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Chapter VI

YEAST THERAPY FOR THE TREATMENT OF CANCER AND ITS ENHANCEMENT BY MGN-3/BIOBRAN, AN ARABINOXYLAN RICE BRAN

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ABSTRACT

Apoptosis constitutes a strictly programmed device for the removal of aged, damaged and abnormal cells. Research in the last decade has revealed a promising future for apoptosis-based cancer therapies. However, chemotherapeutic agents exhibit an indiscriminative killing that involves both cancer cells and many normal cells. It is therefore of particular interest to find agents that have the ability to induce apoptotic activity specifically in cancer cells and with minimal side-effects. Interest in microbe-based approaches to cancer therapy has recently reemerged. The use of S. cerevisiae, the baker's yeast, as a novel approach for the treatment of cancer is based on the concept that tumor cells undergo apoptosis upon phagocytosis of yeast. Our data shows that: 1) Heat killed yeast induces apoptosis in multiple human cancers [e.g. breast, tongue and colon] *in vitro*, 2) yeast has no toxicity against normal cells, 3) induction of apoptosis is yeast-specific, 4) apoptotic activity of yeast in cancer cells is enhanced by MGN-3/biobran, a biological response modifier, and importantly, 5) the phenomenon of yeast induced apoptosis was observed *in vivo* in athymic nude mice model. Electron microscopic studies of tissue samples obtained from *in vivo* yeast-treated tumors clearly show tumor cells phagocytize yeast and then undergo apoptosis. The molecular mechanism underlying this phenomenon is currently under investigtion in our laboratory. Further investigation into this area may present clinical implications for the treatment of cancer.

INTRODUCTION

Major Pathways of Apoptosis

Apoptosis (programmed cell death) is a physiologic form of cell death that plays an important role in normal development, tissue homeostasis, and pathological situations [1,2]. There are three major pathways of apoptosis: 1) death receptor pathway, 2) a mitochondrial pathway, and 3) endoplasmic reticulum (ER) stress pathway [3,4].

1- Death Receptor Pathway

CD95 is a death receptor that belongs to the tumor necrosis factor receptor (TNF R)/nerve growth factor receptor (NGFR) gene superfamily [5,6]. 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) [6,7]. 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 [8]. 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 which 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.

2- Mitochondrial Pathway of Apoptosis

The mitochondrial pathway of apoptosis is triggered by a wide variety of conditions such as nutrient deprivation, cytoskeletal disruption, oxidative stress and DNA damage [9]. This pathway is dependent on the process of mitochondrial outer membrane permeabilization (MOMP) which leads to the release of proteins such as cytochrome C, apoptosis-inducing factor (AIF) and endonuclease G from the mitochondrial intermembrane space into the cytosol [10]. The proteins released from mitochondria induce apoptosis by caspase dependent and caspase independent pathways. In caspase dependent pathway, cytochrome C which is released from mitochondria, binds to cytosolic, monomeric apoptotic protease activating factor–1 (APAF-1) [11], and forms an "apoptosome." The apoptosome then binds to and oligomerizes the proform of caspase 9 and activates the protease. The activated caspase 9 then cleaves and activates caspase 3 and caspase 7. These molecules execute apoptosis through the cleavage of key substrates within the cell. Caspase-independent pathway of apoptosis is mediated by AIF and endonucleaseG, which cause chromatin condensation and large-scale DNA fragmentation [12,13].

Mitochondrial membrane permeabilization is regulated by members of Bcl-2 family [14-16]. The proapoptotic Bcl-2 family members, Bax and Bak, are present in most cells in inactive form, and their activation causes MOMP. The antiapoptotic molecules Bcl-2 and

BclXL inhibit MOMP by preventing the action of propapototic molecules, and inhibited by the antiapoptotic Bcl-2 family proteins. The BH3-only proteins of this family regulate MOMP either by activating Bax and Bak or by antagonizing the antiapoptotic Bcl-2 proteins [17].

3- Endoplasmic Reticulum Stress-dependent Apoptosis

The endoplasmic reticulum (ER) is an intracellular organelle. It is the major site for intracellular calcium and a site for synthesis, post-translational modification and delivery of biologically active proteins to their proper target sites. Perturbation in the calcium homeostasis or redox status, elevated secretory protein synthesis, sugar/glucose deprivation, altered glycosylation, and overloading of cholesterol disrupt ER homeostasis and they can cause accumulation misfolded proteins in ER. This leads to an evolutionarily conserved cell stress response. This response is initially aimed to restore the ER homeostasis. However, persistent or excessive, ER stress triggers cell death, typically apoptosis. The mechanisms for ER-induced apoptosis are not fully elucidated. Several pathways appear to contribute to ER-mediated cell death [18-20].

One pathway is through the release of ER Ca++ into the cytosol. When improperly regulated, Ca2+ has been shown to play a role in several cell death pathways. Cytoplasmic proteases such as calpains are activated by calcium [21,22], whose substrates include Bax and Bid (which are activated), Bcl-2 and Bcl-xL (which are inhibited), and several caspases [23-25]. Phospholipase A can be activated by influx of extracellular Ca2+ to produce intracellular signaling lipids (e.g. arachadonic acid, which can induce reactive oxygen species (ROS) and ER Ca2+ release, followed by mitochondrial Ca2+ uptake. High levels of Ca2+ uptake by mitochondria can activate the permeability transition pore (PTP), a membrane channel that can facilitate mitochondrial swelling and disruption of mitochondrial membrane integrity, leading to cytochrome *c* release and cell death [26,27]. Other downstream effectors of Ca2+-induced cell death include activation of the protein phosphatase calcineurin, which dephosphorylates the proapoptotic protein Bad, allowing it to dimerize with and antagonize Bcl-xL [28].

A second ER stress-induced apoptosis pathway is the activation of the ER-localized cysteine proteases, Caspase 2 in rodents and caspase 4 (in humans) [29,30]. These caspases appear to be activated by ER stress, but not by death receptor-mediated or mitochondria-targeted apoptotic signals. The mechanisms responsible for caspase may be indirect, involving calpains activated by Ca2+ released in the vicinity of the ER [31]. Caspase 7 also may activate caspase 12 by translocating from cytosol to ER [32].

A third pathway through which ER stress triggers apoptosis is through the activation of kinase, Ask1. The kinase pathway initiated by Ask1 leads to activation CJUNNH2-terminal kinase (JNK), and JNK-mediated phosphorylation activates the proapoptotic protein Bim while inhibiting the anti apoptotic protein Bcl-2 [32].

Another pathway involves the activation of transcription factor CHOP also known as growth arrest and DNA damage-inducible gene 153 (GADD153) [33]. Enforced overexpression of CHOP has been reported to lead to cell cycle arrest and/or apoptosis and

CHOP-/- cells display increased resistance to ER stress-mediated apoptosis. The downstream targets of CHOP remain unknown, but CHOP-mediated apoptosis has been coupled to a pathway that suppresses Bcl-2 expression, depletion of intracellular glutathione, and an increase of free radicals.

In brief, there are multiple pathways by which ER stress can trigger apoptosis and there is strong evidence that Ca2+ release from ER stores and subsequent caspase activation mediates programmed cell death under ER stress conditions.

Induction of apoptosis in tumor cells is a promising approach to curing cancer. Many anticancer drugs function by inducing apoptosis in cancer cells [34-40]. Current available treatments for cancer are toxic not only for cancer cells but also for normal cells. As a result, cancer patients often suffer from side effects that decrease their quality of life. New treatment methods that target cancer cells specifically are therefore highly sought after. The phenomenon of induction of apoptosis after phagocytosis of certain microorganisms may offer an effective and alternative method for the treatment of solid tumors. In this article, we will introduce and review the cumulative data collected in our laboratory on the phenomenon of yeast-induced apoptosis, the role of the biological response modifier MGN-3/Biobran in enhancing this effect and possible mechanism underlying this phenomenon.

4- Yeast-Induced Cancer Cell Apoptosis

The induction of apoptosis after phagocytosis of certain microorganisms has been studied using human phagocytic cells. Elevated levels of apoptosis has been observed in neutrophils and monocytes/macrophages post-phagocytosis of microorganisms such as *Escherichia coli* [41], *Mycobacterium tuberculosis* [42], *Staphylococcus aureus* [43], *Candida albicans* [44] and *Shigella Flexneri* [45]. Earlier studies suggest that many tumor cells exhibit phagocytic activity. These include: phagocytosis of titanium particles by sarcoma L929 cells [46], of lymphocytes by carcinomas of the cervix [47,48], of erythrocytes and bacteria by adenocarcinomas [49,50], of elastic fibers by dermatofibroma cells [51], and of *C. albicans* by lymphatic tumor cells [52], and latex beads, fluorescent Matrigel, and yeast by human breast cancer cells (BCCs) [53-55]. In addition, other cancer cell lines (oral and colon) [56], prostate (LNCap) and erythroleukemic cells (K562) (data not shown) likewise have the ability to phagocytize *S. cerevisiae*. Interestingly, a recent study carried out in our laboratory showed that phagocytosis of yeast, *S. cerevisiae,* induces apoptosis in multiple human cancers (e.g. breast, tongue and colon). Heat-killed *S. cerevisiae* was cultured with cancer cells for 4h, and apoptosis of the cancer cells was evaluated using flow cytometry and cytospin preparations.

Breast Cancer

Breast cancer is the leading cause of mortality and morbidity among women in western countries including United States of America. In this study, human BCC line, MCF-7 in suspension, were cultured with *S. cerevisiae*, then phagocytosis of yeast and apoptosis of the cancer cells was evaluated. Results showed that upon phagocytosis of yeast, MCF-7 cells underwent apoptosis that peaked at 4 h (38%) (Figure 1). Data also revealed that other BCC,

ZR-75-1 and HCC7, undergo apoptosis post-phagocytosis of yeast, but there was a differential response among different BCC lines toward the apoptotic effect of *S.cerevisiae*:[55,57]. Subsequent experiments were undertaken to examine monolayer MCF-7 cells that more closely model cancer cell growth. Results showed that monolayer MCF-7 cells grown on cover glass that were cultured with *S. cerevisiae* are able to phagocytize yeast. Again, MCF-7 cells underwent apoptosis following phagocytosis of yeast in a time-dependent manner that maximized at 4 hr (14%) [58].

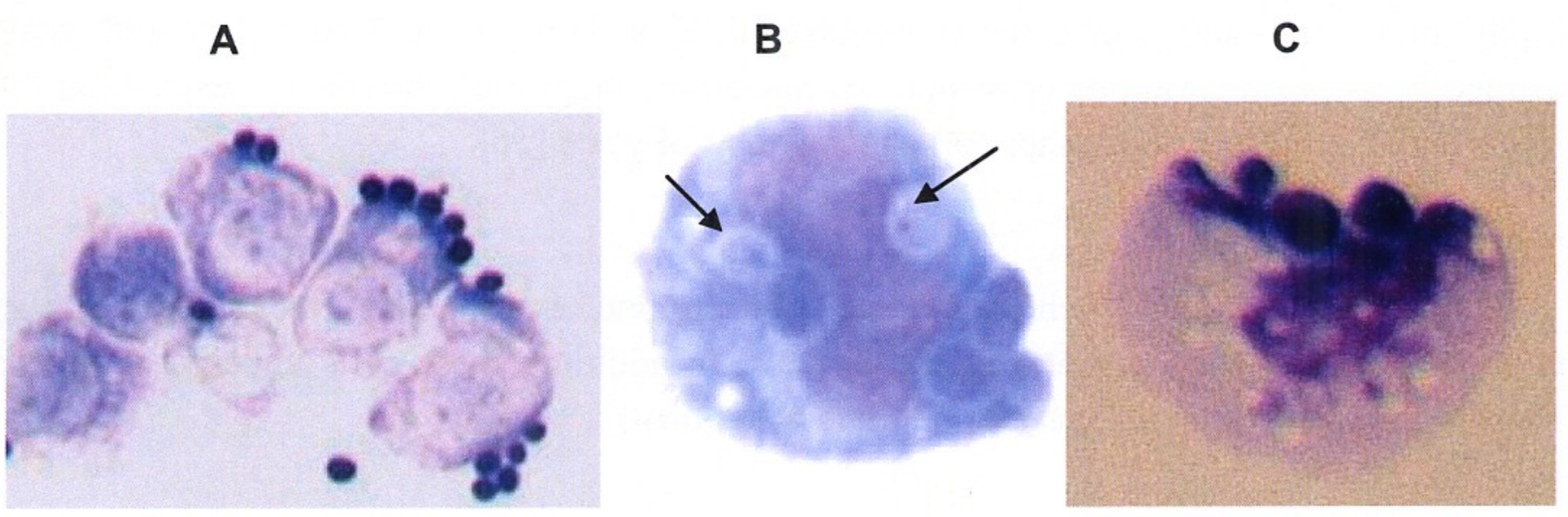

Figure 1. *In vitro* yeast- induces apoptosis in breast cancer cell. Preparation showing MCF-7 cells attached to yeast (dark blue) examined at 10 min post-culture of cancer cell with yeast (a), followed by complete phagocytosis of yeast by MCF-7 cells at 2 hr post culture of MCF-7 cells with yeast. (b). Notice, cancer cell have digested 2 yeast (←).Finally cancer cell underwent apoptosis with fragmented nucleus. Figure 1A Giemsa x 400, B&C x 740.

These studies [55,57-59] revealed a striking difference between levels of phagocytosis and yeast-induced apoptosis in MCF-7 cells in suspension compared to monolayer. Cells in suspension, prepared from trypsinization, demonstrated greater magnitudes of phagocytosis and apoptosis as compared to monolayer. Trypsin stimulates the integrin $\alpha_5\beta_1$-dependent adhesion of human gastric carcinoma cells [60]. $\alpha_M\beta_2$ integrin receptors on myeloid cells mediate the phagocytosis of diverse ligands including cooled platelets [61,62] and non-opsonized pathogens [63]. Since MCF-7 cells carry $\alpha_M\beta_2$ integrin receptors [64,65], it is possible that trypsin stimulates integrin receptors of MCF-7 cells that mediate phagocytosis of *S. cerevisiae.*

Tongue Cancer

Squamous cell carcinoma (SCCA) of the tongue is one of the most common malignant tumors of oral cavity. Oral SCC-4 cells were cultured with *S. cerevisiae* and signs of apoptosis including nuclear fragmentation and membrane blebbing were detected in cytospin preparations stained with Giemsa. Flow cytometry analysis indicated a significant decrease in SCCA survival post-culture with yeast: 21% for SCC-4 as compared with 6% background. The addition of a known caspase inhibitor during SCC-4 co-culture with yeast resulted in the suppression of yeast-induced apoptosis, suggesting the role of caspases in the triggering of apoptosis post tumor cell phagocytosis of yeast [56].

Colon Cancer

Adenocarcinoma (ADENOCA) of the colon and rectum are the most common gastrointestinal (GI) neoplasmas, with an incidence of approximately 44 per 100,000 per year [66,67], and is the second leading cause of cancer death among adult Americans. ADENOCA cell line Caco-2 cells incubated with *S. cerevisiae* showed signs of apoptosis following phagocytosis of yeast. The level of apoptosis continued to increase post co-culture with yeast to a maximum of 76.2% at 4 h. Flow cytometry analysis indicated a significant decrease in Caco-2 cells survival post culture with yeast: 21% for Caco-2 cells as compared with 6% background. The addition of a known caspase inhibitor during Caco-2 co-culture with yeast resulted in the suppression of yeast-induced apoptosis, suggesting the role of caspases in the triggering of apoptosis post tumor cell phagocytosis of yeast [56].

Other Cancers

Other types of cancer such as prostate (LNCap) and erythroleukemic cells (K562) also have been shown to be potent phagocytic cells. In addition, these cells undergo apoptosis following phagocytosis of *S. cerevisiae* (data not shown).

5- Yeast, But Not Other Fungi Induces Apoptosis in Cancer Cells

Further study [68] was undertaken to examine the apoptotic effect of different strains of fungi in breast cancer MCF-7 cells. Eight strains of yeast at different developmental stages were used. These include small yeast cells: *Candida albicans, Candida glabrata, Saccharomyces cerevisiae, and Yarrowia lipolytica;* large yeast cells*: Cryptococcus neoformans* and *Rhodotorula rubra;* and pseudohyphae: *Candida kefyr* and *Candida krusei.* Conidia from four strains of fungal mycelia: *Aspergillus, Aspergillus species, Trichophyton rubrum*, and *Trichophyton tonsurans* were also used. Results showed that phagocytosis and fungi-induced apoptosis of cancer cells was yeast specific and followed a graduated manner in which *Y. lipolytica* had the lowest level of apoptotic effect. In contrast, MCF-7 did not phagocytize or undergo apoptosis post-culture with conidia. Causal factors responsible for the distinguished difference in MCF-7 cell phagocytic ability of conidia compared to yeast could be attributed to differential exposure of cell surface components, such as β-glucan, involved in recognition [69-71].

6- Yeast Does Not Induce Apoptosis in Non-malignant Normal 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-tumorgenic MCF-10A cells derived from human fibrocystic mammary tissue [72]. In contrast, MCF-7 cells from human breast adenocarcinoma have been shown to be tumorigenic in athymic nude mice [73-75]. Monolayers of both MCF-7 and MCF-10A cells were cultured with *S. cerevisiae*. MCF-7 cells phagocytized yeast in a time-dependent manner. On the other hand, there was virtually no phagocytosis of yeast by MCF-10A cells. Similarly, MCF-7 cells

undergo apoptosis while virtually no yeast-induced apoptosis was observed in MCF-10A cells [58]. This demonstrates that monolayer 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 in monolayer. It is of interest to note that trypsin is able to expose these surface receptors. An example of this belief is based on the following observations: 1) MCF-7 cancer cells in suspension, prepared from trypsinization, demonstrated greater magnitudes of phagocytosis and apoptosis as compared to monolayer; and 2) monolayer normal MCF-10A cells showed virtually no phagocytosis of yeast, however MCF-10A cells in suspension do phagocytize yeast (data not shown).

In Vivo Studies Examining the Anti-Cancer Effects of *S. cerevisiae* Using Athymic Nude Mice Model

a) *Tumor Transplantation.* Twenty nude mice (4-5 weeks old) were singularly injected subcutaneously (sc) into the right axillary region with human BCC (MCF-7 cells). A dose of 5 x 10^6 tumor cells/mouse in 0.1ml serum-free RPMI-1640 with matrigel/mouse was applied. Eighty percent of the injected mice developed palpable tumors (50-100 mm) (Figure 2). Mice were weekly injected intratumorally for 45 days with either heat-killed S. cerevisiae (100 µl containing $1x10^8$ yeast) or with HankS' balance solutions (100 µl) as a control. Mice were sacrificed post-final injection, their tumors removed, and levels of BCC apoptosis was determined by electron and light microscopy.

Figure 2.

[b] *Histopathological Changes.* Results showed significantly increased cancer cell apoptosis in yeast treated mice. No noticeable adverse side effects from the yeast treatment were observed; all animals displayed normal feeding/drinking and life

activity patterns. Tumor treated with yeast on a weekly basis for 45 days revealed that tumor cells phagocytized yeast and subsequently underwent apoptosis and fibrosis (79%) *in vivo*. Conversely, saline-injected control mice showed negligible amounts of tumor cell apoptosis. Ultra structural analysis of cancer cells *in vivo* using Electron microscopy revealed phagocytosis of yeast by cancer cells. In addition, cancer cells showed signs of apoptosis such as chromatin condensation, nuclear segregation, convolution and fragmentation (Figure 3).

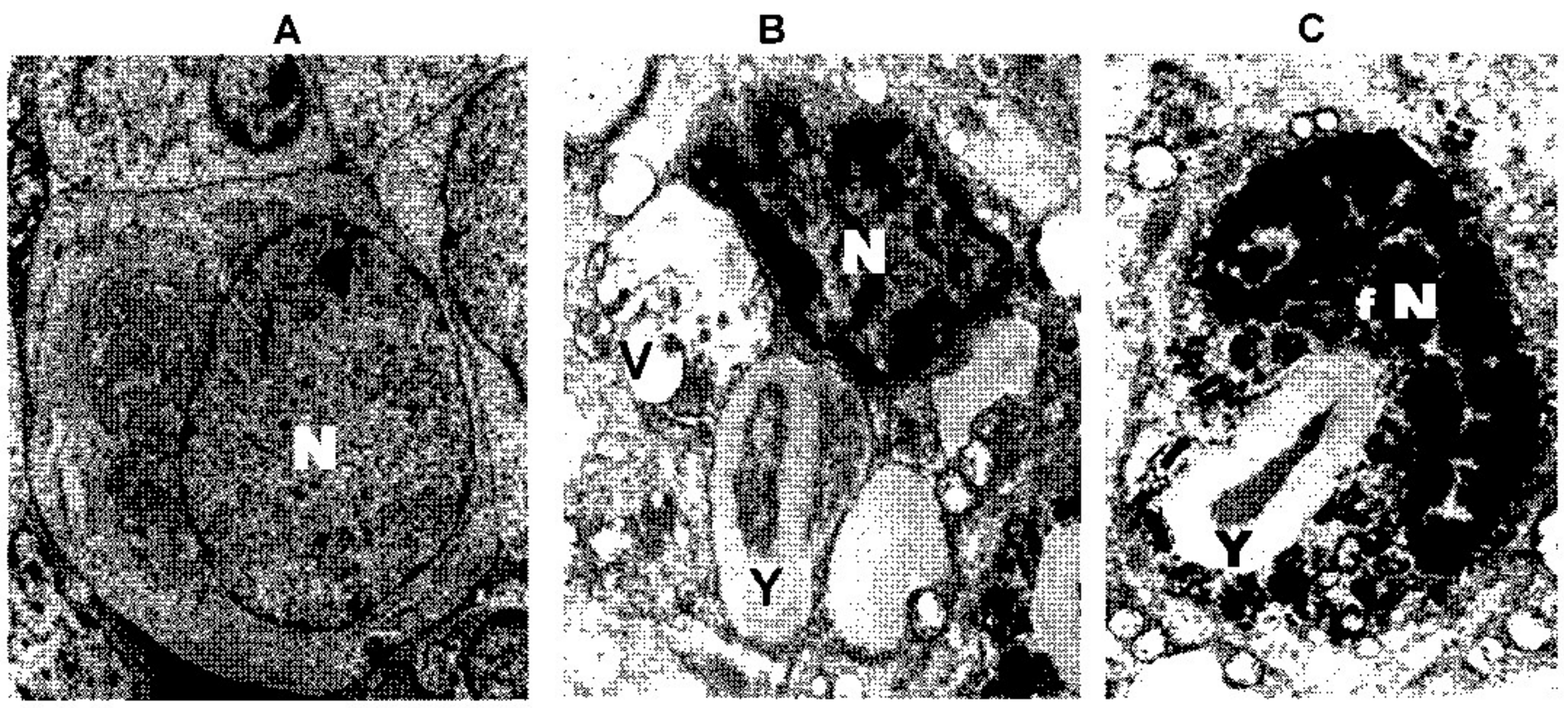

Figure 3. *In vivo* EM preparation showing yeast (Y) induces apoptosis of malignant cells. A- shows control cancer cells with no signs of apoptosis in the nucleus (N) or the cytoplasm. B- shows MCF-7 cell with the early apoptosis with margination and condensation of the nucleus (N). Notice presence of vacuoles (V), and C- shows MCF-7 cell displays the next stage of apoptosis with nuclear segregation, convolution and fragmentation (fN) including numerous residual bodies (A -C x3, 500 TEM).

The Mechanisms of Apoptosis

The mechanisms of apoptosis of BCCs post culture with yeast were directed towards investigating the important role of reactive oxygen intermediates (ROI) as well as the role of caspases. Phagocytosis is associated with the disruption of mitochondrial membrane potential and activation of initiator and effector caspases 8, 9 and 3. However, inhibitors of these caspases did not inhibit yeast-induced apoptosis in cancer cells, suggesting that yeast induces apoptosis in breast cancer cells by a mechanism that is independent of caspase activation [57]. Similar reports with apoptosis of other cells was reported, such as B cells [76] and fibroblast cell lines [77]. A number of potential mediators of caspase-independent cell death have recently been identified; these include mitochondrial proteins AIF, an NADH oxidoreductase, endonuclease G, a mitochondrial DNA repair enzyme and HtrA2/Omi, a serine protease [76-82]. In response to apoptotic stimuli, these mediators are released from mitochondria and transfer death signals to the nucleus in a caspase-independent manner. In the present study, we showed that phagocytosis led to disruption of mitochondrial membrane potential. It is possible that the release of some or all of the above-mentioned mediators might have led to the apoptosis of BCCs. While we have shown that caspases may be involved in

yeast-induced apoptosis of colonic adenocarcinoma calls [56]. Apoptotic effects by yeast are associated with tumor cells, but the mechanism is unclear. The precise mechanisms by which yeast induces apoptosis in cancer cells need to be further investigated.

MGN-3

MGN-3/Biobran is a denatured hemicellulose that is obtained by reacting rice bran hemicellulose with multiple carbohydrate hydrolyzing enzymes from the Shiitake mushrooms [83]. 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 (Figure 4). MGN-3 is a biological response modifier (BRM) that has the ability to boost the activity of different arms of immune system. MGN-3 is a potent activator of natural killer (NK) cells in mice [84], immune-compromised humans [85] and cancer patients [86] *in vivo* . The mechanisms by which MGN-3- induce NK activation were shown to involve increasing the levels of both TNF-alpha and IFN-gamma secretions and also elevating the expression of key cell surface receptors such as CD69, an early activation antigen, the interleukin-2 receptor CD25 and the adhesion molecule ICAM-1 (CD54) [87]. Human ingestion of MGN-3 also resulted in a significant increase in T and B cell mitogen response at 2 months after treatment [83]. Further studies showed that MGN-3 augments macrophage phagocytosis [88]. In addition to immune modulatory activity, we show that MGN-3 enhances yeast-induced apoptosis of MCF-7 cells [58,59]. BCCs treated with MGN-3 exhibited an increased percentage of attachment (200%) and uptake of yeast by MCF-7 (313%). MGN-3 also increased apoptosis of BCCs in a dose-dependent manner and was associated with increased activation of caspases 8 and 9 in MCF-7 cells and caspases 9 and 3 in HCC70 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 [89]. 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.

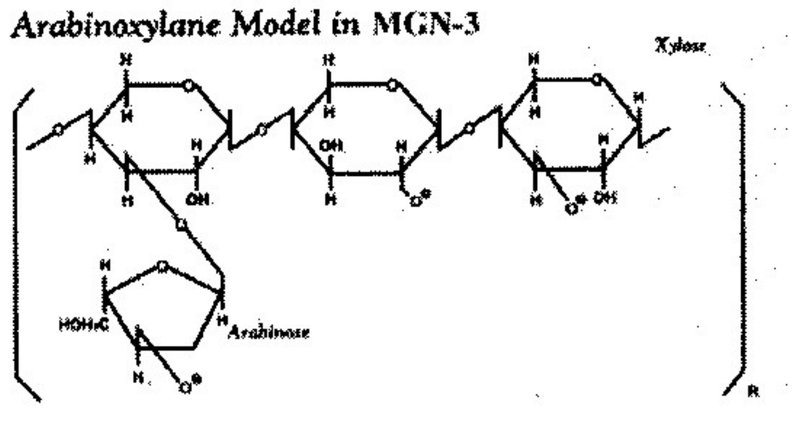

Figure 4.

CONCLUSION

The National Cancer Institute strongly encourages the exploration of cancer treatments with greater specificity for cancer cells and less toxicity for normal tissue. In the present study, we introduced a novel approach to BCC therapy using non-pathogenic yeast, *S. cerevisiae*. Data showed that human BCC undergo apoptosis following phagocytosis of yeast. On the other hand, yeast has no toxicity against normal breast epithelial cells. Our studies will focus on optimizing the efficacy of yeast or yeast cell wall (YCW) component(s). We are attempting to increase the uptake of yeast by the tumor-bearing nude mice using a treatment protocol that examines frequency- and dose-responsiveness, and peak tumor stage sensitivity. We selected MCF-7 because they are a model cell line for the establishment of treatment efficacy prior to patient use, and they undergo yeast-mediated apoptosis both *in vitro and in vivo*.

Results of this study will potentially lead to the development of a novel anti-cancer agent that is safe for human consumption. We propose to introduce *S. cerevisiae* as a treatment for human cancers via IT/IV administration of yeast/YCW components. Both drug delivery methods have been successfully administered for patients. IT administration of ethanol for hepatocellular carcinoma [90,91] and scFv(FRP5)-ETA for metastatic breast cancer [92] have been applied. Additionally, IV administration of β-glucans derived from *S. cerevisiae* in regards to immunomodulation have been applied in patients for postoperative infections [93], or from paracoccidioidomycosis [94]. It is viewed by the human population to be non-toxic. *S. cerevisiae*, a necessary component for the production of fermented foods such as bread and beer, is sold as food supplements for human consumption for constipation relief [95,96]. Moreover, *S. cerevisiae* has been employed as a vehicle for the delivery of chemo-preventative agents in clinical trials of prostate cancer [97,98]. *S. cerevisiae* provides an advantage over many anticancer drugs since it is a universal agent that has been found to be potent against several cancer types.

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