Evaluation for the Effect of Heat Stable Enterotoxin (a) Produced by Enterotoxigenic Escherichia coli on Different Cancer Cells In Vitro

Maha F. Al-Taee * Abdul Wahed B. Al-Shaibany**
Mohamed R. Al-Hadeithy**

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Abstract
This study was conducted for evaluating the cytotoxic effect of heat stable enterotoxin a (STA) produced by enterotoxigenic Escherichia coli on the proliferation of primary cancer cell cultures, obtained from tumor samples that were collected from (13) cancer patients and as follows: five colon cancer patients, two bladder cancer patients, two breast cancer patients, two stomach cancer patients and two lung cancer patients, and on normal cell line (rat embryonic fibroblast / REF) (in vitro) with the use of different concentrations starting from (1) mg/ml and ending with (0.0002) mg/ml by making two fold serial dilutions by using the 96- well microtiter plate, and in comparison with negative (PBS) and positive (MMC, at concentration of 10 μg/ml) controls.

Results showed that, after (24) hours of exposure to STA, the growth of all primary cancer cell cultures obtained from colon cancer patients was inhibited by STA treatment and this inhibition was concentration dependent. Also it was shown that the cytotoxic effect of the high concentration of STA was close to that seen after MMC treatment. While no differences were seen in the growth of all primary cancer cell cultures that were obtained from the other cancer patients, which mean that STA treatment neither inhibit nor enhanced their growth. At the same time STA did not show or has any cytotoxic effect on the normal cell line (REF).

Key word: heat-stable toxin, Escherichia coli, primary cancer cell culture

Introduction
Colorectal cancer is considered to be one of the main causes of death all around the world. Various methods and strategies have been used to treat such kind and others of cancer. The traditional methods include: radiation, chemotherapy and surgery [1]. Recently new approaches have been suggested and developed; one of these using Escherichia coli toxin, for the treatment of colorectal cancer [2].

It was found that E. coli produces a toxin, which mimics a natural colon process and provoked diarrhea. However, the toxin also causes a flood of calcium into the affected cells, stopping colorectal cancer cells from replicating rapidly. Unfortunately there is unexplained inverse relationship between the incidence of colorectal cancer and enterotoxigenic Escherichia coli (ETEC) infection [3]. The toxin that produced by ETEC, which is heat stable enterotoxin a (STa), that cause one of the serious forms of food poisoning may be used in the treatment of one of the most deadly types of cancer [4, 5].

*Biotechnology Department, College of Science, Baghdad University.
**Biotechnology Department, College of Science, Al-Nahrain University.
Enterotoxigenic *E. coli* (ETEC) is an important cause of bacterial diarrheal illness. Infection with ETEC is the leading cause of traveler’s diarrhea and a major cause of diarrheal disease in the undeveloping nations, especially among children [6]. ST is a small monomeric toxin that is closely related to *Shigella* toxins [7]. The heat-stable enterotoxins are a family of closely related peptides. They are classified into two structurally, functionally, and immunogenetically unrelated types, namely STA and STB [8]. STA is methanol-soluble and infant mice-active peptide toxin, while STB is methanol-insoluble and active in pigs, but inactive in infant mice. The toxic activity of STA is resistance to protease [9], while that of STB is inactivated by treatment with trypsin [10].

The authors provided a convincing evidence for the presence of a novel intracellular signaling pathways initiated by STA that prevented the proliferation of colorectal cancer cells. Chemically, STA binds to the guanylyl cyclase-C (GC-C) receptors specifically expressed in the intestinal cells. Ligand binding to GC-C activates the intracellular synthesis of the second messenger cyclic guanosine monophosphate (cGMP) [11]. STA hyperactivates this signaling receptor causing large increases in the intracellular cGMP. In fact, GC-C and its ligands have been implicated in the regulation of the balance of proliferation and differentiation along the crypt-to-villus axis in the intestine. As a result, subsequent loss of the initiation of GC-C signaling may represent one key mutational event underlying neoplastic transformation in the colon [12, 13].

However, intestinal GC-C and its downstream intracellular effector molecules are conserved in colorectal cancer. Thus the presence of STa / GC-C induced cGMP-dependent signaling pathway, through cyclic nucleotide-gated (CNG) channels and calcium was responsible for the anti-proliferation action of STA enterotoxin on human colon cancer cells because control using colon cancer cells devoid of GC-C were without effect [14].

Cancer has been recognized for more than 200 years, it was reported by Hippocrates and Galen. However, Al-Zahrawi was considered as the first physician who described cancer to look a little like crab because of ”finger like projection” [15]. Cancer is a disorder of cell growth that leads to invasion and destruction of healthy tissue by abnormal cells. Although cancer has affected human since earliest time, it was a rare disease until the twentieth century. Cancer now ranks second only to heart disease as a major cause of death in the world [16]. Colorectal cancer is a malignant neoplasm of epithelial cell origin affecting the large bowel. It is a common type of malignancies that affect gastrointestinal tract and it is of favorable prognosis provided that, it is diagnosed and treated in an early stage. Cancer detection at an early stage and identifying susceptible individuals can result in reduced mortality from this prevalent disease [17].

In Iraq, at the present time, cancer is considered as one of the most important causes of death especially after the Gulf war in 1991. For this reason, a large number of studies have been carried out on various forms of cancer with a view to understand the biology, diagnosis and treatment of this disease.

This study aims to open up a new approach in the development of anti-cancer drugs. An attempt to provide anti-cancer agent (STA) for the treatment of colorectal cancer with less or no cytotoxic effect on normal cells.
and with more cytotoxic effect on cancer cells.

**Materials and Methods:**
All the chemicals were obtained from Sigma Chemical Co. (USA) and BDH (England).

**Solutions for detecting the cytotoxic effect of STA on cell cultures:** They were prepared according to the methods used by [18].

**Collection of tumor tissue samples:**
Tumor tissue samples of 5 different types of tumor (colon, stomach, lung, breast and bladder) were collected into sterile tubes containing a transport medium. They were obtained from the operations theatre of Gastroenterology and Hepatology Teaching hospital and Baghdad Teaching hospital.

**Detecting the cytotoxic effect of STA on primary cancer cell cultures:** It was done according to [18].

a. **Management of tumor tissue samples to obtain primary cancer cell cultures:** All the work was done under sterile conditions in the laminar airflow hood as follows: The collected tumor tissue sample was put in a sterile petry dishes and all the necrotic tissue and fat were removed. Then the tissue was washed at least five times with the culture media. After that tissue was transferred into a sterile tube that contains the culture medium and minced very carefully by a sharp curved scissor, with maintaining the tissue in the culture media. Then the tube was centrifuged at 1000 rpm for 5min, to get rid of the large tissue pieces. Finally, the supernatant was then transferred into another sterile tube and before seeding into a 96-well microtiter plate, the number of the cells must be counted and the number of cells at seeding should be $10^7$ cell/ml.

b. **Viable cell count:** Cells were counted by using trypan blue stain working solution (1:10). Only the dead cells will take up the dye and appear blue under the microscope while viable cells exclude the dye and appear white, which make it very easy to distinguish dead cells from viable cells, and this done by mixing 0.2ml of cells and 0.2ml of the stain with 1.6ml of PBS. Then after mixing well sufficient volume was aspirated to fill the Neubaur haemocytometer.

c. **Cytotoxic assay:** 50μl of complete culture media was added to each well in the microtiter plate, except the first vertical line. Then 100μl of STA (after isolation and purification from bacterial suspension) was added to first well in the first and second vertical line only at a concentration of (1) mg/ml. 6 repetitives were made. Mixed well and then 50μl from the first well in the second vertical line was transferred to the second well and two-fold serial dilutions were made until the well number (12). A positive MMC (at a concentration of 10μg/ml) and negative PBS controls was added to each well in the seventh and eighth line of the microtiter plate, respectively. Then 150μl from cell suspension (cancer cells suspension), after their counting, was added into each well in the microtiter plate and incubated at 37°C in 5% CO2 incubator. Later, after 24hr of exposure time, the microtiter plate was removed from the incubator, and 50μl of neutral red dye was added, then incubate at 37°C for 2hr. The viable cells will acquire the dye, while the dead cells will not. After that, all the contents of the microtiter plate were removed and the cells were washed with PBS. Then 50μl from neutral red extraction solution (ethanol : PBS) at a ratio (1:1) was added. This solution elutes the dye from the viable cells that taken the dye. The absorbency was determined by using the ELISA Reader at 492 nm. All the previous steps were done with all tumor types (colon, stomach, lung, breast and bladder) that were used in
this study. Then the cytotoxic concentration 50 (CC50) was
determined by plotting the STa concentrations against the absorbency.

**Cytotoxic effect of STa on normal cell lines**

The detection of cytotoxic effect of STa on normal cell line was done
according to the method of [19]. It was carried out on the normal cell line Ref
(Rat embryo fibroblast), at passage (52) that was provided by Dr. Ahmed
M. AL-Shamery / Iraqi Center for Cancer and Medical Genetic Research/
Baghdad, and as follows: 2ml of trypsin-versene solution was added to
tissue culture bottle (falcon) of 25cm²
that contain the cells (Ref) after the
removal of old tissue culture media and
washing with PBS, the bottle was
shaked smoothly and then inoculated at
37°C for 2min to disaggregate cells and
obtain more single cells as much
as possible. After that, cells were
suspended in a new fresh tissue culture
media and counted at a concentration
of 10⁶ cell/ml by trypan blue. Then
50μl of complete culture media was
added to each well in the microtiter
plate, except the first vertical line.
Later 100μl of STa was added to first
well in the first and second vertical line
only at a concentration of (1) mg/ml. 6
repertatives were Made. Mixed well and
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washed with PBS. Then 50μl from
neutral red extraction solution (ethanol : PBS) was added at a ratio (1:1). This
solution elutes the dye from the viable
cells that taken the dye. The
absorbency was determined by using
the ELISA Reader at 492 nm. The
cytotoxic concentration 50 (CC50) was
determined by plotting the STa
concentrations against the absorbency.

**Results and Discussion:**

**Cytotoxic effect of STa on primary cancer cell cultures.** The cytotoxic
effect of STa on primary cancer cell cultures was determined by evaluating
its effect on: five tumor tissues obtained from patients with colon
cancer, two tumor tissues obtained from patients with bladder cancer, two

tumor tissues obtained from patients with breast cancer, two tumor tissues
obtained from patients with stomach cancer and two tumor tissues obtained
from patients with lung cancer.

Results of the cytotoxic effect of STa on tumor tissues obtained from
five different colon cancer patients showed that STa caused an obvious
inhibition in the growth of cancer cells compared with the negative control
and this inhibition was close to the
inhibition that was seen with the
positive control (MMC, at
concentration of 10(μg/ml), as shown in
figure (1, A, B, C, D and E). These
results that associated with growth
inhibition of primary colon cancer cell

culture after STa treatment were
similar to those obtained by many
authors [20, 21, 3, 1, 5, 22], and in fact
some of them were able to reduce the
growth of cancer cell lines with less
concentrations of STa. They showed
that STa induce colorectal cancer
cytostasis without cell death by targeting guanylyl cyclase-C (GC-C) signaling and the anticancer action of this toxin is mediated by cGMP that dependent influx of Ca^{2+} through the cyclic nucleotide-gated channels. Thus, GC-C is the specific receptor for STA, and without it, STA will be unable to exhibit its cytostasis effect [23, 24, 25, 26]. In fact some chemotherapeutic drugs show their cytotoxic effect by activation of cGMP [27].

Previous studies showed that STA penchant for intestinal cells only, indicating that, as a drug, it would focus just on these cells and leaves others alone, and upon its injected into the blood it might even specifically combat colon cancer cells that had been migrated to other parts of the body. Thereby, derailing metastasis which is a serious problem in this type of cancer. Pitari and coworkers predicted that even if this toxin only slow the growth of colon cancer cells without killing them, it speculate that it may lead to possible therapy that would control colorectal cancer spread.

Figure (1): Cytotoxic effect of STA on primary cancer cells cultures obtained from five patients with colon cancer (A, B, C, D, E).

Thus, if the sacrifice for one is to have occasional diarrhea and prevent the tumor in the colon from ever forming or progressing, it worth's it [5].

At the same time the cytotoxic effect of STA on primary cancer cell cultures obtained from other organs have been also studied. Results showed that STA had no inhibitory effect on the growth of cancer cells obtained from (bladder, breast, stomach and lung cancer) as shown in figures (2, 3, 4, 5, A and B) respectively.

The most reasonable explanation for the absence of STA effect on these primary cancer cell cultures is that STA does not bind to these cells due to the
absence of the expression of GC-C receptors to which STa binds and exerts its effect [26]. These results were in close agreement with those declared by [28, 29, 30], in which they examined the binding affinity of STa to extraintestinal tissues by using many different types of cancers and with the using of more precise techniques including reverse transcription-polymerase chain reaction (RT-PCR), they showed that STa binding were not detected in all these extraintestinal cancers, and thus no cytostasis effect for STa was detected.

Figure (3): Cytotoxic effect of STa on primary cancer cells cultures obtained from two patients with breast cancer (A, B).

Figure (2): Cytotoxic effect of STa on primary cancer cells cultures obtained from two patients with bladder cancer (A, B).

Figure (4): Cytotoxic effect of STa on primary cancer cells cultures obtained from two patients with stomach cancer (A, B).
Cytotoxic effect of STα on normal cell line (REF): When any substance was suspected to be used in cancer therapy, it must show a selective toxicity on cancer cells but not on normal cells in order to say it is safe. So, when cancer cell lines are used in any study, normal cell lines should be also used as a control for comparison. In this study, REF (rat embryo fibroblast) cell line was used as a normal cell line. Results shown in figure (6) indicated that STα has no cytotoxic effect on normal line (REF) as compared with negative control, and there is an increase in the growth of cells as the STα concentration decrease. This inverse relationship may be explained by the increase in the growth medium concentration at the time of STα concentration decrease, since growth medium concentration play a significant role in the cell culture technique [18]. Similar results were indicated by [30], who examined the binding and function of STα in normal extraintestinal human tissues and colorectal tumors. They found that STα was able to bind specifically to all colon and rectum tumors that were examined, while neither STα binding nor STα activation of GC-C was detected in all examined normal extraintestinal tissues. In addition, DNA of tumor cells was found in a relaxant shape, and the DNA molecule was found in unstable figure because of the far away between the H-bonds which connect the both strands of DNA and this makes easy for compounds to interfere or associated to both DNA strands. On the other hand, DNA of normal cells has strong H-bonds that connect the both strands to each others and thus making it more stable, so by this way, the compounds can not interfere or associate with DNA strands [31]. However, inhibition of normal cell line growth was detected with MMC treatment at a concentration of (10) μg/ml, as compared with the negative control, and this result came in agreement with [32], in which they showed that MMC has toxicity to both normal and tumor cell lines and this toxicity could be increase or enhance by certain substances.
References:
on patients with chronic myelocytic leukemia. M. Sc. Thesis. College of Medicine, University of Al-Nahrain.


تقييم تأثير السم المعوي الثابت حاريا (Enterotoxigenic E.coli) في تأثيره على الخلايا السرطانية المختلفة في الزجاج

*محمد آدميح الطهيبي**
**عبد الواحد باقر الشيباني***

**قسم الشئون الإدارية** كلية العلوم جامعة بغداد
**قسم الشئون الإدارية** كلية العلوم جامعة الديوانية

الخلاص:

أجريت هذه الدراسة لتقديم تأثير السم المعوي الثابت حاريا (Enterotoxigenic E.coli) في تأثيره على الخلايا السرطانية المختلفة في الزجاج.

نستنتج من هذه الدراسة وجود تأثير السم المعوي الثابت حاريا (Enterotoxigenic E.coli) في تأثيره على الخلايا السرطانية المختلفة في الزجاج.