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Cholesterol affects gene expression of the Jun family in colon carcinoma cells using different signaling pathways

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ABSTRACT

Hyperlipidemia and hypercholesterolemia have been found to be important factors in cancer development and metastasis. However, the metabolic mechanism and downstream cellular processes following cholesterol stimulation are still unknown. Here we tested the effect of cholesterol on MC-38 colon cancer cells. Using Illumina gene array technology we found a number of genes that were differentially expressed following short term (20–40 min) and longer term (between 2 and 5 h) cholesterol stimulation. Three genes were consistently increased at these time points; c-Jun, Jun-B and the chemokine CXCL-1. We have previously shown that cholesterol stimulation leads to PI3K/Akt phosphorylation, and now demonstrated that cholesterol inhibits ERK1/2 phosphorylation; both effects reversed when cholesterol is depleted from lipid rafts using methyl-β-cyclodextrin (MBCD). In addition, vanadate, an inhibitor of phosphatases, reversed the cholesterol inhibition of ERK1/2 phosphorylation. Specific inhibition of p-Akt by wortmannin did not affect cholesterol's stimulation of the expression of c-Jun and Jun-B, however the vanadate effect of increasing p-ERK1/2, inhibited c-Jun expression, specifically, and the MBCD effect of increasing p-ERK and inhibiting p-Akt reduced c-Jun expression. In contrast MBCD and vanadate both enhanced Jun-B gene expression in the presence of cholesterol and elevation of ERK phosphorylation. Thus there is apparently, a differential signaling pathway whereby cholesterol enhances gene expression of the Jun family members.

1. Introduction

Colorectal cancer is the third leading malignancy for at least the last two decades and is the second leading cause in cancer mortality (Giovannucci, 2002; Jacobs et al., 2012; Parkin et al., 1999). It was found in several studies that lifestyle and diet have a major role in the development of colorectal cancer. Thus the western diets lead to an increased risk for the development of colorectal cancer (Ahmed et al., 2006; Frezza et al., 2006; Jacobs et al., 2012; Keys et al., 1985) as well as other cancers. On the other hand, other diets such as the Mediterranean diet and vegetarian diet, which is characterized by low cholesterol consumption, were demonstrated in several studies to lower the risk of developing cardiovascular diseases, obesity, diabetes (Demarin et al., 2011; Ordovas et al., 2007) and reduced the risk for colorectal cancer (Fraser, 2009). The western diet is characterized by high fat and high carbohydrate content leading to the metabolic syndrome (Ahmed et al., 2006; Kim et al., 2007) which results in heart disease, type 2 diabetes and cancer. Many studies have suggested a direct link between overweight and obesity, hyperlipidemia and cancer development and cancer-related mortality (Calle et al., 2003; Lim et al., 2012).

Cholesterol is a sterol that has as a major role in the structural integrity of the plasma membrane and maintains its fluidity by creating microdomains named lipid rafts (Broitman et al., 1993; Edidin, 2003). Cholesterol is considered to have survival effects on cells. Animal models with elevated plasma cholesterol accumulate cholesterol in lipid rafts, leading to stimulation of the EGFR, which is needed for proliferation and survival (Markowitz et al., 1994; Oh et al., 2007; Zhuang et al., 2005). Downstream targets of EGFR are the phosphatidylinositol 3-kinase (PI3K) pathway, and Akt that is downstream of PI3K, and the MAPK pathway that is upstream of ERK (Samuels et al., 2004; Tsao et al., 2004; Wang et al., 2003). Both in prostate cancer as well as in breast cancer cholesterol stimulation *in vitro* or high plasma cholesterol levels *in vivo* results in increased phosphorylation of Akt and increased tumor growth (Alikhani et al., 2013; Zhuang et al., 2005). ERK stimulation on the other hand was down-regulated due to activation of a serine/threonine phosphatase complex (Wang et al., 2003). Depletion of cholesterol from the plasma membrane resulted in reduced Akt phosphorylation and restored ERK phosphorylation and activation to basal levels, leading to apoptotic cell death, while addition of cholesterol to the cells restored EGFR stimulation and hence Akt and ERK phosphorylation (Alikhani et al., 2013; Oh et al., 2007).

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Although some studies have tried to determine the role of cholesterol stimulation on tumor proliferation and metastasis as well as downstream pathways of cholesterol stimulation in cells, it is as yet unknown which genes are regulated by cholesterol stimulation. Here we describe both signaling pathways that are activated and inhibited by cholesterol and the family of Jun transcription factors (c-Jun and Jun-B) whose expression are regulated by cholesterol addition to MC-38 colon cancer cells.

2. Materials and methods

2.1. Cell culture

Mouse colon 38 (MC-38) adenocarcinoma and MVT-1 (derived from c-Myc/VEGF tumors) cells were cultured in Dulbecco's modified Eagle's medium (Biological Industries, Beit Ha-Emek, Israel) completed with 10% fetal bovine serum (Biological Industries) and 1% penicillin–streptomycin (Biological Industries). Cells were grown to 70% confluence followed by starvation (serum free medium supplemented with 0.1% BSA (Biological Industries)) for 4 h before the proliferation test and overnight before other treatments. The starved cells were incubated with 1 mM cholesterol (Sigma–Aldrich, St. Louis, MO, USA) for various time points ranging from 20 min to 5 h. To investigate whether the phosphatidylinositol 3-kinase (PI3K) is implicated in cholesterol-induced activation of Akt, the starved MC-38 cells were pre-incubated with 100 nM of wortmannin (Sigma–Aldrich), a PI3K inhibitor, for 30 min before addition of cholesterol. In order to evaluate the role of MAPK following the stimulation of cholesterol, the starved cells were pre-treated with 1 mM of U0126 (Sigma–Aldrich), a MEK inhibitor, 30 min before the addition of cholesterol. To investigate the effect of cholesterol depletion from the lipid rafts, cells were treated with 5 mM of methyl-β-cyclodextrin (MBCD) (Sigma–Aldrich), a cholesterol depleting agent, 1 h prior to cholesterol addition. In order to block phosphatase activity the cells were pre-treated with 100 μM of vanadate (Sigma–Aldrich) for 1 h prior to cholesterol addition. In order to investigate how p38 MAPK is affected by cholesterol stimulation we incubated the cells with 0.4 μM of SB203580 (A.G. Scientific, San Diego, CA, USA), a p38 MAPK inhibitor, 1 h prior to cholesterol addition.

2.2. Isolation of proteins and western blot analysis

Cells were lysed in protein extraction buffer (pH 7.4) containing 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1.25% CHAPS (Roche, Indianapolis, IN), 1 mM sodium orthovanadate, 2 mM sodium fluoride, 10 mM sodium pyrophosphate (Sigma–Aldrich), 8 mM β-glycerophosphate (MP Biomedicals, LLC, Santa Ana, CA, USA) and Complete Protease Cocktail (Roche). 10 μg of the protein content were analyzed on SDS–PAGE (12% Tris–Glycine gel) followed by transfer to nitrocellulose membrane (Amersham/GE-Healthcare, Uppsala, Sweden) using Trans-Blot Turbo blotting system (Bio-Rad, CA, USA). The membrane was thereafter blocked followed by probing with primary and secondary antibodies. The membrane was analyzed by direct infrared Imaging System (ImageQuant LAS4000, GE-Healthcare). The primary antibodies used in the study were: mouse anti-Akt (pan)(40D4), rabbit anti-phospho-Akt (Thr308), mouse anti-p44/42MAP kinase and rabbit anti-phospho p44/42MAP kinase (Thr202/Tyr204) (Cell signaling) and mouse anti-β-actin (Sigma–Aldrich).

2.3. RNA extraction from tissue culture

RNA was extracted using the total RNA purification kit (Norgen, Biotek Corp., Ontario, Canada) according to manufacturer instructions. Briefly, 3×10^5 cells were seeded on 6 cm plate. The next day the cells were washed two times with PBS and starvation medium was added to the cells overnight. After cell stimulation cells were washed two times with ice cold PBS and 350 μl of lysis buffer was added to the cells. The cells were incubated for 5 min and cells were than collected into 1.5 ml test tubes. 200 μl of absolute ethanol was added to the lysate and mixed by vortexing. The lysate was loaded on column, washed and eluted into clean tube. RNA concentration was measured in A260 and A280.

2.4. Microarray chip gene expression analysis

Microarray expression profiling was performed in the Genomics Core Facility (BioRap Technologies, Rappaport Research Institute, Technion). The RNA was amplified into cRNA and biotinylated by *in vitro* transcription using the TargetAmp Nano-g Biotin-aRNA Labeling kit for the Illumina system (Epicentre Biotechnologies) according to the manufacturer's protocol, with 100 ng of total RNA as input material. Biotinylated cRNAs was purified, fragmented, and subsequently hybridized to an Illumina MouseWG-6 v2.0 BeadChip according to the Direct Hybridization assay (Illumina Inc.). The hybridized chip was stained with streptavidin-Cy3 (Amersham™) and scanned with an Illumina beadarray reader. The scanned images were imported into GenomeStudio (Illumina Inc.) for extraction and quality control. Using JMP® Genomics V5 software (SAS Institute Inc., Cary, NC), two types of filtering was performed: filtering by signal to remove all probes with signal intensity $\leqslant 2^6$ (background noise elimination), and filtering by variance to exclude all probes with low variance that is $\leqslant 2^{0.1}$. Subsequently principal component analysis was performed to detect outliers and identify major trends. Two different sets of time points were compared, short exposure of 20 and 40 min respectively, and longer exposure of 2.5 and 5 h after cholesterol addition. Each set was analyzed using one-way ANOVA with cutoff for differentially express genes (DEG) at an uncorrected *p*-value of 0.001 and a difference of 1.6-fold change between time points. The 12 and 64 DEG between the short and the longer time points respectively were clustered using the centroid method, where the distance between clusters is defined as the squared Euclidean distance between their mean (Milligan, 1980), each column represents a time point and each row a different transcript, blue are underexpressed and red are overexpressed transcripts, with the scale as defined in the figure. The scree plot beneath the dendrogram point is the distance that was bridged to join the cluster at each step with a line at the natural break where the distance jumps up suddenly.

2.5. Reverse transcript PCR and qtPCR

About 200 ng of RNA were reversed transcribed (RT-PCR) into cDNA using the Verso cDNA kit (Thermo scientific, ABgene, UK) in T3000 Thermocycler (Thermo scientific, Biometra, Germany). The cDNA was than diluted 1:9 and qtPCR reaction was performed using the Absolute Blue SYBR-Green ROX mix (Thermo scientific, ABgene, Rochester, NY) according to manufacturer instructions in Rotor-Gene TM 6000 (Qiagen, Corbett Research, Sydney, Australia). Relative quantity (RQ) was calculated as $2^{-(Ct_{(n)}-Ct_{(normalayser)})}$ for each treatment. Primers used were β2 microglobulin (B2M) forward: TTCTGGTGCTTGTCTCACTGA reverse: CAGTATGTTCGG CTTCCCATTC; c-Jun forward: CCTTCTACGACGATGCCCTC reverse: GGTTCAAGGTCATGCTCTGTTT; Jun-B forward: TCACGACGACTCTT ACGCAG reverse: CCTTGAGACCCCGATAGGGA.

2.6. Cell proliferation assay

About 10,000 MC-38 cells per well were plated in 24-well plates for 24 h followed by replacement of the regular medium to