Evaluation of Apoptotic Activity of Modified Carvacrol and Anticancer Peptide Against AGS Gastric Cancer Cell Line

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Abstract

Introduction

Treating stomach cancer remains a major challenge. There have been many reports of the positive effect of modified carvacrol and anti-cancer peptides on gastric cancer. The main purpose of this study was to determine and design a fusion modified carvacrol and anti-cancer peptide against AGS gastric cancer cell line using flow cytometry technique.

Method

Determination of cell lethality was conducted with different concentrations of the modified carvacrol and anti-cancer peptides against AGS cell line by MTT technique. Treatment of AGS cells with different concentrations of components to evaluate the apoptosis of AGS cells was performed by flow cytometry. In this study used. The MTT test performed according to protocol with different concentrations of components.

Results

The results showed that the percentage of cells treated with modified carvacrol and anti-cancer peptides at a concentration of 80$\mu$g/ml with total apoptosis was 46.91% and increased by 36.25% compared to untreated cells.

Conclusion

Due to the anticancer properties of modified carvacrol with anticancer peptide, the above combination can be used to treat and induction apoptotic activity against gastric cancer cell line.

1. Introduction

Gastric cancer is a growth that is beyond the control of malignant cells in the stomach and is a type of cancer that grows slowly over the years, but before the cancer actually develops, there are changes in the layers of the stomach (Sotoudeh et al., 2002). Gastric cancer is one of the most common cancers, so that in our country, according to the report of the Ministry of Health with an incidence of 93.3 out of every 100,000 people, currently the incidence of gastric cancer, especially cancers is decreasing in western countries, but this is increasing in some countries such as Iran, China, Ireland and Chile. (Malekzadeh et al., 2009; Babaei et al., 2010; Krejs., 2010; Anderoly et al., 2007).

Gastric cancer is the second leading cause of cancer death in the world. Its incidence varies in different parts of the world, but in general it is a major problem in developing countries. According to published statistics, it is estimated that approximately 9.9% of cancer cases in the world are gastric cancer. This cancer is rarely seen before the age of 40, but after the age of 40, its incidence steadily increases. (Babaei et al., 2010; Carcas et al., 2014) Researchers showed that men are almost twice as likely as women to
have cancer. Gastric cancer is the most common cancer in men and the second most common cancer in women after breast cancer in Iran (Tsugane et al., 2007; Steward et al., 2003). Gastric cancer is the most common in men and the third most common in women (Steward et al., 2003). More than 750,000 people die each year from stomach cancer. In the latest study conducted at the Medical and Health Education Center of Iran, the results showed that 39% of the causes of cancer deaths are related to gastric cancer (Rastaghi et al., 2019; Malekzadeh et al., 2009; Moore et al., 2010).

The prevalence of this disease is more common in the lower socioeconomic classes, people with pernicious anemia, people with blood type A or a positive family history. High-fat, high-salt, high-nitrate diets, a history of Helicobacter pylori infection, EBV virus, genetic factors (COX2, P53 involvement), precancerous gastric lesions, and tobacco use are all risk factors (Babaei et al., 2010; Rastaghi et al., 2019; Kamangar et al., 2006). Weight loss and reduced food intake due to anorexia and premature satiety are common symptoms of the disease. In addition to host-related risk factors, tumor characteristics including primary tumor size, lymph node invasion, and distant metastasis play a role in prognosis (Anderoly et al., 2007 & Zhang et al., 2010).

To prevent cancer, more accurate identification of its causes is needed. Unfortunately, there are not many symptoms in the early stages, which is why gastric cancer is so difficult to diagnose in the early stages. Gastric cancer is one of the cancers that can be cured if detected and treated in the early stages. If diagnosed early, the disease is completely curable, and conversely, if diagnosed late, it may go beyond the stomach and invade other parts of the body. Therefore, paying attention to the clinical signs of this disease can help to detect the disease earlier (Harrison et al., 1998; Jin et al., 2015).

There are several treatments available for patients with gastric cancer. Some treatments are standard and others are tested in clinical trials. Six standard treatments for stomach cancer are:

- **Surgery** is the most common treatment for all stages of stomach cancer.
- **Chemotherapy** is a treatment for cancer that uses drugs to stop the growth of cancer cells.
- **Radiation therapy** uses high-energy X-rays or other types of radiation to kill cancer cells or stop them from growing.
- **Radiation chemotherapy** is a combination of chemotherapy and radiation therapy to increase the effectiveness of treatment.
- **Immunotherapy** is a treatment that uses the body's immune system to fight cancer.
- **Targeted therapy** is a type of treatment that uses drugs or substances to identify and attack cancer cells without damaging the body's normal cells.

Fortunately, in recent years, the use of substances derived from the metabolism of probiotics, such as bacteriocins, has become another emerging option in the treatment of gastric cancer (Acuña et al., 2011). Bacteriocin was observed on eukaryotic cells in 1976 by Farkas-Himsley and Chung (1976). Therefore, due to the low side effects of bacteriocin use, they can be used as an independent treatment or as an adjunct to other treatments (Farkas-Himsley et al., 1976).
There have been many reports of the positive effects of bacteriocins in treating infections. Clinical studies have also shown the role of bacteriocins associated with anti-cancer sequences in the prevention, control and treatment of cancers, especially gastrointestinal cancers. Bacteriocins are cationic peptides and therefore attach to the membrane of cancer cells by negative charge instead of binding to normal cells (Dobrzyńska et al., 2005). The other reason is that the membrane of cancer cells has a higher number of microvilli than normal cells and therefore their contact surface is higher and the binding of antimicrobial peptides to the cell membrane Cancer rates are higher than normal cells. Also, cancer cells are more sensitive than cancer cells due to faster proliferation and are affected with a lower dose (Sok et al., 1999; Chan et al., 1998). Flow cytometry is an accurate and high-performance method used to identify cells and evaluate their properties. This technique is based on the scattering of light by the cells under test and the emission of fluorescence from them. Fluorescence diffusion is achieved by the direct use of fluorochromes that bind to cellular components or a combination of fluorescent dyes with monoclonal antibodies. These fluorescence-conjugated antibodies can detect and attach to surface molecules or intracellular compounds of cells and enable the detection of cell types in a diverse cell population by flow cytometry. (Ftahizade et al 2021)

Apoptosis or programmed cell death is a conserved pathway during evolution to remove dysfunctional and damaged cells. Markers at the cell surface are needed to distinguish healthy cells from apoptotic cells. Among the various lipids found in biological membranes are phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylethanolamine phospholipids, sphingolipid sphingomyelin (SPH) and cholesterol.

With the onset of apoptosis, phosphatidylserine, which is normally present on the inner surface of the cell membrane, is transported to the outside of the membrane. Research has shown that annexin V can bind specifically to phosphatidylserine in the presence of calcium. In this method, annexin V protein is conjugated to the fluorescent compound FITC. Used to dye propidium iodide (PI). This dye enters only cells whose plasma membrane structure has been destroyed and binds to DNA. Therefore, it makes it possible to distinguish cells that are in the early stages of apoptosis from cells that are in the late stages of apoptosis. Using this method, the type of cancer cell death is determined as the percentage of necrosis, the percentage of primary apoptosis and the percentage of secondary apoptosis. (Ftahizade et al 2021).

2. Material And Methods

Bradford protein assay

Preparation of recombinant protein concentration

Cell culture

Treatment of cells carried out with different concentrations of recombinant protein.
Treatment of cells performed with different concentrations of protein to study gene expression and flow cytometry

Evaluation of AGS cell apoptosis against recombinant protein performed by flow cytometry.

**Treatment of cells with recombinant protein to evaluate gene expression and flow cytometry**

To evaluate cell apoptosis, prepared two 24 cell culture plates at the same time. After 24 hours, when the cells adhered to the bottom of the plates, were removed the top medium with a pasteurizer pipette, then to all three wells of the plates (triplicated) the concentration of 80 μg / ml of recombinant protein was freshly prepared, added, and also consider wells as controls that are not treated with recombinant protein. Then prepared the final volume to 1 ml with the culture medium. The plates were placed in a CO2 incubator at 37 °C for 24 hours to treat the cells. After 24 hours of incubation, the plates were removed from the incubator.

After removing the supernatant with a pasteurizer pipette and washing with PBS, trypsin was added to remove cells from the bottom of the plates. An inverted microscope was used to examine cell separation. Untreated and treated cells with the recombinant protein were then poured into a Falcon tube at a concentration of 80 μg / ml and centrifuged at 1500 rpm for 5 minutes. Then plate cell sediments were isolated for flow cytometry.

**Apoptosis method**

To evaluation of Apoptosis with flow cytometry, cell deposits from cultures of cells treated with recombinant protein at a concentration of 80 μg / ml as well as untreated cells as controls were transferred to separate falcons. After this stage, 2 ml of culture medium was added to them. Falcon containing cell suspension was sent to laboratory to evaluate the extent of cell apoptosis by flow cytometry and the results, which are in the form of diagrams, were analyzed.

**3. Results**

Results of apoptosis by flow cytometry

In this study, it was found that the percentage of cells treated with recombinant protein that underwent primary apoptosis (Q4 region) as well as necrosis and secondary apoptosis (Q3 region), increased compared to untreated cells in these two regions and the amount of cells Healthy cells (Q1 region) in this group are significantly more than treated cells. These results showed that treatment of gastric cancer cells with recombinant protein at a concentration of 80 μg / ml induced apoptosis in this cell line.

The results of flow cytometry were reported as tables and Figures. Annexin region V- / PI-: In this region, living cells are located. Annexin V + / PI- region: In this region, primary apoptotic cells are located. 3-Annexin V + / PI + region: In this region, dead cells are located by secondary necrosis and apoptosis.
Anxin V- / PI + region: In this region, damaged cells are present during the preparation process (considered as necrosis and no apoptosis has occurred in them).

**Discussion**

Given the importance of cancer and efforts to find effective therapeutic solutions as well as knowledge of the anti-cancer role of herbal compounds and anticonvulsant peptides can be studied by combining modified carvacrol-antipeptide sequence and their therapeutic effects (Sotoudeh et al., 2002).

Apoptosis is a natural process in cells that causes the removal of old, damaged and harmful cells. Any disturbance in this pathway will cause disease. Reducing this pathway can sometimes lead to the growth of abnormal cells, such as cancer cells. Apoptosis activates a special group of aspartate-dependent proteases called caspases, especially caspase 3, both internally and externally. Externally, intracellular caspases are activated by increasing the amount of necrosis factor in the tumor (TNF-α secreted by macrophages and T cells and binding to cell surface receptors and trimming them, while intracellular caspases are activated). In the internal pathway, also known as the mitochondrial pathway, the permeability of the mitochondrial membrane to cytochrome C increased by the relative change of proapoptotic (such as Bax) and anti-apoptotic (such as Bcl-2) mediators. (Ftahizade et al 2021)

In general, in the present study, 24-hour treatment of human gastric cancer cells (AGS) with a concentration of 80 μg / ml of recombinant protein induced apoptosis in the studied cell line and it can be stated that this recombinant protein induces apoptosis and activate internally and leads to cell death.

Flow cytometry results also showed that the percentage of cancer cells treated with modified carvacrol and anti-cancer peptide was significantly apoptosis higher than untreated cells. Treatment of gastric cancer cells with the following combination at a concentration of 80 μg / ml induces apoptosis in this cell line. (Ftahizade et al 2021).

In this study, investigated the effect of modified carvacrol and anti-cancer peptide on the internal pathway of apoptosis and evaluated the apoptotic induction of this recombinant protein. In conclusion, our results showed that treatment with recombinant protein has cytotoxic effects on gastric cancer cells. These findings suggest that the recombinant protein can activate apoptosis through the internal pathway, leading to the death of cancer cells.

This compound can be introduced as an effective inhibitor of the growth and proliferation of gastric cancer cell line (AGS) and in certain conditions may be used as adjuvants in chemotherapy drugs and as a tool to be used to manipulate the expression of genes induced in apoptosis and ultimately control the growth and proliferation of cells, which is a key issue in cancer. (Harrison et al., 1998; Jin et al., 2015).

The above results can be the basis for more extensive studies to comprehensively study the effects for use in the treatment of cancer. In vitro results show that this protein has the necessary properties for in vivo studies.
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**Figures**
Figure 1

A. Negative control. In this assay 96% cell line is living cells. B. Positive control. PBS used for treatment that induced 63% apoptotic activity on cell line. C. Treatment with mixture of Anticancer peptide and carvacrol that induced 99% apoptotic activity. D. Treatment with carvacrol that induced 96.2% apoptotic activity. Treatment with mixture of Anticancer peptide and carvacrol that induced 99% apoptotic activity, Q3, 88.4% delay apoptosis, Q2, primary apoptosis, Q1, 0.82% necrosis, Q4, 0.95% living cells.
Figure 2

Treatment of AGS cell with Carvacrol, PBS, Negative control and Anticancer peptide plus carvacrol. Red color is negative control. Green color is positive control with PBS, Blue color is carvacrol plus Antipeptide cancer and Yellow color is carvacrol result.Q1 is living cell and Q2 is cell death (Apoptosis plus necrosis).