annexin V was then added to the cell suspension and incubated at room temperature in the dark. After a 15-min incubation, 400 µl of Annexin V binding buffer was added, and Annexin binding was analyzed by flow cytometry. Ten thousand cells were acquired and analyzed using Cell Quest software.

Flow cytometry analysis: Flow cytometry analysis was used to examine the percentage of dead non-adherent MCF-7 cells. Cancer cells were cultured with yeast cells at a 1:10 ratio in the presence or absence of MGN-3 (100 µg/ml). Non-adherent cancer cells were collected after 4 h, and the percentage of dead cells was examined by the propidium iodide (PI) technique using flow cytometry. Briefly, non-adherent cells were fixed in 70% methanol, resuspended in the DNA extraction buffer, washed in PBS and incubated with 50 µg/ml of PI for 30 min at room temperature in the dark, and analyzed by FACScan (Becton Dickinson, San Jose, CA, USA).

Giemsa stain: Apoptosis is morphologically defined by cell enlargement, membrane blebbing and chromatin condensation. These criteria were used to identify the apoptotic adherent MCF-7 and MCF-10A cells in cytospin preparations stained with Giemsa on the prepared slides. The percent of apoptotic adherent cells was

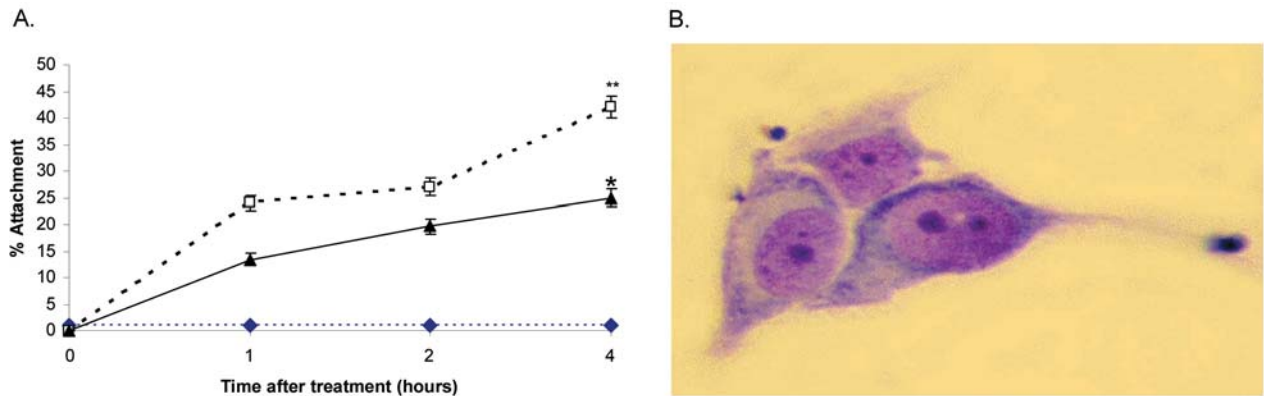


Figure 1. Effect of MGN-3 on the attachment of monolayer MCF-7 cells to yeast. Tumor cells were cultured with yeast at a ratio of 1:10 in the presence (□) or absence of MGN-3 (▲). At 1, 2 and 4 h post-culture cancer cells with yeast, cells adherent to the cover glass were removed, stained with Giemsa and the percentage (%) of attachment (A) was calculated. Data represent the mean \pm SE of 5 separate experiments. Results were compared with MCF-10A cells as control (◆). * $p < 0.03$, ** $p < 0.02$ as compared to MCF-10A cells. (B) Giemsa-stained cytocentrifuge preparation showing yeast attaching to multiple MCF-7 cells at 1 h post-culture of cancer cells with yeast. Notice yeast, stained dark blue, attached to long extensions from MCF-7 cell. Giemsa x 400.

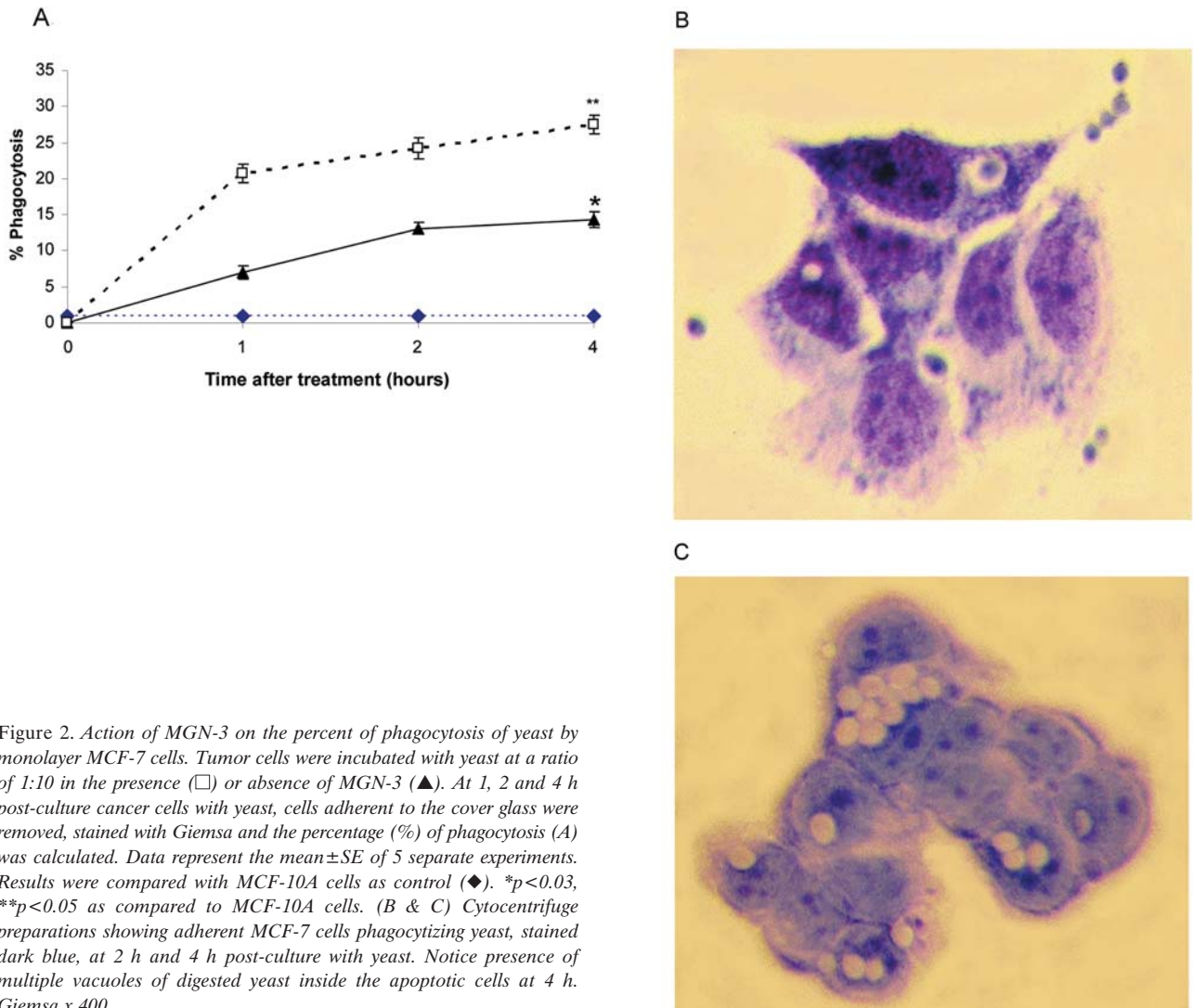


Figure 2. Action of MGN-3 on the percent of phagocytosis of yeast by monolayer MCF-7 cells. Tumor cells were incubated with yeast at a ratio of 1:10 in the presence (□) or absence of MGN-3 (▲). At 1, 2 and 4 h post-culture cancer cells with yeast, cells adherent to the cover glass were removed, stained with Giemsa and the percentage (%) of phagocytosis (A) was calculated. Data represent the mean \pm SE of 5 separate experiments. Results were compared with MCF-10A cells as control (◆). * $p < 0.03$, ** $p < 0.05$ as compared to MCF-10A cells. (B & C) Cytocentrifuge preparations showing adherent MCF-7 cells phagocytizing yeast, stained dark blue, at 2 h and 4 h post-culture with yeast. Notice presence of multiple vacuoles of digested yeast inside the apoptotic cells at 4 h. Giemsa x 400.

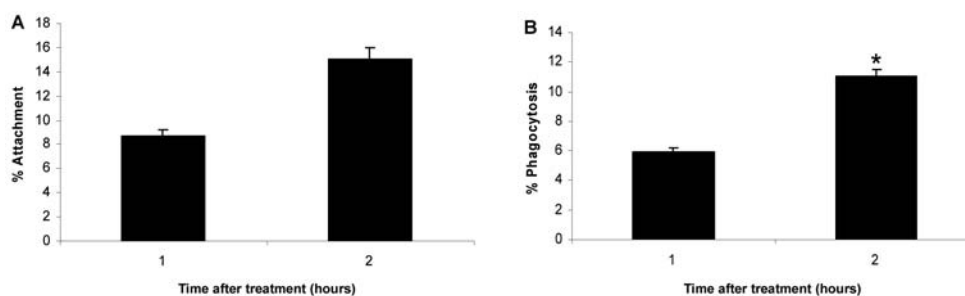


Figure 3. Percentages of attachment and phagocytosis of fluorescent yeast by monolayer MCF-7 cells. MCF-7 cells were cultured with fluorescent yeast and percentages (%) of attachment (A) and phagocytosis (B) were calculated after 2 h post-culture of cancer cells with yeast. Data represent the mean \pm SE of 3 separate experiments. * $p < 0.01$ as compared with the same cells at 1 h.

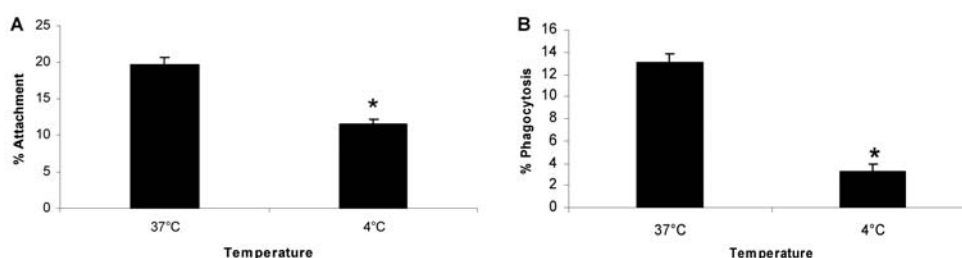


Figure 4. Effect of cooling on percent of attachment and phagocytosis by monolayer MCF-7 cells. Cancer cells were cultured with yeast at a ratio of 1:10 and kept at 4°C and 37°C for 2 h. The percent of attachment (A) or phagocytosis (B) of yeast by adherent MCF-7 cells was determined in cytospin preparations. Data represents mean \pm SE of 3 separate experiments. * $p < 0.01$ as compared to cells at 37°C.

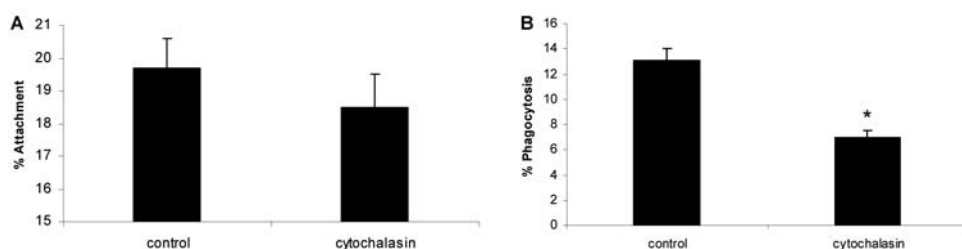


Figure 5. Effect of cytochalasin B on percent of attachment and phagocytosis by monolayer MCF-7 cells. Cancer cells were treated with cytochalasin B (10 μ g/ml) and then were cultured with yeast at a ratio of 1:10 for 2 h. The percent of attachment (A) and phagocytosis (B) of yeast by adherent MCF-7 cells was determined in cytospin preparations. Data represents mean \pm SE of 3 separate experiments. * $p < 0.05$ as compared to cells without treatment.

calculated from observations calculated at 10 different sites on the cover slip, each containing 100 cells. The percent of apoptotic non-adherent cells was also calculated using cytospin preparations stained with Giemsa previously prepared.

Trypan blue stain: Non-adherent apoptotic MCF-7 cells were treated with trypan blue stain and counted using a light microscope and hemocytometer. The percent of dead cells = % dead cells in the supernatant / total number of cells $[100,000] \times 100$.

Statistical analysis. In order to compare the means of treatment 1 (yeast) and treatment 2 (combination of yeast and MGN-3), the analysis of variance design was used.

Results

Phagocytosis studies.

Percentages of attachment and phagocytosis: Monolayer MCF-7 cells were cultured with yeast in the presence or absence of MGN-3, and the percentages of attachment and phagocytosis of yeast by MCF-7 cells were examined at 1, 2 and 4 h. The data in Figure 1A show that MCF-7 cell attachment to yeast occurred in a time-dependent manner, 13.4% at 1 h and 25% at 4 h. Treatment with MGN-3 increased the level of attachment at all time-periods. The

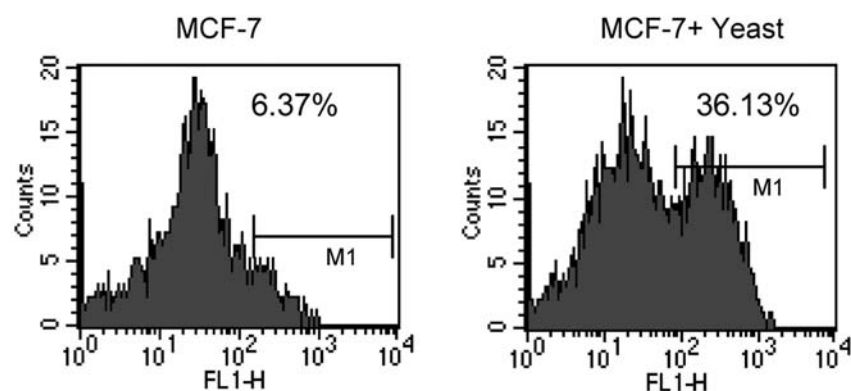


Figure 6. Annexin V staining. MCF-7 cells were incubated with or without yeast (1:10 ratio) for 2 h at 37°C. Cancer cells that were detached from the monolayer were collected, and the percentage of apoptotic cells was measured by Annexin V staining and flow cytometry. Ten thousand cells were acquired and analyzed using Cell Quest software.

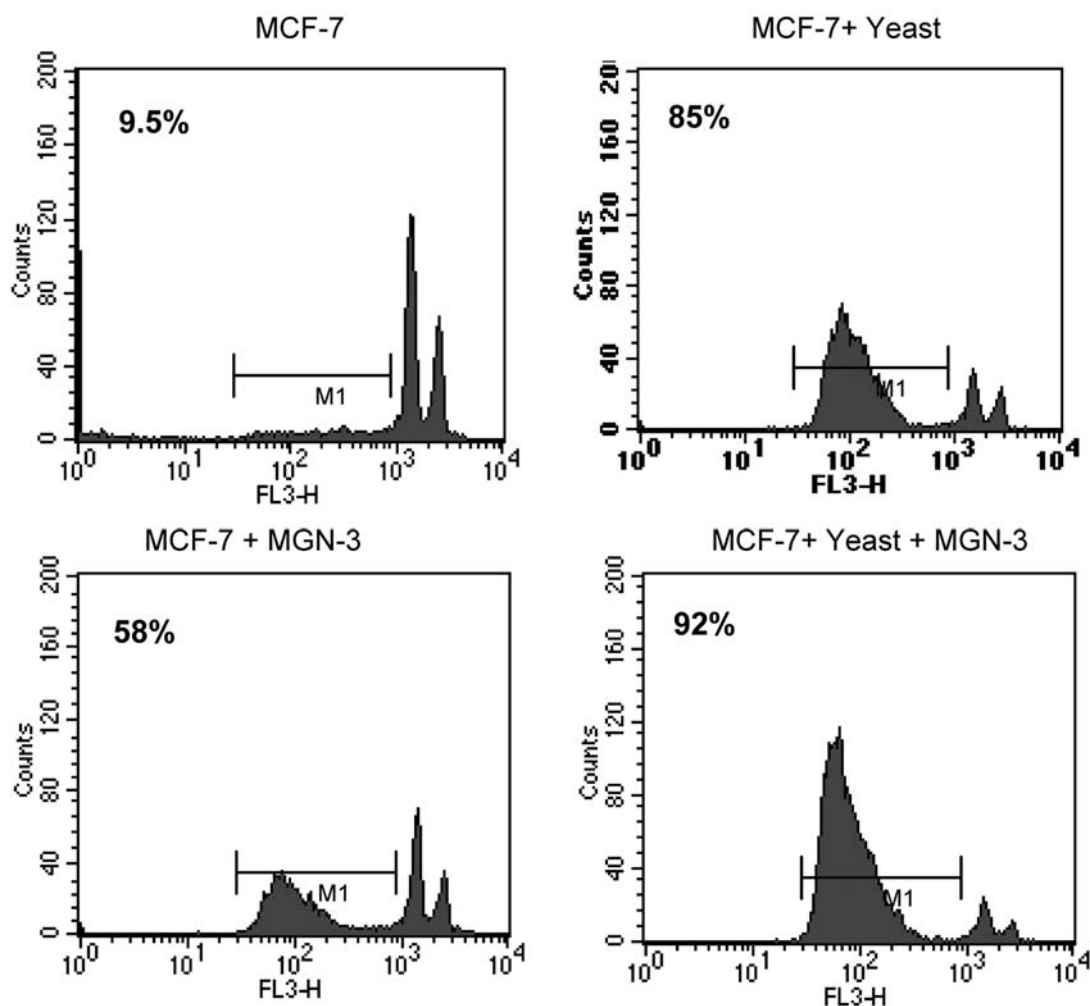


Figure 7. Percent of dead non-adherent MCF-7 cells as determined by flow cytometry. MCF-7 cells were cultured with yeast in the ratio of 1:10 in the presence or absence of MGN-3 (100 µg/ml) for 4 h, and survival of non-adherent cells was determined by flow cytometry using the propidium iodide (PI) technique. The number in the histograms represents the percent of dead cells.

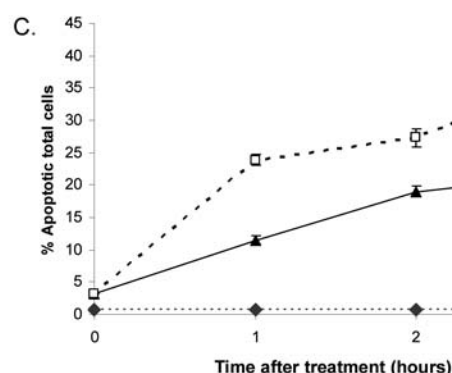
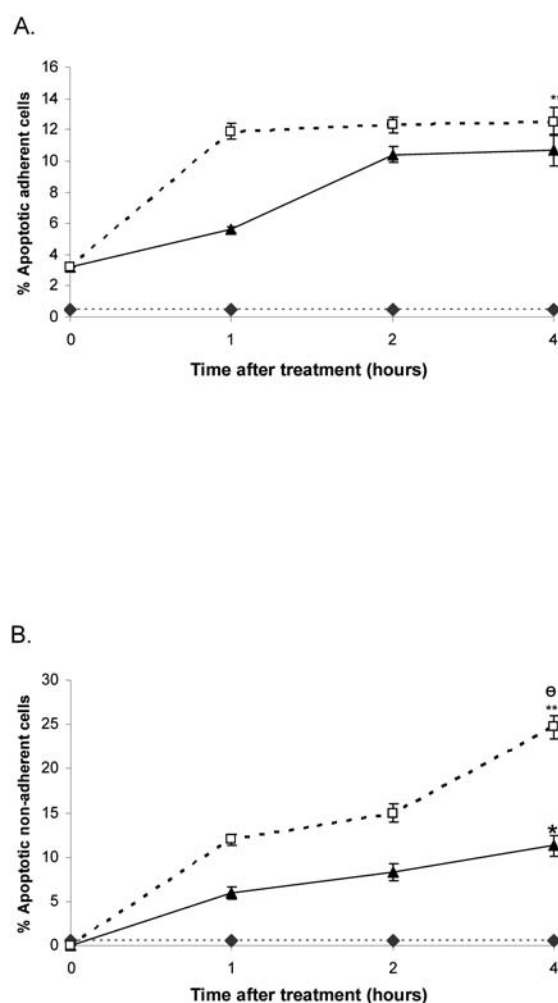


Figure 8. (A). Action of MGN-3 on the yeast-induced apoptosis of adherent MCF-7 cells using Giemsa stain. Tumor cells were cultured with yeast at a ratio of 1:10 in the presence (□) or absence of MGN-3 (▲). At 1, 2 and 4 h post-culture cancer cells with yeast, cells adherent to the cover glass were removed, stained with Giemsa, and the percentage (%) of apoptotic cells was calculated. Data represent the mean \pm SE of 5 separate experiments. Results were compared with MCF-10A cells as control (◆). * $p < 0.02$, ** $p < 0.02$ as compared to MCF-10A cells. (B) Yeast-induced apoptosis of non-adherent MCF-7 cells. Monolayer MCF-7 cells were cultured with yeast in the presence (□) or absence of MGN-3 (▲) for 1-4 h. The supernatant was collected, cytocentrifuge preparations were performed, stained with Giemsa and the percentage (%) of apoptotic cells was calculated. Data represent the mean \pm SE of 5 separate experiments. Results were compared with MCF-10A cells as control (◆). * $p < 0.02$, ** $p < 0.02$ as compared to MCF-10A cells. Θ $p < 0.03$ as compared to yeast-treated MCF-7 cells. (C) Yeast-induced apoptosis of the total apoptotic cells. MCF-7 cells were cultured with yeast in the presence (□) or absence of MGN-3 (▲) for 1-4 h. The percentage (%) of total apoptotic cells = the % of apoptotic adherent cells in Figure 8A + the % of apoptotic non-adherent cells in Figure 8B. Results were compared with MCF-10A cells as control (◆). * $p < 0.001$, ** $p < 0.003$ as compared to MCF-10A cells.

illustration in Figure 1B shows a yeast attached to MCF-7 cells at 1 h. Similarly, phagocytosis of yeast by MCF-7 cells increased as time passed: 6.9% at 1 h and 14.3% at 4 h (Figure 2A). MGN-3 increased the magnitude of phagocytizing cells by 2- to 3-fold at 1-4 h. The illustration in Figure 2B shows a number of MCF-7 cells engulfing several yeast at 2 h. Notice the presence of multiple vacuoles of digested yeast inside the apoptotic cells at 4 h (Figure 2C). On the other hand, the data show that MCF-10A in monolayer virtually did not attach (3%) nor phagocytize (1%) yeast (Figures 1A, B).

Verification experiments of phagocytosis: To differentiate between phagocytic targets attached to the cell surface and those that have actually been phagocytosed, 3 different experiments were conducted. Phagocytosis was examined using fluorescent yeast. The number of adherent cancer cells attached/phagocytized to fluorescent yeast was examined. After 2 h, adherent MCF-7 cells showed 15%

attachment to yeast (Figure 3A) and 11% phagocytizing activity (Figure 3B). Monolayer MCF-7 cells cultured with yeast and kept at low temperature (4°C) demonstrated a significantly low level of phagocytosis (Figure 4B). Similar results were obtained post-treatment of MCF-7 cells with cytochalasin B, a known inhibitor of phagocytosis (33, 34) (Figure 5).

Apoptosis studies. To determine the effects of yeast and MGN-3 treatments on cell survival, apoptotic adherent and non-adherent MCF-7 cells were identified at different intervals, and cancer cell apoptosis was evaluated by 4 different methods:

Annexin V staining: Whether yeast would induce apoptosis in monolayer MCF-7 cells was investigated. For this purpose, cancer cells were cultured with yeast at a ratio of 1:10 for 2 h, and apoptotic non-adherent MCF-7 cells were measured by the Annexin V staining. The data in Figure 6 show that

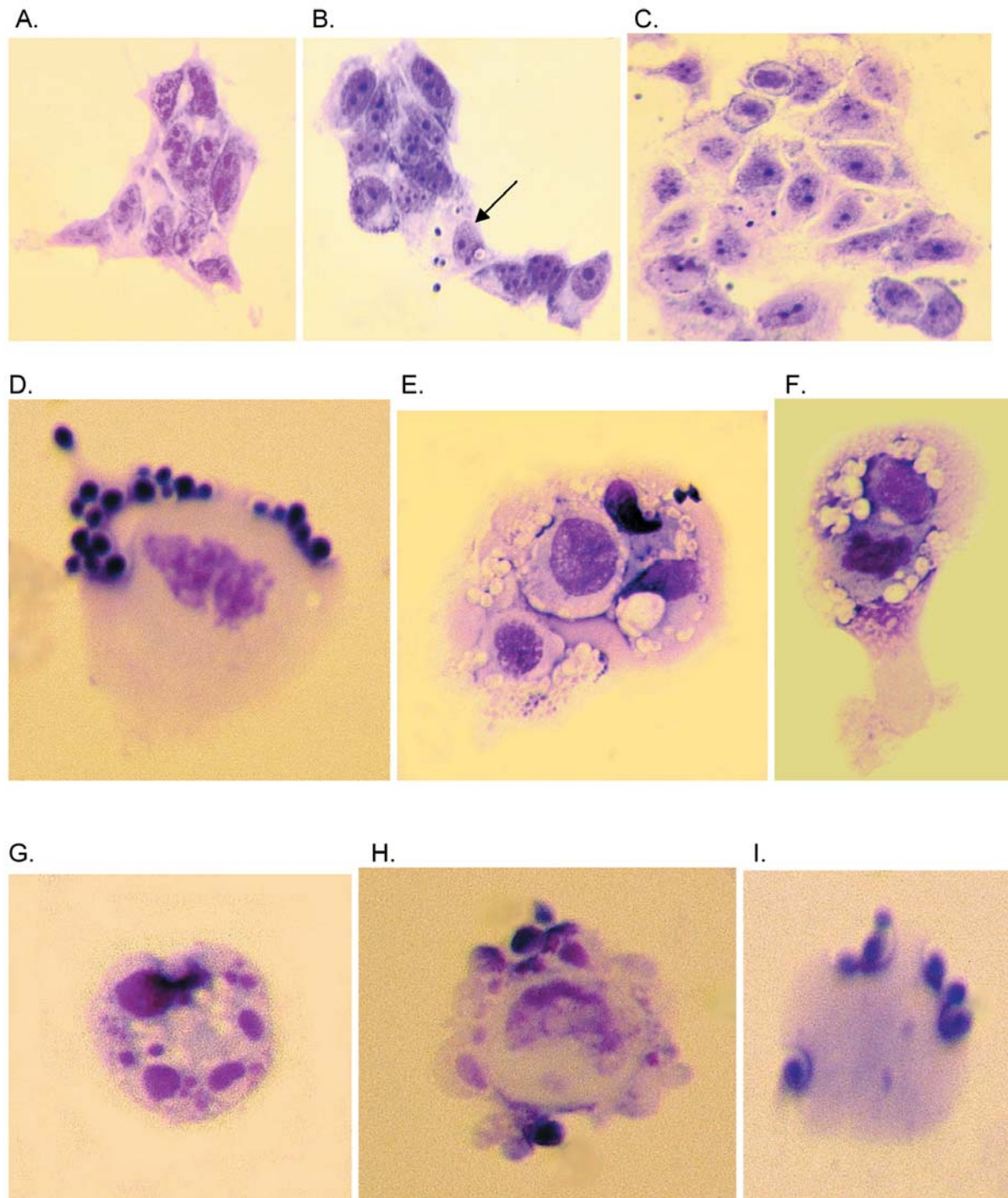


Figure 9. Morphological examination of apoptotic non-adherent and adherent MCF-7 cells. Monolayer MCF-7 cells grown on cover glass were cultured with heat-killed *S. cerevisiae* at a ratio of 1:10, respectively. Supernatants were collected and cytospin preparations were performed and stained with Giemsa. Preparation shows control adherent MCF-7 cells without treatments (A), apoptotic adherent MCF-7 cell (arrow) with chromatin condensation at 4 h post-treatment with yeast (B), and apoptosis accompanied with enlargement and disruption of the architecture of many adherent MCF-7 cells at 4 h post-treatment (C). Preparations of single (D) and multiple (E, F) non-adherent cells at 2 h post-treatment with yeast, show that apoptosis is accompanied with chromatin condensation. Preparations showing apoptotic non-adherent MCF-7 cell with nuclear fragmentation (G) and subsequent disintegration into multiple membrane-enclosed vesicles containing nuclear fragments (H). Finally, (I) shows dead cell that acquired the trypan blue stain. Notice (A-F) presence of yeast, stained dark blue, and multiple vacuoles of ingested and digested yeast inside apoptotic cells in preparations. Figures 9 A-C, E and F Giemsa x 400, D, G, and H x 1000.

culture of MCF-7 cells with yeast resulted in a significant increase in the number of apoptotic cells (36.1% as compared with 6.4% of control untreated cells).

Flow cytometry analysis: Survival of MCF-7 cells at 4 h post-treatment with yeast, MGN-3, or yeast + MGN-3 was examined by flow cytometry. The results depicted in Figure 7 indicate a significant increase in non-adherent MCF-7 cell apoptosis upon treatment with MGN-3 (58% dead cells as compared to control 9.5%). Yeast alone also caused a substantial increase in cell death (85%) as compared to background. Yeast treatment in the presence of MGN-3 further increased tumor cell apoptosis (92%).

Giemsa staining: Apoptosis is morphologically defined by enlargement of the cell, membrane blebbing and chromatin condensation. These criteria were used to identify the apoptotic MCF-7 and MCF-10A cells in cytospin preparations stained with Giemsa. Apoptosis occurred post-culture of yeast with MCF-7 cells and was detected in adherent cells. The data in Figure 8A show 5.6% of adherent MCF-7 cells undergo apoptosis at 1 h post-culture with yeast that was increased to 10.7% at 4 h. MGN-3 treatment increased the percentages of apoptotic cells at 1-4 h. Apoptosis was also examined in non-adherent cells (Figure 8B). Cytospin preparations show increased levels of apoptosis over time. Addition of MGN-3 caused a 2-fold increase in the percentage of apoptotic cells at all intervals. When the data of apoptotic adherent cells are combined with that of non-adherent cells, significant levels of apoptosis are detected in Figure 8C: 11.5% at 1 h and 21.7% at 4 h. MGN-3 increased the magnitude of apoptosis by 207% and 134% at 1 and 4 h, respectively. In contrast, the data also show only 0.2% apoptotic non-adherent MCF-10A cells post-culture with yeast.

Morphological changes of yeast-induced apoptosis of cancer cells are illustrated in Figures 9A-I. Apoptotic cells that were induced by a few or a large number of yeast were noted at different intervals post-culture of cancer cells with yeast. Apoptotic MCF-7 cells with chromatin condensation at 4 h were noted in non-adherent and adherent cells. Preparations also show apoptotic cells with DNA fragmentation. This was followed by membrane blebbing and nuclear fragments situated inside the blebs. Notice, cancer cells contain multiple vacuoles of ingested and digested yeast. Finally, the nucleus disappears and the cells acquire the color of trypan blue stain.

Trypan blue staining: Non-adherent MCF-7 cells were treated with trypan blue stain and counted using a hemocytometer. It can be seen in Figure 10 that yeast caused apoptosis of MCF-7 cells, which increased with time and maximized at 4 h (17%). MGN-3 treatment enhanced the apoptotic effect of yeast, and showed 182% increase in the percentage of apoptotic cells at 4 h. On the other hand, only 0.2% apoptotic non-adherent MCF-10A cells were seen at 1-4 h post-culture with yeast.

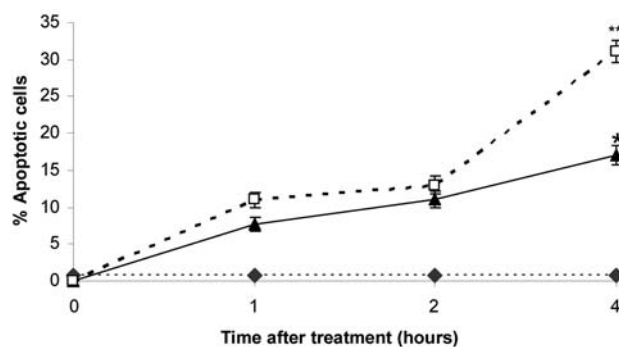


Figure 10. Yeast-induced apoptosis of monolayer MCF-7 cells using trypan blue stain. MCF-7 cells were cultured with yeast for 1-4 h. The supernatant was collected, mixed with 100 μ l trypan blue, and the percentage of dead cells was examined using a light microscope fitted with a 60x objective. The percent of dead cells = % dead cells in the supernatant/total number of cells $[100,000] \times 100$. Data represent the mean \pm SE of 3 separate experiments. Results were compared with MCF-10A cells as control (\blacklozenge). * $p < 0.03$, ** $p < 0.02$ as compared to MCF-10A cells.

Discussion

Neoplastic cells develop various strategies to escape immune surveillance and chemotherapeutic agents. The present study was undertaken to examine the apoptotic effects of *S. cerevisiae* on monolayer BCC, which more closely models cancer cell growth. The data showed that monolayer MCF-7 cells phagocytize *S. cerevisiae*. Results confirmed by fluorescence microscopy, treatment with cytochalasin B and lowering of the temperature excluded the possibility that non-specific adherence accounts for this observation. The results also demonstrated that *S. cerevisiae* is a potent inducer of apoptosis in monolayer MCF-7 cells, as indicated by the Annexin V staining and flow cytometry. The morphological analysis with Giemsa stain revealed the presence of multiple vacuoles of ingested and digested yeast inside the apoptotic cancer cells.

The lack of evidence regarding whether the apoptotic effects of yeast are selective for cancer cells prompted us to examine the effects of *S. cerevisiae* against non-tumorigenic MCF-10A cells derived from human fibrocystic mammary tissue (35). In contrast, MCF-7 cells from human breast adenocarcinoma have been shown to be tumorigenic in athymic nude mice (36-38). We show, here, that MCF-7 cells in monolayer phagocytize yeast and undergo apoptosis, while virtually no phagocytosis or yeast-induced apoptosis was observed in monolayer MCF-10A cells. The apoptotic processes vary according to the malignant nature of the cells, as non-tumorigenic cells in monolayer appear to be more resistant to yeast-induced apoptosis. This demonstrates that normal cells are not phagocytic and, during transformation to cancer cells,

subsequently acquire phagocytic ability. One possible explanation is that cancer cells express surface receptors for attachment/phagocytosis that become exposed during the course of malignancy and are otherwise masked in non-transformed cells.

The present and previous studies (28, 29, 39) revealed a striking difference between levels of phagocytosis and yeast-induced apoptosis in MCF-7 cells in suspension compared to those in monolayer. Cells in suspension, prepared from trypsinization, demonstrated greater magnitudes of phagocytosis and apoptosis as compared to those in monolayer. Trypsin stimulates the integrin $\alpha_5\beta_1$ -dependent adhesion of human gastric carcinoma cells (40). $\alpha_M\beta_2$ integrin receptors on myeloid cells mediate the phagocytosis of diverse ligands including cooled platelets (41, 42) and non-opsonized pathogens (43). Since MCF-7 cells carry $\alpha_M\beta_2$ integrin receptors (44, 45), it is possible that trypsin stimulates integrin receptors of MCF-7 cells that mediate phagocytosis of *S. cerevisiae*. Apoptotic effects by yeast are associated with tumor cells, but the mechanism is unclear. While we have shown that caspases may be involved in yeast-induced apoptosis of colonic adenocarcinoma cells (30), breast cancer cell apoptosis appears to be mediated through a caspase-independent mechanism (39).

MGN-3 is an arabinoxylan extracted from rice bran (46), proven to be a potent biological response modifier (BRM), and has the ability to boost the immune function of NK cells (47, 48), T and B cells (46) and macrophages (49). In addition to immune modulatory activity, we showed that MGN-3 enhanced yeast-induced apoptosis of monolayer MCF-7 cells. This finding, coupled with our earlier results, suggests that MGN-3 may contribute to the exposure of the receptors involved in attachment/phagocytosis. MGN-3 sensitizes human leukemic HUT cells to CD95-induced apoptosis by decreasing the expression of Bcl2 (50). It is possible that MGN-3 acts through a similar mechanism in MCF-7 cells. Further studies need to be carried out in order to elucidate the exact mechanism underlying MGN-3's effect on enhancing yeast-induced apoptosis.

This study confirmed our earlier observation that human cancer cells of the breast, tongue and colon undergo apoptosis following phagocytosis of heat-killed *S. cerevisiae* *in vitro*. We are not aware of any clinical trials on the effectiveness of yeast against cancer; however, tests using selenized yeast as a chemo-preventative agent have been conducted in multiple clinical cancer trials (51-53) in which the effect of daily selenium supplementation against cancer was examined. Identification of the active component(s) in yeast that induces apoptosis might present a therapeutic approach in cancer drug development. The specificity and safety of both heat-killed yeast and MGN-3 provide an advantage over currently available anticancer agents and may establish the foundation for *in vivo* studies with important therapeutic implications.

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