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ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorpjn@gmail.com

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Biological Response of Epithelial Cells line (CMT-93) Induced by β-Carotene

Katrin Roosita[1], Rimbawan Sukandar Martopuro[1], Ita Djuwita[2],
M. Rizal Damanik[1], Clara M. Kusharto[1] and Fumito Tani[3]
[1]Department of Community Nutrition, Faculty of Human Ecology,
Bogor Agricultural University, Bogor, Indonesia
[2]Laboratory of Embryology, Veterinary Faculty, Bogor Agricultural University (IPB), Bogor, Indonesia
[3]Laboratory of Food and Environmental Science, Division of Food Science and Biotechnology,
Faculty of Agriculture, Kyoto University, Japan

Abstract: β-carotene is the most widely studied carotenoid that has provitamin A properties beside its great promise for cancer chemoprotective and human physiological functions. β-carotene can be cleavaged to retinal by some enzymes in wide variety of cells especially in intestinal cells. The retinal is further metabolized to retinoic acid by Aldehyde Dehydrogenase (ALDH) family of enzymes. In this study we use CMT-93 cell line as a cell model of intestinal epithelial cell to study the effect of β-carotene on cells morphology, proliferation and Aldehyde Dehydrogenase (ALDH) gene expression. These cells were grown in DMEM supplemented with 10% heat-inactivated Fecal Bovine Serum (FBS), 4 mM L-glutamine, 50 U/ml penicillin and 50 mg/ml streptomycin. Tetrahydrofuran (THF) (1.25%) was used to solve β-carotene (Sigma). Final concentration of β-carotene in medium for proliferation were 0.5, 1.0, 1.5 and 5.0 μM. The results showed that in higher concentration (1.5 μM), β-carotene can alter the morphologycal structure of monolayer epithelial cells of CMT-93. Meanwhile, β-carotene in supra physiological concentration (5.0 μM) significantly ($p<0.05$) decreased proliferation, exhibited *ALDH1A2* gene expression and changed morphological structure.

Key words: β-carotene, *ALDH1A2* gene expression, CMT-93 cell line, proliferation

INTRODUCTION

Nutritional surveys from various countries consistently report β-carotene intake to be essential to meet vitamin A requirements (Grune *et al*., 2010). Beyond its provitamin A carotenoids properties, β-carotene has a lot of physiological functions, including lipid radical scavenger and as a singlet oxygen quenched, as demonstrated *in vitro*, antioxidant activity, cell development and cancer Protectants (Gropper *et al*., 2009; Bertram, 1994; Livny *et al*., 2002). β-carotene also plays a significant role in gene regulation and gap junction development (Frey and Vogel, 2011; Bertram, 1999; Elliot, 2005; Livny *et al*., 2002; Donaldson, 2011). Each β-Carotene molecule can be converted to two molecules of retinal by cleavage, specifically at its central double bond, catalyzed by β-carotene-15,15-dioxygenase. The enzyme activity is expressed specifically in intestinal epithelium and in liver (Duester, 2000; Nagao, 2004). To be functioned as ligands for nuclear Retinoic Acid Receptors (RAR) that regulate gene expression at cellular level, retinol and retinal should be further metabolized to retinoic acid. There are three major families dehydrogenases of retinoid metabolic pathways that catalyzing the reversible oxidation/reduction of retinol and retinal, i.e., the alcohol dehydrogenase (ADH), short-chain dehydrogenase/ reductase (SDR) enzyme families and aldehyde dehydrogenase (ALDH) family. Genetic studies indicate *in vivo* roles for two ADHs (ADH1 and ADH4), one SDR (RDH5) and two ALDHs (ALDH1 and RALDH2) all of which are conserved between humans and rodents (Duester, 2000).

A wide variety of cells, cultured in vitro, are able to convert β-carotene to retinol (Wei *et al*., 1998). Meanwhile, only limited papers explain about the conversion of β-carotene to retinoic acid in intestinal epithelial cells, that catalyzed by aldehyde dehydrogenases (Gropper *et al*., 2009; Grune *et al*., 2010). Aldehyde dehydrogenases which convert retinal to retinoic acid is regulated by ALDHs'gene. In this study we used CMT-93 to study the effect of β-carotene on intestinal epithelial cells morphological characteristic, proliferation and ALDH1A2 gene expression.

MATERIALS AND METHODS

Cell culture: The CMT-93 is a murine cancer cell line with a rectal epithelial cell-like phenotype (Iwakiri and Podolsky, 2001; Rosbottom *et al*., 2002) was obtained from culture collection of Laboratory of Food and Environmental Science, Division Food Science and

Corresponding Author: Katrin Roosita, Department of Community Nutrition, Faculty of Human Ecology, Bogor Agricultural University, Bogor, Indonesia

Biotechnology, Faculty of Agriculture, Kyoto University, Japan. These cells were grown in DMEM supplemented with 10% heat-inactivated Fecal Bovine Serum (FBS), 4 mM L-glutamine, 50 U/ml penicillin and 50 mg/ml streptomycin. The cells were incubated within a humidified incubator with 5% CO_2 at 37°C.

Tetrahydrofuran (THF) was used to solve β-carotene (Sigma) until 1.25% of final concentration. The final concentration of β-carotene in medium were 0.5, 1.0, 1.5 and 5 μM. Each treatment (β-carotene) and control were conducted in 3 to 4 plates of cell culture. The cells were incubated for 2 x 24 hours for each treatment. The morphological characteristic of CMT-93 was observed under fluorescent microscope (Olympus).

MTT assays: MTT (3- (4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays was performed by MTT Kit from Roche and according to manufacturer's instructions. For MTT assays, 2.0 x 10^4 cells/well were plated in 100 μl of culture media in 96 wells plates. Cells were incubated overnight at 37°C in a humidified atmosphere of 5% CO_2 for cells attachment. The following level of β carotene concentration were applied, i.e., 0.1, 0.5, 1.5 and 5 μM pure β-carotene (Sigma). As a negative and normal control of proliferation Triton-114 solution (1.25%) and standard medium (DMEM+10% heat-inactivated FCS) were used respectively. After 24 hours incubation, 10 μl of MTT solution (MTT reagent in 5 mg/ml PBS) was added into the plate. The result of MTT assays was determined by absorbance value in spectrophotometer at 550 nm (UV-240 Shimadzu).

Reverse Transcription (RT)-PCR analyses: Total cellular RNA was prepared using Qiagen RNAase-Mini Kit according to the manufacturer's instructions. The following cDNA probes were generated by Takara RT-PCR Kit. The number of cell for RT PCR were 5 x 10^5 cells to get appropriate result. The pellet of cells were preserved at -80°C before analyzed.

The primer sequence are as follow, ALDH1A2-sens (mouse): TGGGTGAGTTTGGCTTA CGG; Antisens: AGAAACGTGGCAGTCTTGGC; GAPDH-sens (mouse): TTCACCACCAT GGAGAA GGC, Antisens: GGCATGGACTGTGGTCATGA. The result of RT – PCR were represented by gel electrophoresis.

Statistical analysis: Significance of the different treatment was analyzed against control using Analysis of Variance (ANOVA) with Duncan post hoc analysis. It was considered to be significantly different when $p<0.05$.

RESULTS

Morphological structure of CMT-93: The normal morphological structure of CMT-93 was shown by cells that was cultured in control medium (Fig. 1a). The morphological structure of CMT-93 was slightly changed

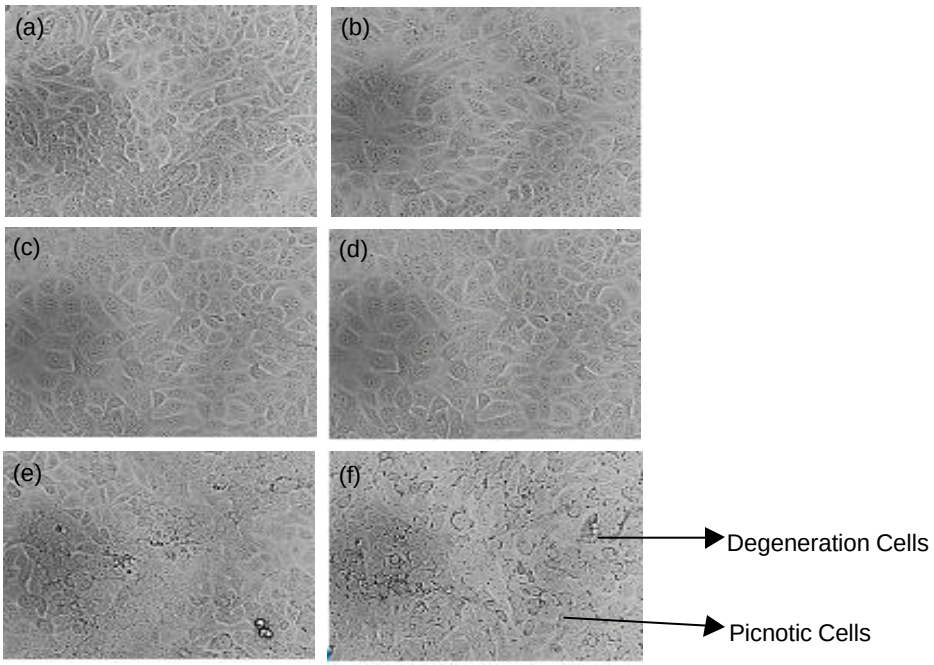

Fig. 1(a-f): Morphological differences between CMT-93 cell line in medium control (a), add medium added by THF (b), β carotene (0.1 μM) (c), β carotene (0.5 μM) (d), β carotene (1.5 μM) (e) and β carotene (5.0 μM) (f)

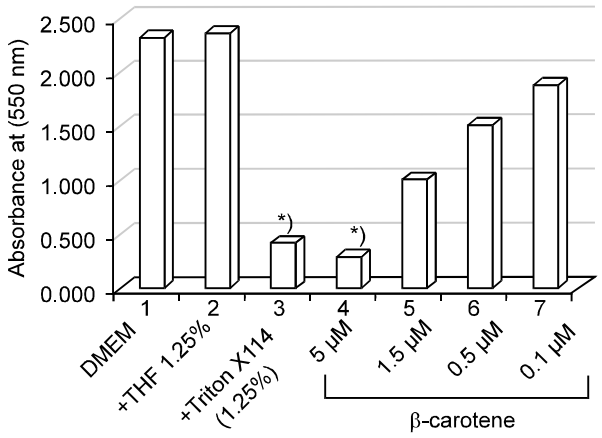

Fig. 2: The absorbance value of CMT-93 cell line after treated by MTT Assays with Triton 114, THF (1.25%) or β-carotene (concentration 0.1, 0.5, 1.5 and 5 µM)

in THF (1.25%) (Fig. 1b) and BCA (0.1 µM) and (0.5 µM) (Fig. 1c and d, respectively), where the CMT-93 cells grew larger. However, in higher and highest concentration of BCA (1.5 and 5.0 µM) showed degeneration and pycnotic of The CMT-93 cells, as shown in Figure 1e and 1f.

Proliferation and mitochondrial function: Figure 2 showed the absorbance value of MTT assays of CMT-93 cell line treated by Triton X-114, THF (1.25%) and β-carotene (concentration 0.1, 0.5, 1.5 and 5.0 µM). The absorbance value shows the relative proliferation of cell. According to Lu *et al.* (2012) MTT method is one of the most widely used methods to analyze cell proliferation and viability. It is taken up through endocytosis and is reduced by mitochondrial enzymes as well as endosomal/lysosomal compartments, then is transported to cell surfaces to form needle-like MTT formazans.

The lowest value of MTT assays is shown by BCA 1 [5.0 µM] and Triton X-114 (1.25%). Triton X-114 is a detergent-phase separation for extraction of lipophilic proteins (Malen *et al.*, 2010). Meanwhile THF (1.25%) does not significantly alter proliferation of CMT-93 cell line and even similar with DMEM that represent a normal proliferation rate of CMT-93 cell line.

The result of MTT assays (Fig. 2) also showed that β-carotene tend to supress proliferation as shown by absorbance values of β-carotene in all concentration that lower than control. The higher concentration of β-carotene the lower proliferation.

ALDH1A2 gene expression: The GADPH gene is a gene of housekeeping enzyme, was included to confirm result of the RT PCR analysis for mRNA target ALDH1A2 gene.

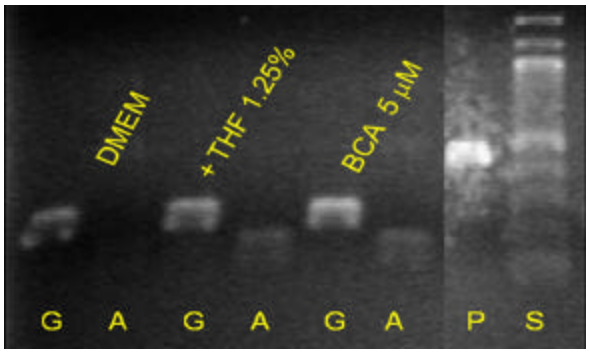

Fig. 3: GADPH and ALDH gene expression in CMT 93 cell line treated with or without THF (1.25%) and β-carotene (5 µM). A = ALDH1a-2, G = GADPH, P = Positif control, S = Standard

GADPH was expressed by all living cells in control medium (DMEM), THF 1.25% and BCA 5 µM. Meanwhile, the ALDH1A2 gene was expressed in THF (1.25) and β-carotene [5 µM].

DISCUSSION

β-carotene is carotenoid that has provitamin A activities with great promise for cancer chemoprotective and physiological functions (Zhang *et al.*, 1992). β-carotene has limited solubility in aqueous solution, thus in our study, THF was used as solvent for β-carotene to increase its solubility in aqueous culture medium. According to Craft and Soares (1992) the solubility of carotenoids including β-carotene was greatest in tetrahydrofuran compare to 17 others organic solvents, while acetonitrile exhibited the least solubility for β-carotene.

Distinct morphologic changes including changing cell-to-cell attachments, rounding and apparent swelling were observed in subconfluent cells treated with high concentration of β carotene [5 µM and 1.5 µM]. Some of these morphological changes, are correlated with study of human intestinal epithelial due to change of action microfilaments and cell volume that may lead to an alteration in tight junction function in the polarized intestinal epithelium (Koshy *et al.*, 1996).

According to Gross *et al.* (1997), supra physiological concentration β-carotene is >2.0 µM and physiological of concentration β-carotene is ~0.3-2.0 µM are consistent with clinical observation of human subjects. Thus this morphologycal structure changes of CMT-93 cell line is due to higher than physiological concentration of β-carotene.

Tetrahydrofurant (THF) is a widely used compound which is considered as not being easily biodegradable. As shown in Fig. 1, compare to cells that grown in control medium, the morphological structure of these cell is only slightly altered and the MTT assays also confirm these finding.

According to Boesch-Saadatmandi *et al*. (2011) THF 1.25% does not affect cells proliferation and viability. THF is superior compare with Fecal Calf Serum and Tween 40 with regard to carotenoid stability. This result also confirmed by morphological structure of CMT-93 cell line induced by β-carotene in physiological concentration [0.1 and 0.5 μM] which also contained 1.25% of THF in its culture medium.

Meanwhile, β-carotene at higher physiological concentration (1.5 μM) tended to decrease proliferation and in supra physiological concentration (5.0 μM) significantly inhibit proliferation (Fig. 2). In line with Wojcik *et al*. (2008) mitochondrial competence of cell determine by MTT assays express the proliferation index. Beta-carotene in the same concentration (5.0 μM) decrease the proliferation of rat oval cell *in vitro*.

Gel electrophoresis of RT PCR analysis showed that ALDH1A2 gene expression also altered by THF. We hypothesize *ALDH1A2* is expresed by CMT-93 cell line as process of THF detoxification. Regarding, THF that commonly used as solvent for carotenoid is not easily biodegradable and may result in cytotoxicity (Boesch-Saadatmandi *et al*., 2011; Yao *et al*., 2013).

In this study we used an acceptable dose of THF 1.25% that can be detoxify by the cells (Boesch-Saadatmandi *et al*., 2011). Our results showed that 1.25% in final concentration of THF was able to induced ALDH1A2 gene expression. We suggest that in this case, the ALDH1A2 gene expression was purposed as detoxification mechanism of cells that lead a normal morphological structure and proliferation of CMT-93 cell line.

ALDH gene super family encodes enzymes that are critical for certain life processes and detoxification via NAD (P)-dependent oxidation numerous endogenous and exogenous aldehyde substrates, including pharmaceuticals and environmental pollutants. The ALDH gene products appear to be multi functional protein, processing catalytic and non catalytic properties (Vasiliou and Nebert, 2005).

This study also revealed that ALDH1A2 gene expression on intestinal cells (CMT-93) induced by β-carotene. Regarding the metabolism β-carotene, *ALDH* plays an important role in formation of molecules retinoid acid. After converting β-carotene to retinal catalyzed by β-carotene-15,15_-dioxygenase, ALDH catalyzes the oxidation of retinal to retinoid acid. The 9-cis-retinoid acid function as ligands for nuclear retinoid acid receptors (RAR) that regulate gene expression (Jackson *et al*., 2011; Duester, 2000).

In conclusion, β-carotene in supra physiological concentration (5 μM) significantly ($p<0.05$) decreased proliferation and changed morphological structure of CMT-93 epithelial cell line. We propose that intestinal epithelial cells can convert β carotene to retinoid acid in the present of *ALDH1A2* expression and CMT-93 cell line can be used as model to study β-carotene conversion from many kind plant base of food.

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