

Original Article

Cytoplasmic and membranous CD24 marker expression has indirect correlation with cAMP/cGMP ratio

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Abstract

Background: CD24 is a cell adhesion molecule that has been implicated in metastatic tumor progression cells. Our aim was to clarify the correlation between CD24 expression and cAMP:cGMP ratio in murine colorectal cancer cell line (CT26) after using cholera toxin.

Materials and Methods: The CT26 cells were cultured in flasks for assaying cAMP and cGMP by ELISA kits; also the cells were cultured in flasks for assaying cytoplasmic and membranous CD24 expression. The Real-Time PCR was done for cDNA that was synthesized from CT26 cells' mRNA. Also, expression CD24 marker of cells was determined by Anti-CD24 antibody and Goat Anti-Rabbit IgG-FITC (flow cytometry).

Results: The cholera toxin increases the cAMP:cGMP ratio and influenced the cytoplasmic and membrane CD24 expression level.

Conclusion: Cholera toxin increased cAMP. After increasing cAMP/cGMP ratio, cytoplasmic and membranous CD24 expression showed decline.

Keywords: CT26 cell line, colorectal cancer, cAMP, cGMP, CD24

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Introduction

The third most common cause of death in the world is colorectal cancer (CRC) ¹. In 2008, about 608,700 deaths from CRC and 1.2 million new cases had been reported throughout the world. Australia, New Zealand, Europe and North America have the highest incidences; whereas Africa, South-Central Asia have the lowest incidence rates ². There are acceptable methods for screening and detecting CRC, including occulted blood in feces, flexible sigmoidoscopy, double-contrast enema and colonoscopy ³.

There are several markers like CD24, CD44, CD133 and CD166 on the surface of cells of different tumors such as breast, lung, colorectal, pancreas, prostate, ovarian and renal ⁴. CD24 is a single chain sialoglycoprotein with 24 KD molecular weights ⁵. CD24 and clinicopathological factors have correlation with the degree of differentiation ⁶. It is expressed in a large variety of malignancies ⁷. CD24 is a ligand for P-selectin which can disseminate CD24 positive tumor cells ⁸.

Cyclic 3', 5'-adenosine monophosphate (cAMP) and Cyclic 3',5'-guanosine monophosphate (cGMP) are

intracellular second messengers. They mediate the action of large numbers of hormones and neurotransmitters ². The cGMP in some tissues acts opposite of cAMP; thus, increase of cGMP concentration is associated with cell proliferation but increasing level of cAMP inhibits cell proliferation ^{10, 11}. cAMP and/or cGMP level alterations had been reported in tissues, blood and urine of patients with cancers and other pathophysiological states ^{12, 13}.

Cholera toxin (CT) belongs to the family of AB₅ bacterial toxins. The total molecular mass of the holotoxin, AB₅, is 86 kDa. The A subunit (28 kDa) of CT is composed of two distinct parts A1 and A2. The A1 component is responsible for the toxic enzyme activity, while the A2 component serves as non-covalent linker of subunit A to subunit B (58.4 kDa). Then subunit A1 is translocated to the cytosol of the host cell, where it catalyses the covalent transfer of an ADP-ribose moiety of the signaling protein G_s. Ribosylated form of G_s stabilizes the GTP bound of protein, which stays continually activated. This modification of the adenylate cyclase system results in an elevated level of cAMP which causes the activation of the sodium pumps in the lumen of the cells. The electrochemical imbalance is then compensated by driving Cl⁻ and H₂O out of the cells. The process of Cholera Toxin action is followed by enormous loss of water from the epithelial cells into the intestinal lumen, causing water diarrhea characteristic for cholera ¹⁴.

Our aim in this study is showing that cytoplasmic and membranous CD24 expression affected by increasing cAMP:cGMP ratio.

Methods

Cholera toxin (CT): Toxin was isolated from *Vibrio cholerae* (Inaba) PTCC1611 that is toxigenic without protease production. This strain was purchased from Iranian Research Organization for Science and Technology.

Cell line: CT26 is an undifferentiated murine colorectal adenocarcinoma cell line that was purchased from Pasteur Institute of Iran (Tehran).

Bacterial culture: Large amount of CT was produced in 500 ml of Modified YEP medium (yeast extract

0.4%, Bacto-pepton 1%, NaCl 0.5%, KCl 0.15% and sodium bicarbonate 0.4%; PH 7.0) at 37°C for about 20 h on a rotary shaker at 90 rpm ^{15, 16}. The toxin was separated from cells by Millipore Stericup filter PVDF membrane (0.22 μm, Sigma Co.). Then it was concentrated by using a 30 KD Amicon Ultra Centrifugal filter. The toxin confirmation by Anti-Cholera toxin antibody (Abcam Co.) was followed by semi-dry western blot method. In addition, the toxin concentration was assayed by PRO-MEASUR Protein Measurement Solution (iNtRON Co, Korea). Appropriate concentration of cholera toxin was determined by MTT ¹⁷.

cAMP and cGMP assay: CT26 cell line was inoculated in two tissue culture flasks (vent 25 cm² Jet Biofil Co.), 7 ml RPMI GlutaMAX, HEPES (Gibco Co.) + 10% FBS (Invitrogen Co.) were added and cells were incubated at 37°C in 5% CO₂, until filling of about 80% of flasks. 10 ng/ml of cholera toxin was added in one of the flasks and was returned to incubate for 36 h. The cells were separated by scrapers and were poured in separate falcon tubes. The tubes were centrifuged in 12000g for 10 min. The supernatants were used for cAMP and cGMP assay by cAMP direct immunoassay and cGMP direct immunoassay kits (Abcam Co.) according to company protocols. The OD of wells was read at wave length 450 by ELISA reader (BioTek).

mRNA extraction: RNA extraction from toxin affected and non-toxin affected cells was done by easy-BLUE Total RNA Extraction Kit (iNtRON Co.) and two groups cDNAs were constructed by Maxime RT PreMix Kit [Oligo (dt)₁₅ Primer] (iNtRON Co.). The program was 45°C for 60 min and 95 °C for 5 min (36 cycles).

Real-time PCR: Amplification primers for CD24 were 5'- TACCCACGCAGAT TACTG -3' (Forward) and 5'- CAGTAGAGATGTAGAAGAG -3' (Reverse) and for GAPDH as a housekeeping gene were 5'- ATGATGACATCAAGAAGG TG -3' (Forward) and 5'- AGTTGCTGTTGAAGTCGCAG -3' (Reverse). The RT-PCR reactions were done in 0.2 μl: 12.5 μl Power SYBR Green PCR Master Mix (Invitrogen Co.) + 1μl forward primer + 1μl reverse primer + 3 μl cDNA + 7.5 μl DDW (Table 1).

Table 1: The Real Time-PCR for cytoplasmic CD24

Time (min or sec)	Temperature (°C)	Steps	No.
5 min	94	Initial denaturation	1
45 Sec	94	Denaturation	2
45 Sec	52	Annealing	3
45 Sec	72	Extension/elongation	4
10 min	72	Final elongation	5
Short-term	4	Hold	6
Stages 2-5, 35 cycles			7

Flow cytometry: Dissociated cells (with and without cholera toxin) with Trypsin/ EDTA (Sigma Co.) were counted and each group transferred to a 5 ml tube. The cells were washed twice with PBS containing 2% FBS each time they were centrifuged at 4°C for 5 min (1500 rpm). Then cells were re-suspended in cold PBS with 2% FBS; for each cell group (10⁶ cells/ml), 100µl were transferred to microtubes. 1µg/ml Anti-CD24 antibodies (Abcam Co.) as a first antibody were added to each tube and were incubated for 15 min on ice. The cells were washed 3 times with cold PBS containing 2% FBS and after each time, they were centrifuged at 400 rpm for 5 min. The pellets were re-suspended in cold PBS with 2% FBS and 1µg/ml Goat Anti-Rabbit IgG-FITC (Abcam Co.) as a second antibody was added to each tube; they were incubated for 15 min on ice. Excess antibody was washed with cold PBS containing 2% FBS in 1500 rpm for 5 min. The cells were re-suspended with cold PBS 1X and flow cytometry was done using Bench-Top Flow Cytometry (Partec PAS III, Germany).

Statistical analysis: Statistically significant difference was determined by Chi square (χ²) analysis and definition as p<0. 05. In addition, the correlation between cAMP:cGMP ratio and CD24 expression were determined by the Pearson correlation coefficient. Also, results of RT-PCR and flow cytometry were analyzed by REST V2.0.13 and FlwJo V10.0.6 softwares, respectively.

Results

The MTT assay (data are not shown) confirmed that 10 ng/ml of cholera toxin could transform fibroblasts CT26 cell line after 36 h (Figure 1). After 36 hours, the

cAMP level was significantly higher in CT26 cell culture with toxin than cell culture without toxin (p=0.04), 8 pmol VS. 9.2 pmol, respectively. However, the cGMP level showed a bit elevation in CT26 with toxin, but this increasing was not significant (p=0.1). In addition, cAMP:cGMP ratio had no significant excess against cholera toxin (p=0.1) (Table2).

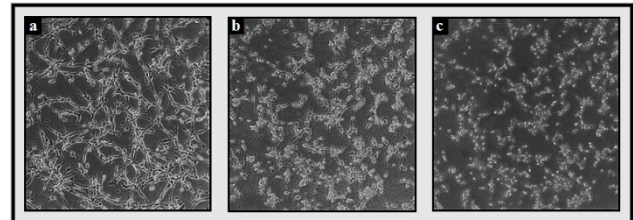


Figure 1: Cholera toxin transformed CT26: a) CT26 culture without toxin; b) CT26 culture with toxin after 24 h; c) CT26 culture with toxin after 36 h

Table 2: cAMP and cGMP changes in CT26 cells without and with toxin

CT (ng/ml)	cAMP (pmol/well)	cGMP (pmol/well)	cAMP:cGMP
0	8	5.8	1.4
10	9.2	4.2	2.2

CT: Cholera toxin; cAMP: Cyclic adenosine monophosphate; cGMP: Cyclic guanosine monophosphate

Cytoplasmic CD24 changes were assessed by RT-PCR. The cytoplasmic CD24 expression showed significant decrease in cell culture with toxin against control cell culture (p=0.00). Also, the flow cytometry for CD24 expression level (CD24^{low+} and CD24^{high+}) on cell surface displayed a significant decline in cell culture with toxin in comparison with control cell culture (p=0.0001) (Figure 2). Albeit, in Q3 area (Figure 2) there was a development in CD24^{high+} expression on cells.

The CD24 expression in cytoplasm and on cell surface had strong indirect correlation with cAMP:cGMP ratio (r = -1).

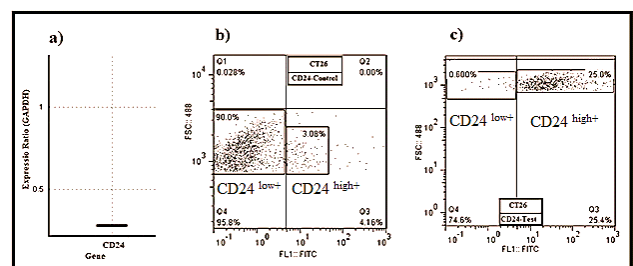


Figure 2: a) Decreasing cytoplasm CD24 expression in cultured CT26 with toxin; b) Flow cytometry of cultured CT26 without toxin; c) Flow cytometry of cultured CT26 with toxin

Discussion

In our study, the cAMP level in cultured CT26 cells without and with cholera toxin had increased to 8 and 9.2 pmol, but cGMP displayed decline to 5.8 and 4.2. The cAMP/cGMP in cell group was higher than cultured CT26 with toxin. In addition, increasing cAMP/cGMP ratio was in parallel with the cell morphology transformation. These results were similar to above studies. In Zhu study the cholera toxin (CT) in 10 ng/ml concentration can induce morphological transformation in glioma cells ¹⁷. CT accumulates cAMP with ADP-ribosylation of Gs protein¹⁸. Increasing cAMP is the cause of glioma cell differentiation^{19, 20}.

In our study, MTT assay confirmed that 10 ng/ml of cholera toxin could transform CT26 cell line. The cyclic nucleotide (cAMP and cGMP) metabolism alterations has often been found in neoplastic tissues ²¹. The plasma cAMP/cGMP in the normal subjects and the acute leukemia patients were 3.56 pmol/ml and 1.51 pmol/ml, respectively ²². Another study showed that urinary level cAMP/cGMP in normal subjects (7 µmol/24h) is higher than abnormal cervical cytology (1.4 µmol/24h) ¹². In rats with hepatoma, tumor size was correlated with cGMP level; it was decreased after chemotherapy, radiotherapy and surgery. Plasma cGMP levels in patients with cirrhosis are significantly higher than control subjects. In the cirrhotic patients' plasma cGMP/cAMP ratio levels were significantly higher than control group ²³. Deregulation of cAMP and cGMP pathways occur during infection, intestinal inflammation and cancer ¹³.

In our study, the cytoplasmic CD24 expression had a significant decline when CT26 cell line was cultured with cholera toxin ($p=0.00$). Also, identical result was seen for membranous CD24 ($p=0.0001$). A small population of cells was converted to CD24^{high+}. CD24 as an alternative ligand of P-selectin and an adhesion receptor on activated endothelial cells and platelets can enhance the metastatic potential of CD24 expressing tumor cells ^{24, 25}. In the gastric cancer (NUGC-2) and colonic cancer (COLO-2001) cell lines, CD24 expression was detected in RNA blot analysis ²⁶. CD24 is an import marker for cancer diagnosis and prognosis. CD24 expression is significantly higher in invasive

breast carcinoma than in precancerous lesions. Cell-surface and cytoplasmic CD24 expression correlates with poor prognosis, histology grades, tumor sizes and lymph node positivity ^{27, 28}. In Weichert study, the normal colon mucosa was negative for CD24, although some cases displayed a weak membranous staining in the brush border; but the CD24 expression in colorectal carcinomas was high. Membranous CD24 staining was positive in 68.7% of tumors and strongly positive in 17% of tumors. For cytoplasmic CD24 staining, 84.4% of cases were positive and 10.2% cases showing a strong expression. Cytoplasmic CD24 was significantly associated with higher tumor stages, nodal or systemic metastasis, and finally tumor grade. The strong cytoplasmic CD24 expression is associated with shortened patient survival times, which shows the biological role of CD24 in colorectal cancer ²⁹. Furthermore, cytoplasmic and membranous CD24 had strong indirect correlate with cAMP:cGMP ratio ($r = -1$). Similar study was not found to compare with our study.

Conclusion

cAMP level and cAMP:cGMP ratio in cultured cells with cholera toxin were higher than control cells. In CT26 cell line, cytoplasmic and membranous CD24 expression declined after using cholera toxin. In addition, There was an indirect correlation between cAMP:cGMP excess and decrease in CD24 expression.

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