Abstract. We have recently demonstrated that non-metastatic human breast cancer cell lines undergo apoptosis following phagocytosis of \textit{S. cerevisiae}. In this study, we investigated the apoptotic effect of heat-killed yeast against human metastatic breast cancer (MBC) cells, MDA-MB-231 \textit{in vitro}, and the underlying mechanistic bases of this effect. Results show that monolayer MDA-MB-231 cells phagocytized yeast (50% at 16 h) and underwent apoptosis (32% compared with 7.6% of untreated cells, representing a 4.2-fold increase). The increase in apoptosis was associated with an elevation of \([\text{Ca}^{2+}]_i\). Addition of 2-aminoethoxydiphenyl borate (2APB), a pharmacological inhibitor of \text{Ca}^{2+} release from the endoplasmic reticulum, effectively diminished yeast-induced apoptosis. Furthermore, yeast caused a substantial decrease in expression of \text{Bcl-2} and an increase in \text{Bax} resulting in alteration in the \text{Bax:Bcl-2} ratio. However, yeast had no effect on NO levels. In conclusion, yeast induces apoptosis of human MBC cells \textit{in vitro} by a mechanism involving intracellular \text{Ca}^{2+} and \text{Bax:Bcl-2}.

Introduction

Breast cancer is the most frequently diagnosed cancer in women, and the incidence of breast cancer is close to 50% in women 65-years-old (1). Two characteristics of breast cancer malignancy are their invasive and metastatic potential. In its most recent issue of Cancer Facts, the National Cancer Institute estimated that 178,480 new cases of invasive breast cancer and 62,030 new cases of \textit{in situ} breast cancer occurred among American women in 2007. These high figures reflect how primary breast tumors metastasize to regional and distant sites. However, despite inroads in the pharmacologic-based treatment of cancer, there are still very limited therapeutic options for the treatment of advanced stages of the disease. Survival statistics are significantly reduced when the cancer metastasizes (2) and the therapy remains essentially palliative.

It is therefore of particular interest to find agents that induce apoptosis of metastatic breast cancer (MBC) cells with minimal side-effects. Previously, we have revealed the ability of Baker's yeast, \textit{S. cerevisiae}, to induce apoptosis in several tumor cell lines, breast (3-5), tongue and colon (6) \textit{in vitro} and \textit{in vivo} (7-9), without inducing a significant effect in normal cells (5). In these studies, cancer cells demonstrated the ability to phagocytize yeast and subsequently undergo apoptosis. The phagocytosis-induced apoptotic effect by yeast is selective for tumor cells, but the molecular mechanism(s) have yet to be determined.

Phagocytosis is associated with the triggering of several apoptotic pathways in professional phagocytes. To determine which pathway is operative in yeast-induced apoptosis, we investigated: i) the apoptotic effect of yeast in MBC, and ii) the molecular mechanisms underlying the apoptotic effect of yeast. Results showed that killed non-pathogenic yeast, \textit{S. cerevisiae}, exerts an apoptotic effect against MDA-MB-231 cells by a mechanism that includes: i) intracellular \text{Ca}^{2+} and ii) pro/antiapoptotic molecules (Bax and Bcl-2).

Materials and methods

\textit{Chemicals and antibodies.} Purified EGCG (>98% pure) and GTP were obtained from Mitsui Norin, Co., Ltd. (Shizuoka, Japan). Annexin V-conjugated Alexa Fluor488 Apoptosis detection kit was purchased from Molecular Probes, Inc. (Eugene, OR, USA). The primary antibodies to Bax and Bcl-2
and anti-rabbit secondary antibodies were purchased from BD Biosciences (Franklin Lakes, NJ).

Complete medium (CM). CM consists of DMEM supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT, USA), 100 μg/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA).

Tumor cell line. The human highly metastatic breast cancer cell line (MDA-MB-231) was used in the present study. The cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Tumor cells were cultured in monolayer in CM and maintained in a humidified incubator at 37°C in 5% CO₂.

Preparation of S. cerevisiae. Commercially available baker’s and brewer’s yeast, S. cerevisiae, was used in suspensions that were washed once with phosphate-buffered saline (PBS). They were heated for 1 h at 90°C to kill the yeast and washed 3 times. Quantification was carried out using a hemocytometer and cell suspensions were adjusted to 1x10⁷ cells/ml.

Phagocytic assay. A previously reported phagocytic assay was employed (3,5,15) to examine cells in suspension and in monolayer. For cell suspension, cells were collected after trypsinization. For cells in monolayer, the medium containing non-adherent tumor cells (1 ml) was transferred to glass tubes and mixed with adherent cells that were collected by trypsinization. At different time-points both cell suspension tubes and mixed with adherent cells were collected by detaching loosely attached yeast from tumor cells. Cell suspensions were adjusted to 1x10⁷ cells/ml.

Similarly, the number of apoptotic cells in the 200 μl was counted.

Apoptosis studies
Detection of apoptotic cancer cells by morphological analysis. Apoptosis is morphologically defined by membrane blebbing and chromatin condensation. These criteria were used to identify the apoptotic cancer cells in cytospin preparations stained with Giemsa (3-6).

DNA content by flow cytometry. The cellular DNA content was measured following extraction of degraded DNA from apoptotic cells by propidium iodide (PI) staining. Cells (1x10⁶/ml) were fixed in 70% ethanol, washed with PBS and re-suspended in DNA extraction buffer (0.2 M Na₂HPO₄ with 0.1 M citric acid, pH 7.8). Following extraction, the cells were washed and incubated in DNA staining solution (20 μg/ml PI in PBS containing 50 μg/ml RNase). The cells were stained for 30 min at room temperature in the dark and were analyzed by FACScan (Becton Dickinson, San Jose, CA, USA). After exclusion of necrotic debris, the sub G0/G1 peak was used to quantify apoptosis.

Cellular Ca²⁺ flux. Cellular Ca²⁺ flux was determined as follows: MDA-MB-231 cells (1x10⁵ cells per 100 μl CM per well) were cultured overnight in a 96-well black well/clear bottom plate (Coster, Cambridge, MA, USA). The growth medium was removed, and the cells were incubated with 100 μl of ScreenQuest™ Fluo-8 NW Calcium Assay Kit (according to the manufacturer’s instructions, ABD Bioquest, Inc., Sunnyvale, CA, USA) for 1 h at room temperature. S. cerevisiae (25 μl/well) was added to make the final ratios of the cancer cell to yeast to be 1:0, 1:10, 1:20, and 1:50. Fluorescence at Ex=490/Em=525 nm was measured by SPECTRAMax M5 (Molecular Devices Corp., Sunnyvale, CA, USA) and Ca²⁺ flux was expressed as RFU (relative fluorescence units).

Nitric oxide (NO) production. Production of NO was determined as the amount of nitrite, a stable end-product of NO, in the culture supernatant obtained at 2, 8, 24 and 48 h post culture of yeast with cancer cells. Nitrite was measured by a colorimetric assay using the Griess reagent (1% sulfanilamide and 0.1% N-1-naphthylenediamine dihydrochloride in 2.5% H₃PO₄ solution) (16). The absorbance at 540 nm was measured and the nitrite concentration was quantified (in μM) using sodium nitrite as the standard in each assay.

Western blot analysis. The expression of Bax and Bcl-2 was determined using Western blot analysis. Cells were cultured with yeast at a ratio of 1:50 for 48 h. Cells were harvested, washed with cold PBS [10 mmol/l (pH 7.4)], and lysed with ice-cold lysis buffer [50 mmol/l Tris-HCl, 150 mmol/l NaCl, 1 mmol/l EGTA, 1 mmol/l EDTA, 20 mmol/l NaF, 100 mmol/l Na₂VO₄, 1% NP40, 1% phenylmethylsulfonyl fluoride, 10 Ag/ml aprotinin, and 10 Ag/ml leupeptin (pH 7.4)] for 30 min and centrifuged at 14,000 g for 20 min at 4°C as detailed previously. The supernatant was collected and used immediately. Cell lysates (75 μg) were subjected to Western blot analysis by 4-12% SDS polyacrylamide gel electrophoresis using 1:500 anti-BAX antibody or 1:500 anti-
Bcl-2 (BD Bioscience Franklin Lakes, NJ, USA) primary antibody. The washed PVDF membranes were incubated with 1:2000 dilution of monoclonal secondary antibodies. Immunoreactive bands were visualized by using ECL detection system (Amersham, Buckinghamshire, UK). To verify equal protein loading and transfer, the blots were stripped and re-probed for β-actin using an anti-actin rabbit polyclonal antibody. The relative intensity of each protein band in a blot was measured by using computerized software program Quantity One software (Bio-Rad, Hercules, CA, USA) and the ratio of Bax:Bcl-2 was analyzed as previously described (17). Briefly, the intensities of Bax and Bcl-2 bands were quantified and normalized to β-actin, and then the Bax:Bcl-2 ratio was calculated. Data are represented as mean ± SEM.

Statistical analysis. Using the Student's t-test, we tested the significance of difference in the percent survival of apoptotic cancer cells, Bax:Bcl-2 ratio post culture with yeast as compared to cancer cells alone, and the difference in the cellular Ca^{2+} flux of the cancer cells in response to yeast stimulation as compared to cancer cells alone. We used the cut-off of p<0.05 as significant.

Results

Phagocytosis studies

Percentage of attachment. MDA-MB-231 cells in suspension and monolayer were cultured with yeast, and the percentage of attachment of yeast to cancer cells was examined at different intervals. Data in Fig. 1A show that attachment of MDA-MB-231 cells in suspension reached 24% at 1 h, then decreased gradually and reached 4% at 16 h, while attachment of cells in monolayer increased steadily as time passed and maximized at 16 h. Similarly, the attachment index (AI) of cells in suspension shows sharp increase at 1 h then decreased at 2.5 h and reached its minimal level at 16 h (0.04%) (Fig. 1B), while the AI of monolayer MDA-MB-231 cells increased slowly in a time-dependent manner and maximized at 16 h (Fig. 1).

Percentages of phagocytosis. Phagocytosis of yeast by MDA-MB-231 cells in suspension increased as time passed and maximized at 16 h (67%), while cells in monolayer also increased but to a lesser extent (Fig. 3A). A similar pattern of increase in the phagocytosis index (PI) by MDA-MB-231 cells in suspension and monolayer was noted as time passed and maximized at 16 h (Fig. 2A and B). The illustration in Fig. 2C shows massive ingestion of yeast by tumor cells. The intensity of color of yeast easily define phagocytosed (dark blue) and ingested yeast (gray color).

Apoptosis studies. To determine the effects of yeast treatment on cell survival, apoptotic MDA-MB-231 cells were identified at different intervals and cancer cell apoptosis was evaluated by two different methods:

Giemsa staining. Apoptosis is morphologically defined by enlargement of the cell, membrane blebbing and chromatin condensation. These criteria were used to identify the apoptotic MDA-MB-231 cells in cytospin preparations stained with Giemsa. Apoptotic cells that were induced by yeast were noted at 16 h post-culture of cancer cells with yeast. Morphological changes of apoptotic cancer cells are illustrated in Fig. 3A and B. Most apoptotic cancer cells were enlarged and demonstrated membrane blebbing and chromatin condensation. Note that the apoptotic cells are heavily packed with yeast. Fig. 3C shows control untreated MDA-MB-231 cells.

DNA content by flow cytometry. We examined the apoptotic effect of yeast against MDA-MB-231 cells in monolayer using flow cytometry. For this purpose, cancer cells were cultured with yeast at a ratio of 1:50 for 3 days and apoptotic MDA-MB-231 cells were measured by PI technique. Data in Fig. 4 show that culture of MDA-MB-231 cells with yeast resulted in a significant increase in the number of apoptotic cells (32% as compared with 7.6% of control untreated cells, representing a 4.2-fold increase). Addition of 100 μM of 2-aminoethoxydiphenyl borate (2APB), an inhibitor of Ca^{2+}, significantly diminished yeast-induced cancer cell apoptosis and brought the levels of apoptosis within the range of values of the control untreated cells.

Yeast-treatment increases cellular Ca^{2+} flux. We determined the role of cellular Ca^{2+} flux associated with phagocytosis in inducing apoptosis of cancer cells by testing the levels of intracellular Ca^{2+} as follows: MDA-MB-231 cells were stimulated with *S. cerevisiae* at different ratios, 1:10-1:50. Control
was cancer cells alone. Cellular Ca\textsuperscript{2+} flux was measured with Screen Quest Fluo-8 NW Calcium Assay Kit according to the manufacturer’s specifications, which is more accurate than the standard Fura-2 assay. Results in Fig. 5 show that intracellular Ca\textsuperscript{2+} levels significantly increased at a cancer cell to yeast ratio of 1:20 (p<0.05), with further increase at 1:50 (p<0.001).

Figure 2. Percent phagocytosis of yeast by MDA-MB-231 cells. Cancer cells in suspension and monolayer were cultured with yeast at a ratio of 1:50, respectively. At different intervals post-culture cancer cells with yeast, 100 μl trypan blue was added for 5 min and cytocentrifuge preparations were performed then stained with Giemsa. (A) The percentage (%) of phagocytosis and (B) phagocytotic index (PI) was obtained using a light microscope fitted with objective x60. The figure represents one of three identical experiments. (C) Cytocentrifuge preparations showing cancer cells phagocytizing yeast, stained dark blue, and ingesting several particles of yeasts, stained light blue, at 6 h post culture with yeast. Giemsa x400.

Figure 3. Morphological examination of apoptotic MDA-MB-231 cells. Cancer cells in monolayer were cultured with yeast at a ratio of 1:50. At 16 h post-culture cancer cells with yeast, 100 μl trypan blue was added to the mixture for 5 min, and cytocentrifuge preparations were performed, then stained with Giemsa. (A) Apoptotic cancer cells with enlargement of the cells, membrane blebbing and chromatin condensation. Most cells have an increased number of ingested yeast, stained light blue, inside the apoptotic cancer cells. (B) DNA fragmentation in the upper cell and 2 long blebs in the lower cell. (C) Control untreated MDA-MB-231 cells. Giemsa x400.

Figure 4. DNA content by flow cytometry. Monolayer MDA-MB-231 cells were incubated with yeast (1:50 ratio) in the absence or presence of 2-aminoethoxydiphenyl borate (2APB) (100 μM). At 3 days, cancer cells (detached and adherent cells removed via trypsinization) were collected and percentage of apoptotic cells was measured by PI and flow cytometry. Cells were analyzed by FACScan. The sub G0/G1 peak was used to quantify apoptosis.
Production of NO. Production of NO by MDA-MB-231 cells was examined post culture with yeast. Results indicated that the production of NO was not detected at any time post culture of yeast with cancer cells (data not shown).

Protein expression of Bcl-2 and Bax. Effect of yeast on protein expression of Bcl-2 and Bax as determined by Western blot analysis. Bcl-2 is known as antiapoptotic protein that has been associated with the inhibition of apoptosis. On the other hand, up-regulation in proapoptotic protein Bax, leads to an increase in apoptosis. Cells were cultured with yeast at a ratio 1:50 respectively for 48 h. Fig. 6A depicts results of Western blot analysis showing that treatment of MDA-MB-231 cells with yeast resulted in downregulation of the antiapoptotic protein Bcl-2 concomitantly with up-regulation of Bax. The ratio of Bax:Bcl-2 was calculated and results in Fig. 6B show that a significant change (p<0.001) in the expression of these apoptotic molecules occurs post treatment with yeast.

Discussion

Metastatic breast cancer (MBC) is the primary cause of death among breast cancer patients. The lack of effective treatment for MBC prompted us to examine the apoptotic effects of *S. cerevisiae* against MBC and to investigate the possible mechanistic bases underlying its effect. Results of this study reveal that human MBC cells (MDA-MB-231) do phagocytize *S. cerevisiae* and subsequently undergo apoptosis. These findings are consistent with our earlier studies *in vitro* using the human breast cancer cell lines (MCF-7, ZR-75-1 and HCC70) (3-5) as well as with *in vivo* studies involving weekly IT injections of yeast into nude mice bearing MCF-7 cells. Results showed significantly increased cancer cell apoptosis upon treatment with yeast (7,8).

Phagocytosis of yeast by MDA-MB-231 cells proceeded after attachment between yeast and cancer cells, and was characteristically different in cell suspension versus cells in monolayer. MDA-MB-231 cells in suspension showed an increase in the level of phagocytosis in association with a decline in the level of attachment, suggesting that the cells have an active mechanism of phagocytizing the attached yeast. However, an increase in the attachment level by monolayer MDA-MB-231 cells was concomitant with an increase in the level of phagocytosis, possibly due to a slower rate of ingestion of the yeast cells relative to cells in suspension. Trypsinized MDA-MB-231 cells in suspension also demonstrated greater levels of phagocytosis than cells in monolayer. These data are in accordance with our earlier studies (3-5), and may be attributed to the stimulating effects of trypsin on the αMß2 integrin receptors which mediate phagocytosis (18-20).

Our data showed that *S. cerevisiae* whole yeast cells induce apoptosis of MDA-MB-231 cells *in vitro*. The morphological analysis with Giemsa-stained preparations revealed the presence of ingested yeast inside the cancer cells which showed signs of apoptosis such as cell enlargement, membrane blebbing and chromatin condensation. These results were confirmed by flow cytometry that showed more than a 4-fold increase in the level of apoptosis over the untreated control cells. MDA-MB-231 has been used as a model to examine the anti-metastatic effect of several agents such as macronutrients.
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the energy derived from the chemiosmotic potential gradient by yeast is Ca\textsuperscript{2+} dependent as indicated by: i) significant of this study showed that apoptosis of MDA-MB-231 cells persistent or excessive, will trigger cellular apoptosis. Results an evolutionarily conserved cell stress response which, if accumulation of misfolded proteins in the ER. This leads to in the calcium homeostasis or redox status can cause the plasma membrane.

With respect to apoptosis, on the other hand, perturbation in the calcium homeostasis or redox status can cause the accumulation of misfolded proteins in the ER. This leads to an evolutionarily conserved cell stress response which, if persistent or excessive, will trigger cellular apoptosis. Results of this study showed that apoptosis of MDA-MB-231 cells by yeast is Ca\textsuperscript{2+} dependent as indicated by: i) significant increased production of intracellular Ca\textsuperscript{2+} flux post culture of cancer cells with yeast; and ii) significant suppression of apoptosis of cancer cells upon addition of a pharmacological inhibitor of Ca\textsuperscript{2+}, 2-aminoethoxydiphenyl borate (2APB). However, the mechanisms by which Ca\textsuperscript{2+} contributes to induction of apoptosis of MDA-MB-231 cells are not fully elucidated. Ca\textsuperscript{2+} often plays an important role in the triggering of apoptotic signals directly via intrinsic (or mitochondrial) pathway of apoptosis (13-15). Our previous study showed that phagocytosis of yeast by human breast MCF-7 cancer cells led to disruption of mitochondrial membrane potential (MMP) (3). Mitochondria regulate Ca\textsuperscript{2+} levels by utilizing the energy derived from the chemiosmotic potential gradient $\Delta \Psi_m$ (33). A key feature of the intrinsic pathway of apoptosis is permeabilization of the outer mitochondrial membrane and a resulting disruption of MMP and release of cytochrome c, which activates the caspase cascade leading to apoptosis. Ca\textsuperscript{2+} appears to facilitate disruption of MMP by different pathways, including activation of permeability transition pores (PTP) (34,35), and modulation of Bax and Bcl-2 levels.

Ca\textsuperscript{2+} may cause cancer cell apoptosis via altering the levels of Bax and Bcl-2. Apoptosis is modulated by antiapoptotic and proapoptotic effectors, which involve a large number of proteins. Therefore, we looked at the effect of yeast on proapoptotic and antiapoptotic proteins of the Bcl-2 family, including Bax and Bcl-2. The proteins of the Bcl-2 family play an important role in induction of apoptosis and are considered as a target for anticancer therapy (36,37). However, Bax is a proapoptotic protein and its predominance over Bcl-2 promotes apoptosis (38,39). Studies have also shown that the ratio of Bax to Bcl-2 proteins increases during apoptosis (40). Western blot analysis revealed that co-culture of MDA-MB-231 cells with yeast showed an increase in the expression of Bax concomitant with a decrease in Bcl-2 protein expression, suggesting that changes in these proteins may be responsible for yeast-induced apoptosis. It is also worth noting that the addition of the Ca\textsuperscript{2+} inhibitor 2APB resulted in a reversal of the inhibitory effect of yeast on Bcl-2 protein levels (data not shown). These data suggest that the rise in calcium may be responsible for yeast-induced apoptosis via Bax and Bcl-2.

It has been shown also that nitric oxide (NO) is produced by activated macrophages. NO is synthesized endogenously by the enzyme nitric oxide synthase (NOS) in activated macrophages (41). NO plays an important role in the killing of intracellular microbial pathogens, and possesses tumoricidal activity (42). It is of interest to note that, co-culture of MDA-MB-231 cells with yeast did not produce any NO levels. This suggests that the apoptosis in human metastatic breast cancer cells operates by NO-independent mechanism.

In conclusion, yeast possesses an apoptotic effect against the human highly metastatic breast cancer cell line, MDA-MB-231, in vitro by a mechanism that includes intracellular Ca\textsuperscript{2+} release and apoptosis associated molecules. Data indicate that Ca\textsuperscript{2+} plays an important role in cancer cell phagocytosis/apoptosis in a manner similar to that of professional phagocytic cells. However, NO is not involved in the process of yeast-induced apoptosis of human cancer cells. By defining the mechanism by which yeast induces apoptosis, we can potentially isolate steps along the pathway that might serve as effective targets for therapeutic intervention. In addition, pharmacological agents that may synergistically enhance this process represent possible therapeutic agents for the treatment of cancer. Examples of these agents include Thapsigargin, an agent known to induce the release of Ca\textsuperscript{2+} from ER.

References


