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Human Squamous Cell Carcinoma of the Tongue and Colon Undergoes Apoptosis upon Phagocytosis of *Saccharomyces Cerevisiae*, the Baker's Yeast, *In Vitro*

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Abstract. *The present study was carried out to evaluate the effect of phagocytosis of killed yeast on the induction of apoptosis in two human solid tumors of the gastrointestinal (GI): the squamous cell carcinomas of the tongue (SCCA) are (SCC -4 and SCC-9) and the adenocarcinomas of the colon (ADENOCA) are (Caco-2 and DLD-1). Cancer cells were cultured with heat killed Saccharomyces cerevisiae, baker's and brewer's yeast, at ratio of yeast to cancer cells = 10:1. The percentage of tumor cells that had attached/phagocytosed yeast and oxidative burst was determined by using oxidative sensitive dye (DCFH-DA) and flow cytometry. SCC-4 and colon Caco-2 cells demonstrated initial high levels of phagocytosis that peaked (35.8-52.8%) at 2 hr. The oral SCC-9 and colon DLD-1 cells demonstrated low phagocytic activity (7-12%). Phagocytosis was not associated with oxidative burst. Upon phagocytosis of yeast, cancer cells underwent apoptosis that was maximized at 4 hr. Yeast-induced apoptosis was significant in SCC-4 (45%), as compared with SCC-9 cells (17%), and Caco-2 (76%), as compared with DLD cells (12%). Apoptosis in cancer cells was inhibited by caspase inhibitor, Z-VAD-FMK; this suggests that caspases may be involved in apoptosis of the GI cancers. This data may have clinical implications for the treatment of solid tumors.*

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Squamous cell carcinoma (SCCA) of the tongue is one of the most common malignant tumors of oral cavity. Surgical therapy alone, or in combination with chemotherapy, or radiation therapy are the mainstay of treatment for SCCA of the tongue. By year 2000, there were 70,000 newly diagnosed patients with head and neck cancer in the US. The 5-year survival rate approximates 46% annually and has not dramatically changed in the last 40 years (2). The high mortality from oral cancer occurs, despite improvements and advances in treatment modalities, diagnosis (3, 4), and in the increased knowledge of the biological characteristics of oral cancers (5).

Cancers of the colon and rectum (ADENOCA) are the most common gastrointestinal (GI) neoplasmas, with an incidence of approximately 44 per 100,000 per year (6, 7), and is the second leading cause of cancer death among adult Americans. Specific genetic defects have been identified which cause hereditary colon cancers in humans and account for about 5% of all cases. In addition, a number of intestinal luminal risk factors for colon cancer have been described. The first-line treatment is radical surgical resection, with local lymph node dissection. In advanced stages of the disease, the effect of palliative chemotherapy is limited (8, 9).

The development and search for more effective treatment modalities, and/or adjuvant therapies to treat early and advanced stages of the disease, have now become an important subject of research. Several reports suggest that extracts from Scutellaria baicalensis (a traditional Chinese herbal medicine) (10), a vegetable, Anastasia Red (11), garlic (12), ginger (13) and kiwifruit (14), selectively and effectively inhibits cancer cell growth *in vitro* and *in vivo*.

The phenomenon of induction of apoptosis after phagocytosis of certain microorganisms may offer an effective and alternative way for the treatment of solid

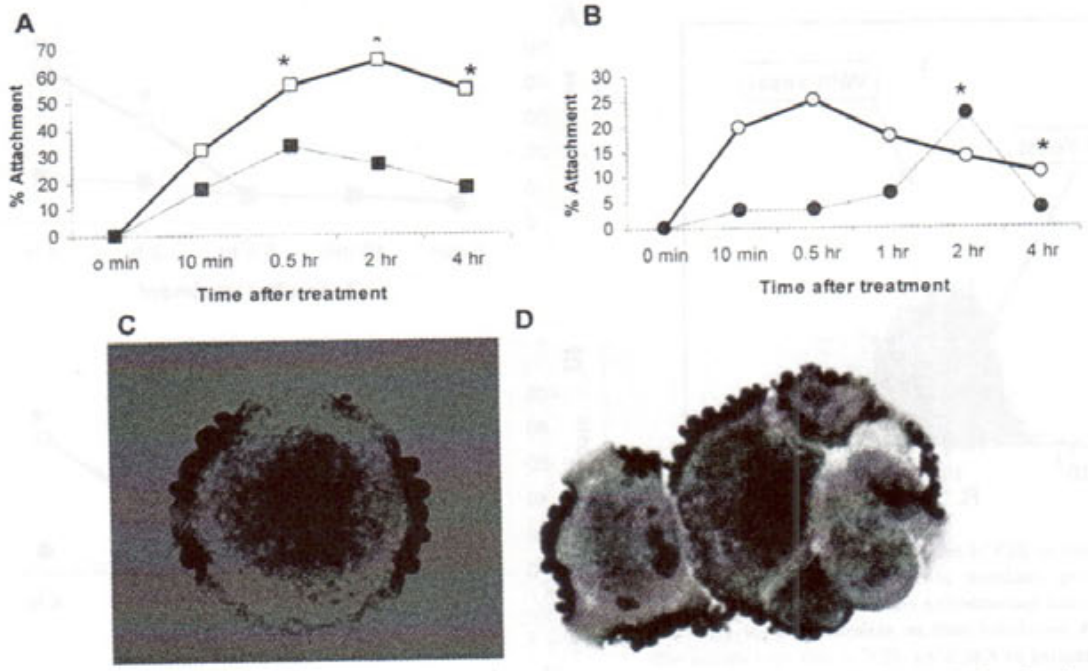

Figure 1. *Percentage of attachment of oral and colon cancer cells to yeast. (A) oral SCC-4 (□) and SCC-9 cells (■) and (B) colon Caco-2 (○) and DLD cells (●) were cultured with yeast and percentages of attachment were calculated at 10 min, 0.5, 2 , and 4 hr post culture cancer cells with yeast. Data represent the mean of 3 different experiments. *p<0.01. As compared with the corresponding cells at 10 min. (C) Cytocentrifuge preparations showing increased number of yeast attached to SCC-4 cell and Caco-2 cells (D) at .5 hr. Giemsa x 740.*

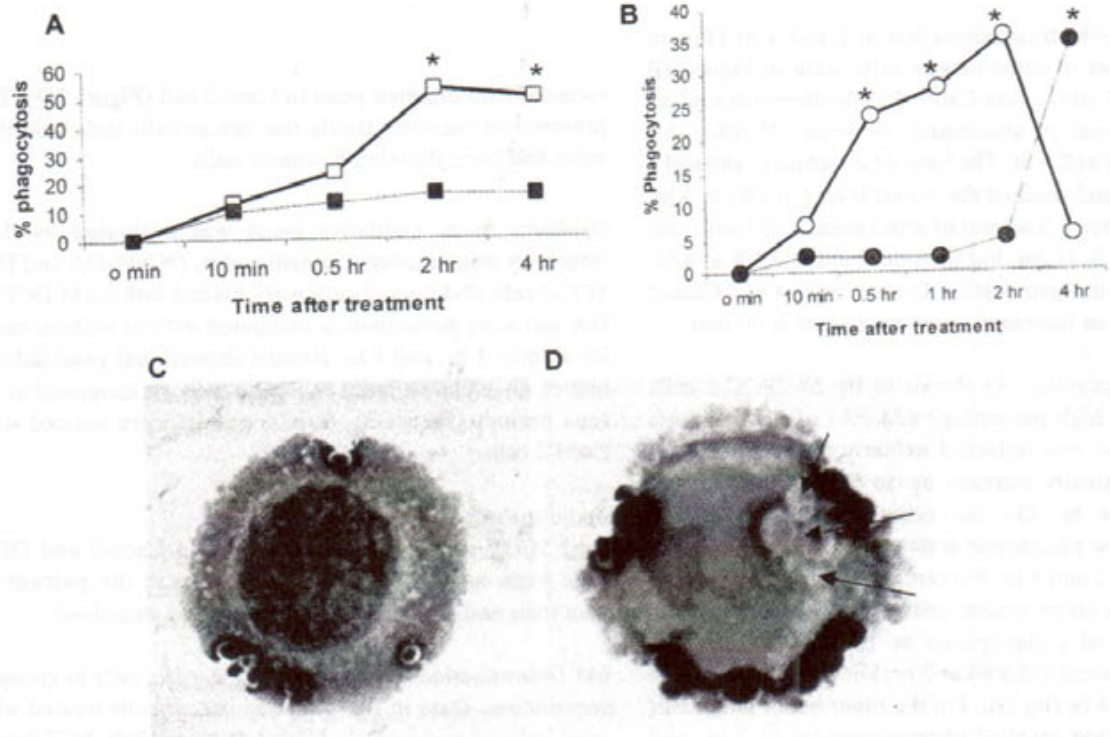

Figure 2. *Percentage of phagocytosis of yeast by oral and colon cancer cells. (A) oral SCC-4 (□) and SCC-9 cells (■) and (B) colon Caco-2 (○) and DLD cells (●) were cultured with yeast and percentages of phagocytosis were calculated at 10 min, 0.5, 2, and 4 hr post culture cancer cells with yeast. Data represent the mean of 3 different experiments. *p<0.01. (C) Cytocentrifuge preparation showing phagocytosis of yeast by SCC-4 cell and Caco-2 cell (D) at 2hr. The presence of vacuoles (arrows) inside the cancer cells indicated that yeast had been digested by tumor cells. Giemsa x 740.*

be involved. Alternatively it is possible that caspase involvement in yeast-induced apoptosis may be tumor cell specific. Further studies are needed to clarify the role of caspases in yeast-induced apoptosis.

Results of these studies may lead to a new therapeutic strategy for the treatment of GI cancer. The translational potential of this study needs to be demonstrated in bio-therapeutic strategy.

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showed a low level of apoptic cells at 10 min; this steadily increased and maximized to 76.2% at 4 hr. In contrast, DLD cells demonstrated an initial low level of apoptosis at 10 min; it remained low through 4 hr. (Figure 4B).

We also examined the apoptic cancer cells morphologically in cytospin preparations. Cell shrinkage, membrane blebbing and chromatin condensation are the criteria used to identify the apoptic cells. Apoptosis maximized at 4 hr post culture of yeast with cancer cells. SCC-4 cells exhibited an increased phagocytic activity against yeast; this was followed by the gradual demise of cancer cells, starting with early chromatin condensation (Figure 5 A), followed by nuclear fragmentation (B&C) and membrane blebbing (D). Preparation also showed cells with severe nuclear condensation, which was located in an eccentric position (E). Notice the yeast inside apoptic SCC-4 cells. Finally, the death of the tumor cells, as confirmed by the trypan blue exclusion (F).

(B) Determination of percentages of dead cells by flow cytometry. Cancer cell survival was determined by flow cytometry using the propidium iodide (PI) technique. Results of cancer cell survival shows that the co-culture of cancer cells with yeast for 2 hr caused a significant decrease in SCCA survival: 21% for SCC-4 cells as compared with 6% of background. On the other hand, SCC-9 cells demonstrated no increase in the percentage of apoptic cells, post culture with yeast, as compared with the background of cancer cells (5%) (Figure 6). Similar results were noticed with colon Caco-2 and DLD cells

(C) The action of caspase inhibitor. To determine whether or not caspases are involved in yeast-induced apoptosis, SSC-4 and Caco-2 cells were incubated with Z-VAd-FMK, a known inhibitor of caspases; they subsequently were allowed to phagocytize yeast. As observed in Figure 7, the data shows that Z-VAD-FMK suppressed yeast-induced apoptosis in SCC-4 cells. Similar results were obtained with the Caco-2 cells. This suggested that caspases may be involved in triggering apoptosis in these two cell lines.

Discussion

Induction of apoptosis in tumor cells is one approach to cure cancer. Many anticancer drugs function by inducing apoptosis in cancer cells (17-23). Specific intercellular damage induced by many therapeutic agents has been characterized and shown to involve Fas/FasL system (20), mitochondria (21), and DNA damage (22, 23). Oral cancer is one of the most disfiguring types of cancer, since the surgical removal of the tumor may result in facial distortion. Colon cancer has been characterized as one of the leading causes of cancer death among adults in many industrial countries. Therefore, identification of new reagents and their targets would present a therapeutic advantage. Our recent study had shown that human breast cancer cells (BCCs) possess high phagocytic activity against heat-killed Baker's yeast, *Saccharomyces cerevisiae in vitro* and that these cells undergo apoptosis upon phagocytosis of yeast (15). In this study, we examined whether GI cancer cells also can demonstrate a similar phenomenon. Data indicated that oral cancer SCC-4 and colon cancer Caco-2 cells are indeed phagocytic cells, as manifested by their high phagocytic activity against yeast *in vitro*. In addition, these tumor cells underwent apoptosis post phagocytosis of yeast.

Many tumor cells exhibit phagocytic activity, including epithelial carcinomas of the cervix (24, 25), adenocarcinoma (26, 27), dermatofibroma cells (28), lymphatic nonphagocytic tumor cells, mouse sarcoma L929 cells (29), and BCCs (30-32). Further studies demonstrated that hyperplastic and dysplastic gut epithelium phagocytized bacteria (33-35), thus indicating that this phenomenon occurs in the early stages of cell abnormality in the gut. In the present study we noted that SCC-4 and Caco-2 cells exhibit two different types of phagocytosis against yeast: a cytoplasmic invagination, and the extension of cytoplasmic folds around yeast which is followed by complete encirclement of yeast. Similar findings are also observed in BCCs (15). Co-culture of SCCA and ADENOCA cancer cells with yeast did not produce H_2O_2 and O_2- as indicated by flow cytometry analysis. We (32) and others (30) have recently reported the lack of an oxidative burst in breast MCF-7 cells following phagocytosis of yeast. These reports suggest that ingested material is digested by a pathway independent from oxidative burst.

It is of interest to note that both oral SCC-4 and colon Caco-2 tumor cells underwent apoptosis post phagocytosis of yeast. Many stimuli can induce apoptosis upon phagocytosis in phagocytic cells. These include staphylococcus aureus (36, 37), Escherichia coli (38), mycobacterium tuberculosis (39), and candida albicans (40). With respect to cancer, scientists have noted that many bacteria have the ability to reduce the growth rate or size of tumors. Around 1893, W. B. Coley observed tumor regression in patients infected with erysipelas bacteria (41, 42). The anticancer activity of many strains of bacteria has been reported: these include Mycobacterium bovis BCG for the treatment of bladder cancer (43-45), Salmonella for plasmacytoma and the melanoma-bearing mice (46-49), and the ability of Salmonella, Clostridia, and other anaerobic bacteria to cause tumor regression (50). Several mechanisms were proposed to analyze the antitumor effect of these bacteria such as immunomodulation and the inhibition of angiogenesis. Since the term "apoptosis" was not coined until recently, the possibility of the apoptotic effect against cancer cells by these microorganisms cannot be excluded.

Results of this study demonstrated oral SCC-4 and colon Caco-2 cells to be more responsive to apoptosis by yeast than SCC-9 and DLD cells. The results also showed non-metastatic Caco-2 cancer cells having higher levels of

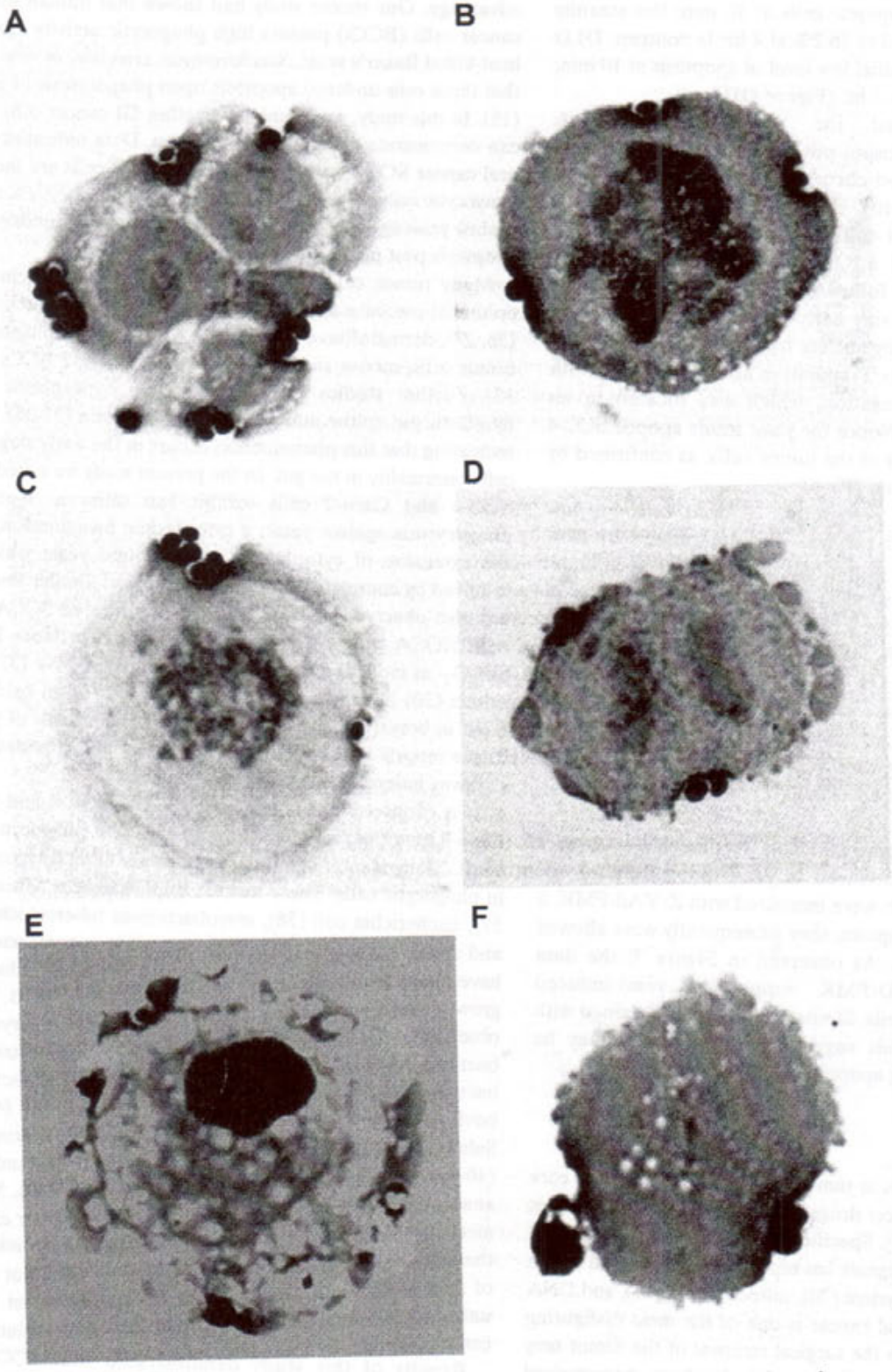

Figure 5. *Cytospin preparation of SCC-4 cells showing signs of apoptosis. (A) early chromatin condensation and the nucleus occupy about half of the cell. Nuclear fragmentation occurs (B&C) and membrane blebbing (D). Preparation also showed cells with severe nuclear condensation which was located in an eccentric position (E). Notice the presence of small and large vacuoles and the yeast inside apoptic SCC-4 cells. Finally, dead tumor cells stained with trypan blue (F). Giemsa x 740.*

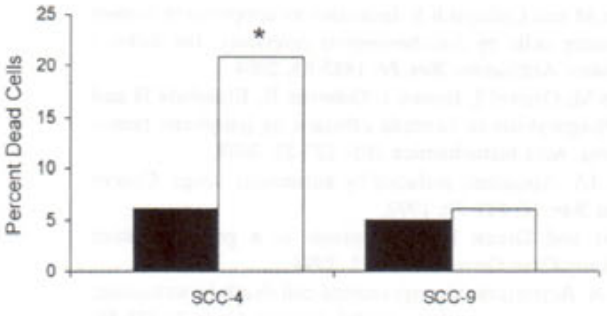

Figure 6.*Percentage of dead cancer cells post treatment with yeast as determined by flow cytometry. SCC-4 and SCC-9 cells were cultured with yeast in a ratio of 1:10 for 2 hr and cancer cell survival was determined by flow cytometry. Data represent the mean of 3 different experiments. *p<0.01. As compared with the background of cancer cells.*

attachment and phagocytosis than metastatic DLD-1 cancer cells. This suggests that the uptake of yeast by cancer cells may trigger apoptosis. A similar observation was noted with BCCs. *S. cerevisiae* induced higher apoptosis in BCCs (MCF-7, ZR-75-1, and HCC70) (15). This finding is in accordance with an earlier report by Glinsky *et al.* (51, 52). He found an increased resistance to apotosis among highly metastatic cancer cells as compared to their poorly metastatic counterparts.

The mechanism(s) by which yeast-induces apoptosis in SCC-4 and Caco-2 cells is not known. We hypothesize that attachment and/or phagocytosis of *S. cerevisiae* lead to activation of caspases and to the triggering of apoptosis in cancer cells. This view is supported by our finding that Z-VAD-FMK, a broad inhibitor of the family of caspases, inhibited yeast –induced apoptosis in Caco-2 and SCC-4 cell lines. Previously we reported that Caspase 3, 8, and 9 inhibitors failed to inhibit apoptosis in BCCs. Taken together, these findings suggest that additional caspases may

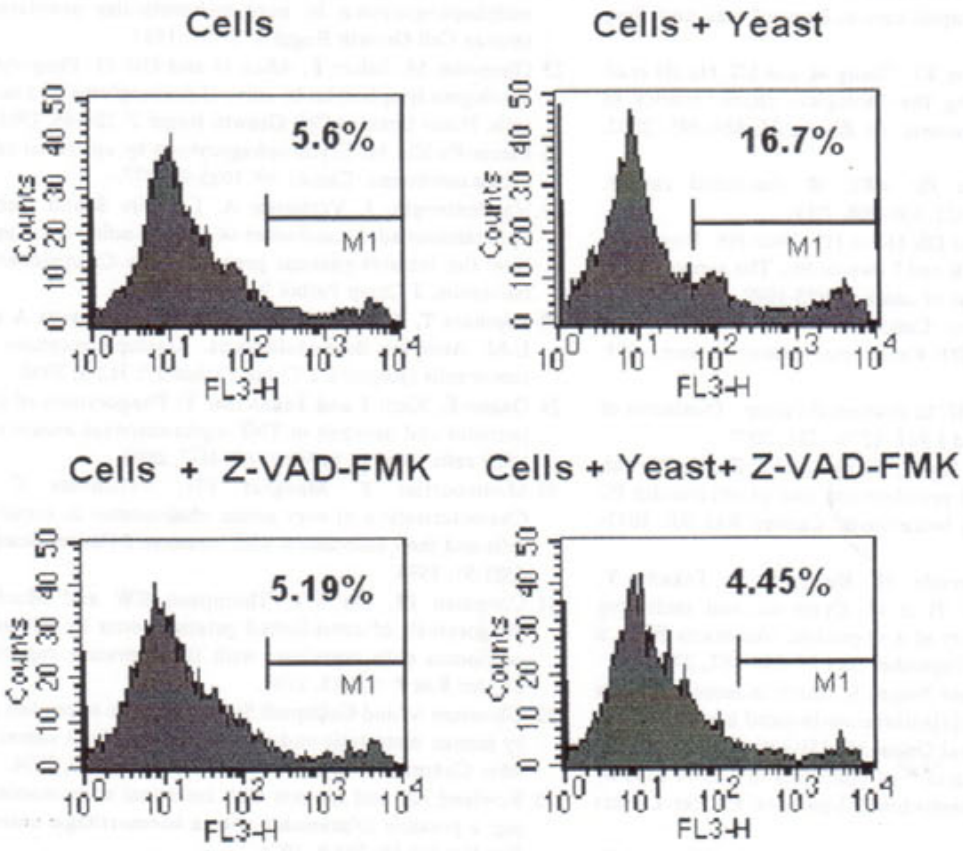

Figure 7. *The action of caspase inhibitor. SSC-4 cells were incubated with Z-VAd-FMK, a known inhibitor of caspases; they subsequently were allowed to phagocytize yeast. Intracellular active caspases were determined with casp glow caspases determination kit using FACScan. Fig. 7 represents a representative dot blot showing increased activation of caspases.*

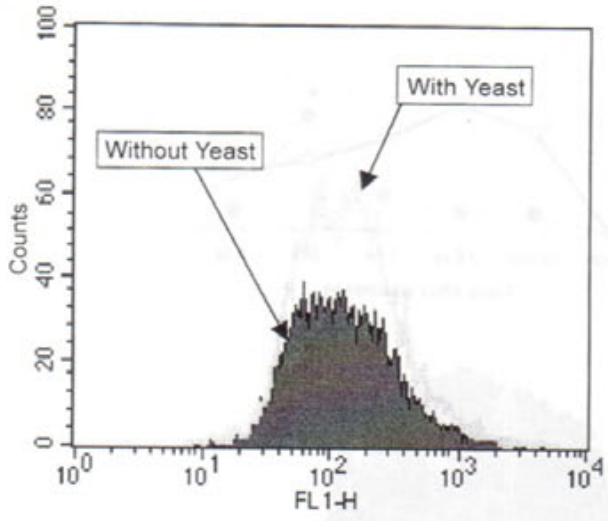

Figure 3. *Oxidative burst in SCC-4 cells. Oxidative burst was evaluated by flow cytometry using oxidative sensitive dye, dichlorofluorescein diacetate (DCFH-DA) and hydroethidine (HE). SCC-4 cells were loaded with 5 μM DCFH-DA, incubated with or without yeast for 1 hr, and oxidative burst was evaluated by FACScan. SCC-4 cells were loaded with HE and superoxide production was assessed by FACScan.*

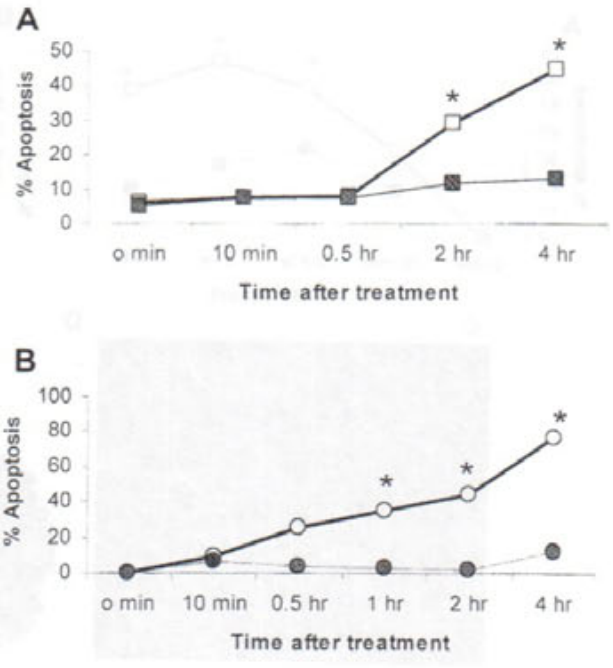

Figure 4. *Percentage of dead cancer cells as determined by cytospin preparation. (A) oral SCC-4 (□) and SCC-9 cells (■) and (B) colon Caco-2 (○) and DLD cells (●) were cultured with yeast in a ratio of 1:10 and cancer cell survival was determined at .5, 2 and 4 hr. *p<0.01.*

(33%) at 0.5 hr which remained low at 2 and 4 hr (Figure 1A). With respect to colon cancer cells, data in Figure 1B shows that at 10 min, colon Caco -2 cells demonstrated an initial high percent of attachment to yeast (19.6%); this increased to 25% at 0.5 hr. The level of attachment showed a steady decrease and reached the lowest level (10.3%) at 4 hr. DLD cells exhibited a low level of attachment at 0.5 and 1 hr, and reached 22.1% at 2hr, but then declined to 3.3% at 4 hr. The illustrations in Figures 1C & D show SCC-4 and Caco-2 cells attached to an increased number of yeast at 30 min.

Kinetics of phagocytosis. As shown in fig 2A, SCC-4 cells demonstrated a high percentage (23.7%) of phagocytosis against yeast that was detected as early as 0.5 hr. SCC-4 cells showed a steady increase up to 52.8% at 2 hr, and maintained at 4 hr. On the other hand, SCC-9 cells demonstrated low phagocytic activity (13%) at 0.5 hr; this remained low at 2 and 4 hr. Percentage of phagocytosis was also examined in colon cancer cells. Caco-2 cells showed a low percentage of phagocytosis at 10 min (7.3%); this increased and peaked (35.8%) at 2 hr, but then declined and reached 5.5% at 4 hr (fig 2B). On the other hand, DLD cells demonstrated a low level of phagocytosis up to 2 hr, and then increased to 34.8% at 4 hr.

The illustration in figure 2C shows SCC-4 cell phagocytizing several yeast at 2 hr. Notice the presence of vacuoles with digested yeast in Caco-2 cell (Figure 2D). The presence of vacuoles inside the cancer cells indicated that yeast had been digested by tumor cells.

Oxidative burst. Oxidative burst was evaluated by flow cytometry using oxidative sensitive dye, DCFH-DA and HE. SCC-4 cells and Caco-2 cells were loaded with 5 μM DCFH-DA and were subsequently incubated with or without yeast for 15 min, 1 hr, and 3 hr. Results showed that yeast did not induce an oxidative burst in SCC-4 cells, as examined at all time periods (Figure 3). Similar results were noticed with Caco-2 cells.

Apoptosis studies

Oral SCC-4 and SCC-9 cells and colon Caco-2 and DLD cells were cultured with *S. cerevisiae*, and the percent of apoptosis and activation of caspases were examined.

(A) Determination of percentages of apoptic cells in cytospin preparations. Data in fig 4A shows cancer cells treated with yeast induced apoptosis at different time periods. SCC-4 cells showed 30% apoptic cells at 2 hr and maximized to 45% at 4 hr. On the other hand, SCC-9 cells showed only 17% apoptic cells at 4 hr. Regarding colon cancer, Caco-2 cells

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tumors. We have recently demonstrated that phagocytosis of heat-killed *S. cerevisiae* by breast cancer cells (BCCs) induces apoptosis in cancer cell lines MCF-7, ZR-75-1 and HCC70 (15). The present study was undertaken to examine whether SCCA of the tongue and ADENOCA of the colon phagocytize yeast, and whether they undergo apoptosis post phagocytizing yeast. This data may have therapeutic implications for the treatment of solid cancer.

Materials and Methods

Tumor cell lines. Five human tumor cell lines were used in the present study. These included two human oral cancer cell lines: SCC-4 and SCC-9; two human colon cancer cell lines: Caco-2 and DLD-1 cells; and a human macrophage cell line (U973) were also used. All cell lines were purchased from American Tissue and Culture Collection (ATCC), Manassas, VA. Oral tumor cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine, and 100 μg/ml streptomycin and penicillin. Colon cancer cells were cultured in Eagles MEM with Earles Salts supplemented with, non-essential amino acids, L-glutamine 2 mM and sodium pyruvate (1 mM) in the presence of 10% FBS. All cells were routinely maintained in log phase in a humidified incubator at 37°C with 5% CO_2.

Preparation of S. cerevisiae. Commercially available Baker's and brewer's yeast, *S. cerevisiae*, was used. Yeast suspensions were washed once with phosphate-buffered saline (PBS) and incubated for 1 hr at 90°C to kill yeast. Following washing, yeast cells were quantified using a hemocytometer and cell suspensions were adjusted at 1 x 10^7 cells/ml.

Phagocytic assay. Phagocytosis was assessed by cytospin preparation and flow cytometry. Phagocytic assay using cytospin preparations was done as previously described with slight modifications (16). In brief, yeast was mixed with tumor cells at a 10:1 ratio (yeast to tumor cell). For this purpose, a 0.5 ml tumor cell suspension in CM containing 1 x 10^6 cells/ml was mixed with 0.5 ml yeast suspension containing 1 x 10^7 organisms/ml. The mixtures were centrifuged in capped plastic tubes (16 x 100 mm; Falcon Plastic, Los Angeles CA, USA) for 5 min at 50 xg, and incubated at 37°C and 5% CO_2. After 10 min - 4 hr incubation, the mixtures were thoroughly re-suspended to detach loosely attached yeast from tumor cells. Cell suspensions (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 and were examined for the levels of attachment and uptake of yeast by tumor cells using oil immersion and a light microscope fitted with a 100x objective (Nikon, Tokyo, Japan).

Assessment of attachment and uptake of yeast by tumor cells. Assessment of attachment of yeast by tumor cells was calculated as the percentage of 500 tumor cells that attached to one or more yeast. The assessment of uptake of yeast by tumor cells was calculated as the percentage of 500 tumor cells that ingested one or more yeast.

Oxidative burst. Reactive oxygen species was determined by utilizing oxidation-dependent fluorescence of dye 2'7'-dichlorofluorescein (DCFH) and/or hydroethidine (HE). DCFH and HE are nonfluorescent, but become highly fluorescent when oxidized by hydrogen peroxide and superoxide anions, respectively, which are produced during oxidative burst in phagocytic cells. SCC-4 cells and Caco-2 cells were incubated with cell permeable DCFH-DA (dichlorofluorescein-diacettate 5 μM, Molecular probes, Eugene OR) or HE (1 μM) at 37°C. After 20 min incubation, cells were trypsinized and were cultured with or without yeast for an additional 15 min, 1 hr, and 3 hr at 37°C. Cells were transferred on ice and analyzed on FACScan using Cell Quest Software; statistical analysis was performed by the Kolmogorov-Smirnov test, also using Cell Quest Software System (Becton-Dickinson, Menlo Park, CA, USA). A D value of >0.2 is considered statistically significant.

Apoptosis studies

(A) Detection of cancer cell viability using flow cytometry. Flow cytometry analysis was used to examine the percentage of dead cancer cells. Cancer cells were cultured in the presence or absence of yeast cells at ratio of 1:10 and the percentage of dead cancer cells was examined by propidium iodide technique. Briefly propidium iodide (PI) was added to cells(1x10^6/ml) to give a final PI concentration of (5 μg/ml); cells were stained for 30 min at room temperature in the dark and analyzed by FACScan (Becton Dickinson, San Jose, CA, USA).

(B) Detection of apoptic cancer cells by morphological analysis. Apoptosis is morphologically defined by cell shrinkage, membrane blebbing and chromatin condensation. These criteria were used to identify the apoptic cancer cells in cytospin preparations stained with Giemsa.

(C)Effect of caspase inhibitor on yeast-induced apoptosis. To study a role of the caspase in apoptosis, 1 x 10^6 cells of SCC-4 and Caco-2 were cultured with two different concentrations (1 and 4 μM) of the caspase inhibitor (Z-VAD-FMK, BioVision, Palo Alto, CA, USA). At 30 min, cells were washed twice with HBSS and cultured with yeast at the ratio of 10:1 for 2 h. The percentage of dead cancer cells was examined by flow cytometry as described above.

Statistical analysis. Using student *t*-test, we tested the significance of difference in the percent changes of apoptic cancer cells post culture with yeast as compared to cancer cells alone. We also tested the significance of difference in the percent changes of attachment and phagocytosis of each cell line at 10 min- 4 hr, as compared to 10 min, as follows:

$$\frac{(4\text{ hr} - 10\text{ min})}{10\text{ min}} \times 100$$

Results

Percentage of attachment. Percentages of attachment were examined at different intervals, post culture of cancer cells with yeast. Oral SCC-4 cells demonstrate an initial high percent of attachment to yeast (56%); this was detected at 0.5 hr and remained at 2 and 4 hr. On the other hand, SCC-9 cells demonstrated a lower percentage of attachment of yeast