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Scientific paper

Keyhole limpet hemocyanin, a novel immune stimulant with promising anticancer activity in Barrett's esophageal adenocarcinoma

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Abstract

Background: Keyhole limpet hemocyanin (KLH) is a recently described immune stimulant and hapten carrier derived from a circulating glycoprotein of the marine mollusk *Megathura crenulata.* We previously reported that KLH has significant antiproliferative effects *in vitro* against breast, pancreas, and prostate cancers. We hypothesized that KLH would be effective against Barrett's esophageal adenocarcinoma in an *in vitro* model.

Methods: Barrett's esophageal adenocarcinoma cell lines (SEG-1 and BIC-1) were cultured using standard techniques. Cells were plated at 1×10^5 and KLH was added at concentrations ranging from 400ng to 100μg/well. After 24 and 72h incubation, cells were assayed for viability using the MTT technique. Statistical analysis was performed using ANOVA. Apoptosis was evaluated using a cell death detection kit after 16 hours of incubation with KLH.

Results: KLH treatment significantly ($p < 0.001$) reduced viability in a dose and time-dependent manner. Apoptosis was increased in treated SEG-1 cells, but no changes in apoptosis were seen in treated BIC-1 cells.

Conclusions: KLH directly inhibits the growth of human Barrett's esophageal cancer *in vitro* by apoptotic and nonapoptotic mechanisms.

Keywords: Keyhole limpet hemocyanin; Esophageal cancer; Barrett's esophagus; Apoptosis

Keyhole limpet hemocyanin (KLH) is a high molecular weight, copper-containing protein with significant antiproliferative effects in vitro against breast, pancreas, and prostate cancers [1]. KLH found in the hemolymph of the sea mollusk *Megathura crenulata* [2]. This extracellular respiratory protein has many immunostimulatory properties, including the ability to enhance the host's immune response by interacting with T cells, monocytes, macrophages, and polymorphonuclear lymphocytes [3]. Since its discovery, KLH has been used primarily as a carrier for vaccines and antigens and as adjuvant treatment in regimens such as antimicrobial therapy. However, progress has been slow over the last 2 decades to study its fundamental role and efficacy in the treatment of lethal cancers.

KLH has been tested in the treatment of superficial cancers of the bladder with noteworthy success. More than 30 years ago, Olsson et al [4] immunized patients with 5 mg of KLH and observed a marked reduction in the recurrence of superficial bladder cancer. Lamm et al [5] reported reduced tumor growth and prolonged survival in mice using the MBT-2 murine model of transitional cell carcinoma. KLH has been tested against mitomycin C chemotherapy in patients and was found to be superior in preventing bladder tumor recurrence with no adverse local or systemic side effects [6,7]. More recently, multicenter clinical trials have confirmed the efficacy of KLH given intravesically for 6 weeks to patients with various stages of bladder cancer [8]. Although KLH is regarded as a safe and highly effective immunotherapy for superficial bladder cancer, its efficacy in the treatment of epithelial derived adenocarcinomas is still unknown.

Adenocarcinoma of the esophagus is one of the most rapidly increasing cancer diagnoses in westernized society. First described in 1950, Barrett's esophagus occurs when the distal esophagus becomes partially lined with columnar epithelium of the intestinal metaplasia subtype. Its complications include stricture, ulceration, and most importantly, dysplasia and cancer [9]. The cancer risk of Barrett's esoph-

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agus is widely debated; even conservative investigators agree that a 20- to 40-fold increase in cancer risk exists [9,10]. About one third of patients with Barrett's esophagus present with malignancy, and most adenocarcinomas of the esophagus arise from Barrett's mucosa, an estimated 6,000 deaths per year in the United States. [9,11]. Devesa et al [12] evaluated incidence trends in the United States and noted a 350% increase in the incidence of esophageal adenocarcinoma between 1976 and 1994. Others have estimated an average annual increase in incidence of 20.6% for the United States, with even higher incidence rates currently noted for Great Britain, Australia, and the Netherlands [9–11]. Surgical and adjuvant therapies are available but are limited in efficacy in advanced disease or in debilitated patients. Until recently, an *in vitro* model of the disease was unavailable. Recently, Beers, et al [13] developed stable tissue cultures of Barrett's adenocarcinoma, allowing basic research into its growth and control.

We previously reported that KLH has significant antiproliferative effects *in vitro* against breast, pancreas, and prostate cancers [1]. However, the effects of KLH on the growth of esophageal adenocarcinoma have not been reported. We hypothesize that KLH might have beneficial effects in Barrett's-associated esophageal adenocarcinoma. Using appropriate doses, we have studied the effects of KLH on cell growth and apoptosis in an *in vitro* model of Barrett's adenocarcinoma.

Material and methods

Keyhole limpet hemocyanin

Keyhole limpet hemocyanin (KLH) was supplied as a lyophilized powder (Cal Bio Chem., La Jolla, California). KLH was resuspended to the desired assay concentrations in tissue culture medium for each cell line. The KLH concentrations tested were 400 ng, 800 ng, 1.6 μg, 3.1 μg, 6.3 μg, 13.0 μg, 25 μg, 50 μg, and 100 μg per well.

Cell culture

Human esophageal adenocarcinoma cells (BIC-1 and SEG-1) were derived from Barrett's-associated adenocarcinomas of the distal esophagus [13] and generously provided as a gift by David G. Beer (University of Michigan). Cells were maintained as monolayers in DMEM media with L-glutamine (Gibco In Vitrogen Corp, Carlbad, California), supplemented with 10% FBS (BioWhittaker, Walkerville, Maryland) and streptomycin (100 μg/mL), and maintained in humidified air at 5% CO_2 in a 37°C environment. For experimental procedures, cells were plated in a sterile 96-well microtiter plate at 1×10^5 cells per mL and incubated for 24 and 72 hours with KLH. An equal volume of media was added representing the controls.

MTT assay

The MTT colorimetric assay was performed to detect tumor cell viability after incubation. MTT, a tetrazolium dye (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, thiazolyl blue; SIGMA, St. Louis, Missouri) was added to the each well as described previously [14]. Plates were incubated in the presence of MTT dye for 4 hours. Mitochondrial dehydrogenase activity reduced the yellow MTT dye to a purple formazan, which was then solubilized with acidified isopropanol and absorbance was read at 570 nm on an enzyme-linked immunosorbent assay (ELISA) plate reader.

Cell death detection ELISA Plus

The Cell Death Detection ELISA (Roche Applied Science, Indianapolis, Indiana) was used to evaluate the presence of apoptosis and necrosis activity in the cells after incubation with KLH for a period of 16 hours. After treatment, the cells were lysed to release cytoplasmic histone-associated-DNA-fragments, an indicator of apoptosis. Cellular supernatants were collected for the measurement of cell lysis material, an indication of cell necrosis. Absorbance (ABS) was read at 405 nm. Higher ABS correlated with increased apoptosis. The data are reported as a percentage of the untreated control.

Statistical analysis

Determination of statistical significance was performed by analysis of variance (ANOVA). *Post hoc* comparison of individual concentration means with the control was completed using the Tukey-Kramer multiple comparison test [15]. All data are reported as means and standard errors.

Results

Cell proliferation

The effects of KLH on esophageal adenocarcinoma tumor cell growth were assessed using concentrations ranging from 400 ng to 100 μg per well after 24 and 72 hours. Both the BIC-1 and SEG-1 cell lines were significantly ($P < 0.001$) inhibited by KLH as compared with untreated controls at 72 hours as shown in Fig. 1 The BIC-1 cell line exhibited a mean growth inhibition of 28.5% $\pm$ 21.2% (range 3.9% to 71.3%, $P < 0.001$) at 72 hours. KLH concentrations of 100 μg, 50 μg, 25 μg, and 13 μg all exhibited significant ($P < 0.001$) antiproliferative effects when compared with untreated control. In addition, the SEG-1 cell line exhibited an average of 29.9 $\pm$ 15.6% growth inhibition at 72 hours (range 13.1% to 53.8%).

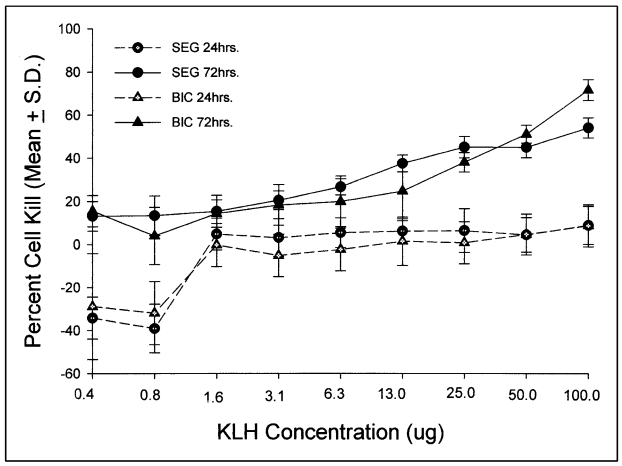

Fig. 1. Antiproliferative effects of keyhole limpet hemocyanin (KLH) *in vitro* on Barrett's esopohageal adenocarcinoma.

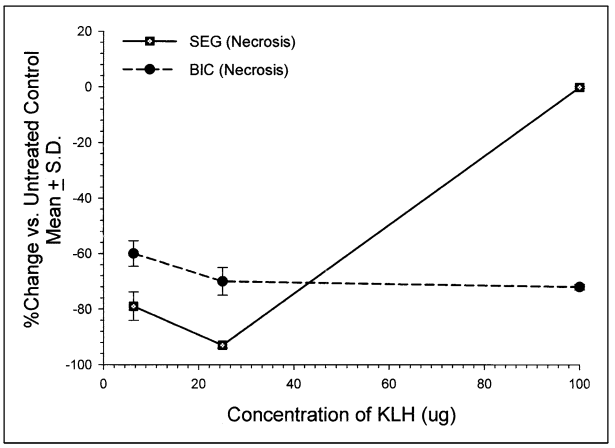

Fig. 3. Evaluation of the necrotic activity of Barrett's esophageal adenocarcinoma after treatment with keyhole limpet hemocyanin (KLH).

Evaluation of apoptosis and necrosis

A cell death ELISA assay was used to determine whether the significant decrease in cell growth observed after treatment with KLH was the result of enhanced apoptosis in Barrett's-associated adenocarcinoma cell lines. Fig. 2 shows the significant ($P < 0.001$) increases in apoptotic activity noted in the BIC-1 cells at a KLH dose of 100 μg per well, a 51.4% ± 9.8% increase compared with the untreated control was observed. However, no change in apoptotic activity was noted in the treated SEG-1 cells. Decreases in cellular necrosis were observed with both cell lines treated with KLH. In the BIC-1 cells, decreases in necrosis were observed at all doses compared to the untreated controls, ranging from 71.9% to 92%. Similar decreases in necrosis were seen in the treated SEG-1 cells at the lower dosages Fig. 3.

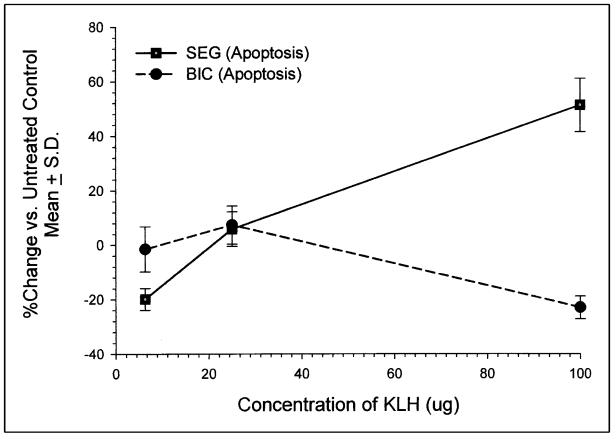

Fig. 2. Evaluation of the apoptotic activity of Barrett's esophageal adenocarcinoma after treatment with keyhole limpet hemocyanin (KLH).

Comments

Keyhole limpet hemocyanin is an alternative immunotherapy regimen for the treatment of cancers. We previously reported that KLH has significant antiproliferative effects *in vitro* against breast, pancreas, and prostate cancers [1]. These studies show that KLH may be useful in reducing the growth of esophageal cancer. We treated esophageal adenocarcinoma cells with increasing concentrations of KLH and evaluated mitochondrial respiration to determine if this would be an effective growth-inhibitory agent. The results of this study, although preliminary, show that KLH inhibits the proliferation of two esophageal cancer cell lines *in vitro* by an average of more than 29% ± 18% after 72 hours of exposure. Overall, the cell growth inhibition by KLH observed in this study equaled or surpassed response rates to drug therapy reported in documented clinical trials [6,7,16].

The tumor-suppressive effects of KLH may be mediated by alterations in apoptosis, a genetically regulated form of cell death. Reductions in apoptotic activity are a hallmark of many malignancies [17]. In addition, the apoptotic responses to DNA damage become more variable with age, thus contributing to the development of degenerative diseases. In particular, the susceptibility to cancer is increased owing to a decline in cell response to apoptotic cues such as the proapoptotic gene P53 [18]. For example, mutations of the p53 protein are common in Barrett's esophagus. Alterations in the tumor suppressor gene p53 may account for premalignant or malignant disease in patients with Barrett's esophagus [19]. In this study, we found a differential apoptotic response to KLH in our esophageal adenocarcinoma cells. The SEG-1 cells are negative for the p53 mutation. Correspondingly, we found an increase in apoptotic activity in this cell type when treated with KLH. The BIC-1 cells are positive for the p53 mutation, and no increase in apoptosis was noted, despite significant cell growth inhibition. That would suggest that the inhibitory effect of KLH might work

through different pathways depending on p53 status of the cell.

In addition to p53 status, the Barrett's-associated esophageal adenocarcinoma cells used in this study are known to differ in their baseline apoptotic rates and their ability to express cyclooxygenase (COX)-2. Souza et al [20] showed that SEG-1 cells express cyclooxygenase (COX)-2 abundantly and have a lower baseline rate of apoptosis. The same authors found that the BIC-1 cells do not express COX-2. The SEG-1 esophageal adenocarcinoma cells used in this study, showed a marked increase in apoptotic activity in response to KLH. Conceivably, this may explain the decrease in cell growth and increase in apoptotic activity to KLH by these cells and not the BIC-1 cells. In addition, we observed decreases in cellular necrosis with both cell lines treated with KLH, indicating the lack of cytotoxic effects even at the highest concentrations.

One of the most remarkable changes in the epidemiology of esophageal cancer is its incidence and cellular origin on a worldwide basis. Although there has been a dramatic shift from squamous cell to adenocarcinoma in the United States, squamous cell carcinoma remains the most common and lethal esophageal tumor in the world [21]. As with bladder carcinoma, esophageal carcinoma is amenable to topical application of potential therapeutic agents, and like bladder cancer, the surgical therapy for esophageal cancer requires large and life-altering resection. Despite many advances in surgical therapy for esophageal carcinoma, the overall prognosis of patients with both cell types of this cancer has not improved greatly during the past three decades. Most patients are diagnosed in the advanced stage of cancer, with short median survival times. Survival may be prolonged by the addition of adjuvant therapies. The use of topical therapies for Barrett's esophagus and even early stage Barrett's adenocarcinoma has increased recently, given the morbidity and mortality of esophagectomy. The use of an immunostimulant and cytotoxic agent, such as KLH, is a promising development that warrants further study in *in vitro* and *in vivo* models.

Conclusions

In summary, we have shown that significant growth inhibition of Barrett's associated esophageal adenocarcinoma cells results upon treatment with hemocyanin from the giant keyhole limpet marine organism. These effects are exhibited by both apoptotic and nonapoptotic mechanisms, depending on cell type. Promising results from the use of KLH in inhibiting growth of cancer cells have led to the initiation of *in vitro* studies to determine if KLH is best suited for use alone or in combination with conventional therapy. Treatment of Barrett's esophageal adenocarcinoma cells with the novel immunostimulant KLH produces significant growth inhibition and warrants further investigation to determine its mechanism of action.

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