

β -carotene Roles in Proliferation and Differentiation, Connexin and β -casein Gene Expression of Mammary Gland Cells Line

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ABSTRACT

Introduction: The objectives of this study were to examine the effect of β -carotene on cells proliferation and differentiation, connexin (Cx43) and β -casein (Csn2) gene expression of mammary gland cells line (HC11). Cx43 involves development of gap junction intercellular communication, while Csn2 induces milk synthesis in differentiated mammary gland cells. **Method:** The HC11 cells were grown in medium RPMI 1640 supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 50 mg/ml streptomycin and enhanced by epidermal growth factor (EGF), insulin, hydrocortisone and prolactin. Final concentration of β -carotene in medium was 0.5, 1.5 and 5.0 μ M respectively. Cell proliferation was determined by a colorimetric assay for assessing cell viability (MTT assays). Gene expression was analysed by reverse transcription polymerase chain reaction (RT-PCR) and gel electrophoresis. **Results:** Triton X-114 as a negative control significantly decreased proliferation of HC11 cell line as compared to its control (<0.05%). Meanwhile, β -carotene even in supraphysiological concentration [5 μ M] did not affect proliferation of HC11 cell line. The structure of mammosphere of HC11 cell line treated with supraphysiological concentration β -carotene [5.0 μ M] was largest with higher density and tightly connected with their neighbouring cells. Cx43 and Csn2 saw higher expression on β -carotene treated HC11 cell lines. **Conclusion:** β -carotene did not inhibit cell proliferation; meanwhile, differentiation of mammary gland cells line (HC11) represented by mammosphere development, and genes expression of connexin (Cx43) and β -casein (Csn2) were induced by β -carotene.

Key words: β -carotene, β -casein (Csn2), connexin (Cx43), genes expression, lactogenesis mammary gland cells line (HC11), mammosphere development

INTRODUCTION

Lactation is one of several important physiological process which determines growth and development of an infant. During this stage, mammary gland cells are

very active on synthesising milk from its precursors, macro and micro nutrients, under hormonal regulation. β -carotene plays a significant roles among micronutrients. With regard to its provitamin A property, β -carotene is a safe source of vitamin A during

lactation period (Grune *et al.*, 2010). Moreover, β -carotene has some role in the physiological functions of the mammary gland with regard to its antioxidant activity, increasing milk yield and mammary gland health (Yang, Li & He (2006), although the mechanism is still unclear.

Several studies have revealed that β -carotene increases gene expression and gap junction intracellular communication (GJIC) which leads to cell differentiation (Frey & Vogel 2011; Elliot 2005, Livny *et al.* 2002; Donaldson 2011). GJIC is a channel like structure in the cell membrane that is developed by connexin. GJIC maintains cell membrane permeability, regulates cell metabolism and differentiation. During lactation, the development of the mammary gland GJIC is very critical for sustainability of milk production (Gropper, Smith & Groff, 2009; Neville, 2009; Solomon, 2001; Talhouk *et al.*, 2005).

Meanwhile, the effect of β -carotene on gene expression and differentiation of the mammary gland during lactation has not been revealed. The objectives of this study were to examine the effect of β -carotene on cell proliferation, connexin-43 and β -casein gene expression, and differentiation of mammary gland cell lines.

METHODS

Cell culture

Mammary gland cell line (HC11) was obtained by Professor Nancy Hynes from Friedrich Miescher Institute for Biomedical Research, Switzerland. These cells were grown in RPMI supplemented with 10% heat-inactivated Fetal 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% CO₂ at 37°C.

Tetrahydrofuran (THF) was used to solve β -carotene (Sigma). THF was only 1.25% of final concentration, meanwhile final concentrations of β -carotene in medium

were 0.5, 1.0, 1.5 and 5 μ M. Number of samples for each treatment to analyse cell differentiation and gene expression were 3 to 4 plates. The cells were incubated for 2 x 24 h for each treatment. The mammosphere structure was observed under a microscope (inverted epifluorescent Olympus).

MTT assays

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is a colorimetric method for assessing cell viability, was performed by MTT Kit from Roche and according to manufacturer's instructions. For MTT assays, 2.0 $\times 10^4$ cells/well were plated in 100 μ l of culture media in 96 wells plates, 6 wells for each treatment (n=6). Cells were incubated 24 h at 37°C in a humidified atmosphere of CO₂ 5% for cell attachment. The following β -carotene concentrations were applied: 0.1, 0.5, 1.5 and 5 μ M of β -carotene 98% purity (Sigma). Triton-114 solution (1.25%) and standard medium (RPMI+10% heat-inactivated FCS) were used as negative and normal control of proliferation, respectively. After 24 h incubation, 10 μ l 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 microplate reader at 550 nm (Biorad).

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. To get appropriate results, the number of cells required for RT PCR was 5 $\times 10^5$ cells. The pellet of cells were preserved at -80°C before analysis.

The primer sequence were as follow: (1) α -casein GTGGCCCTTGCTCT-TGCAAG (forward) and AGTCTGAGGAAAAGCCTG AAC (Reverse); (2) Connexin-43: GTTCAGCCTGAGTGCCGTCTAC(Forward) and GGCTCT-GCTGGAAGGTCCGCTGATC (Reverse); (3) GAPDH: ACGACCCCTTCAT

T-GACCTC (Forward) and CTTTCCAGAG GGGCCATCCAC (Reverse).

Statistical analysis

Significance of the different treatments was analysed against control using Analysis of Variance (ANOVA) and Duncan Multiple Range Test (DMRT) post hoc analysis. It was considered to be significantly different when $p < 0.05$.

RESULTS

Proliferation of HC11 cell line

As shown in Figure 1, Triton X-114 significantly decreased proliferation of mammary gland (HC11) cell line compared to its normal control ($p < 0.05\%$). Meanwhile, β -carotene even in supraphysiological concentration [$5\mu\text{M}$] did not affect proliferation of HC11 cell line.

Differentiation of HC11

The mammosphere structure of mammary gland cells (HC11) treated by different concentrations of β -carotene is shown in Figures 2A, B, C, D. The morphology of HC11 that was treated by β -carotene is larger, of higher density and more tightly connected with neighbouring cells. Figure 2D shows that the mammosphere structure of HC11 cells treated with upraphysiological concentration of β -carotene has a largest single mammosphere with a lot of fluid inside its bubbles-like (dome) structure.

In response to lactogenic hormones, HC11 cells are differentiated, develop mammosphere structure and synthesize milk proteins. Mammosphere is a formation of dome shape cell structures in mouse mammary epithelial cell cultures that are commonly used to determine the degree of lactogenic differentiation (Morrison & Cutler, 2010).

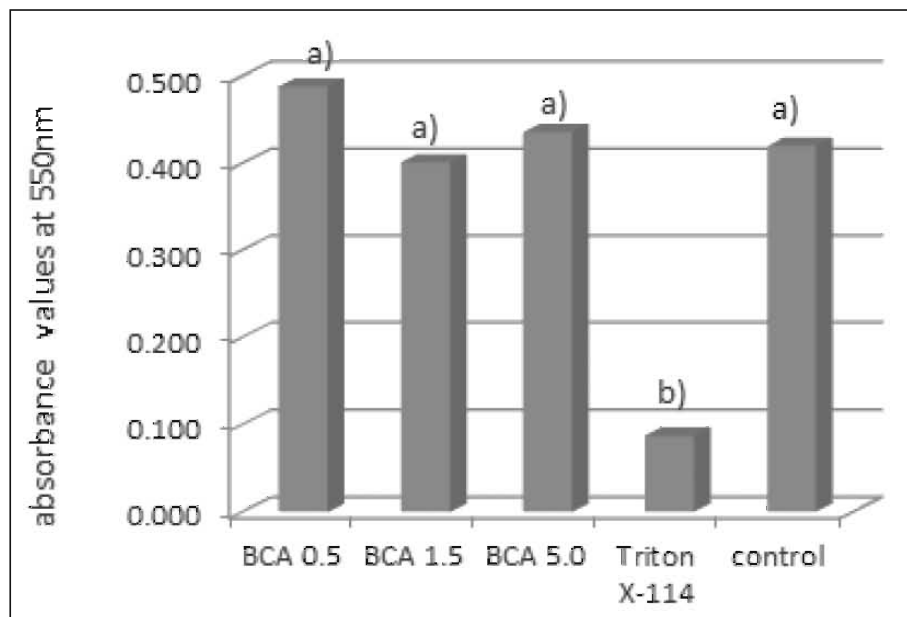


Figure 1. The absorbance value of HC11 cell lines proliferation treated by β -carotene (BCA) 0.5, 1.5 or $5.0\mu\text{M}$ as compared to Triton X-114 and its control ($n=6$). Significant difference ($p < 0.05$) between treatments is shown by different alphabets on each bar. [Standard deviation (SD) for BCA 0.5, BCA 1.5, BCA 5.0, Triton X-114 and control are 0.041, 0.032, 0.058, 0.042 and 0.042 respectively].

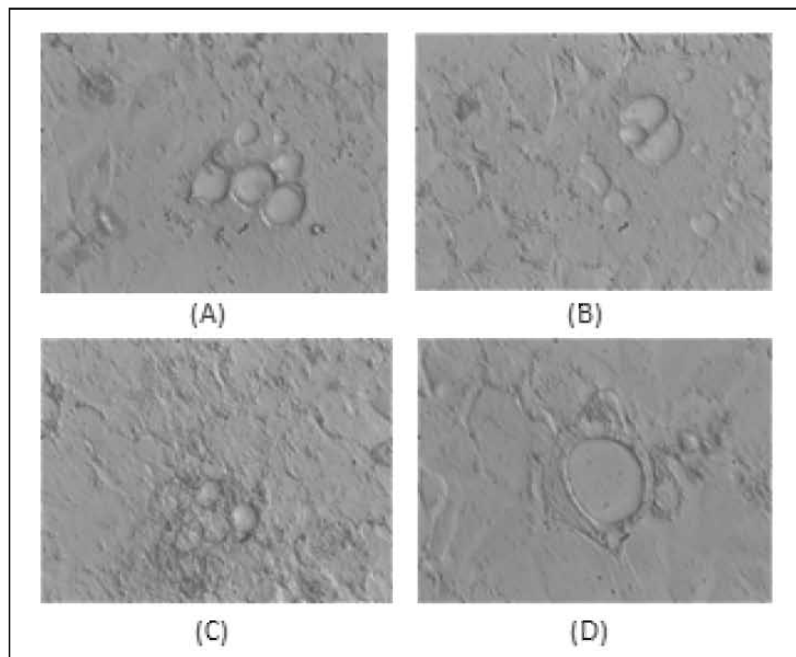


Figure 2. The morphology of control (A), β -carotene 0.5 (B), 1.5 (C) dan 5.0 (D) μM of differentiated HC11cells. (40x magnification)

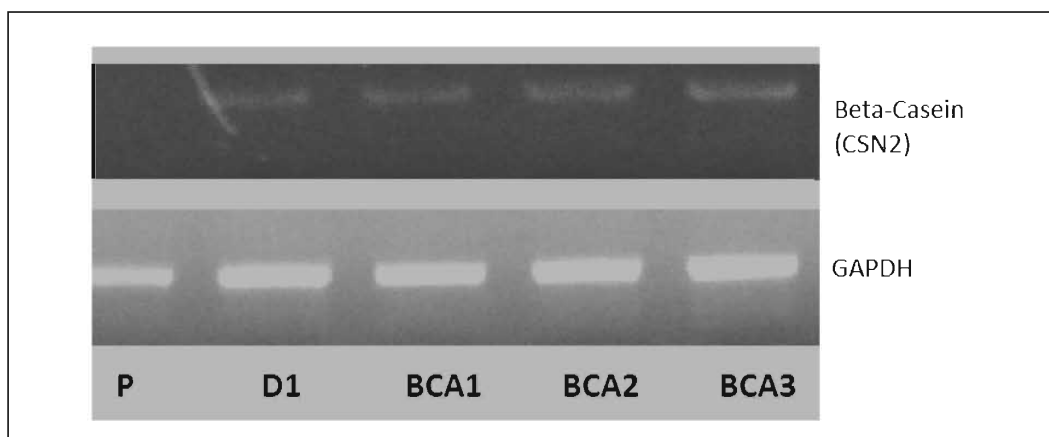


Figure 3. Gene Expression of β -casein (*Csn2*) and *GADPH* of proliferated (P), differentiated (D1), and differentiated with β -carotene treatment BCA1 (0.5 μM); BCA2 (1.5 μM), and BCA3 (5.0 μM) of HC11 cell line.

Gene expression of connexin (Cx43) and *Csn2*

Figure 3 shows differentiated HC11 cell line with (BCA1, BCA2, BCA 3) and without (D1). β -carotene treatment induced β -casein

(*Csn2*) gene expression but the proliferated (P) HC11 cell line did not. Meanwhile, *GADPH* gene were expressed in all HC11 cells lines, both in proliferated or differentiated cells. It can also be seen in the figure that in

higher concentrations of β -carotene treatments, that is, BCA2 and BCA3, their concentrations are 1.5 and 5.0 μ M respectively, and the RT PCR bands are thicker and brighter. From these results, it is proposed that in higher concentrations of β -carotene, the gene expression of β -casein (*Csn2*) is higher.

The mechanism of β -carotene effect on differentiation and lactogenesis of mammary gland epithelial cells was studied by analysing gene expression of *connexin* (*Cx43*). We used the same HC11 cell lines samples that expressed β -casein gene. The results are shown in Figure 4. *Connexin* (*Cx43*) gene was expressed by all differentiated HC11 cell lines. The bands of *Connexin* (*Cx43*) gene of β -carotene treated HC11 cell lines on gel electrophoresis were thicker and brighter compare to its control (Figure 4D1).

DISCUSSION

Synthesized β -carotene was the treatment used in this study. According to Grune *et al* (2010), there is no difference between naturally occurring or chemically synthesised β -carotene.

The results showed that β -carotene did not influence proliferation of mammary gland cell lines, unlike the results of Wojcik, Bobowick & Martelli (2008) that indicated β -carotene inhibits proliferation in oval cell lines. Our previous study (Roosita *et al.*, 2013) also showed that β -carotene in supraphysiological concentration (5.0 μ M) inhibits proliferation development of intestinal epithelial (CMT-93) cells lines.

We propose that the effect of β -carotene was countered by epidermal growth factor (EGF). EGF was applied in culture medium of mammary gland cells for normal growth as shown *in vivo*. Meanwhile, the EGF was not used in other previous studies (Wojcik *et al.*, 2008; Roosita *et al.*, 2013).

According to Pena & Rosenfeld (2001), EGF induces mammary gland cells

proliferation *in vivo*. As the EGF effect is stronger than that of β -carotene, the effect of supraphysiological concentration of β -carotene on inhibition of cells proliferation could be countered by EGF that induces cells proliferation.

The structure of mammosphere as a specific marker of mammary gland cell differentiation is correlated with *Csn2* gene expression. We suggest that mammary gland differentiation, as represented by development of mammosphere and *Csn2* gene expression, is influenced by β -carotene. According to Lemay *et al.* (2007) and Maningat *et al.* (2008), *Csn2* gene is highly expressed during lactogenesis. *Csn2* gene expression is needed for milk synthesis.

Connexin (*CX43*) gene was expressed by all differentiated HC11 cell lines that express *Csn2* gene. The bands of RNA of *Connexin* (*Cx43*) gene of HC11 cell line treated by β -carotene looked thicker and brighter as compared to its control. We suggest that β -carotene increases the gene expression of *connexin* (*Cx43*). This result is similar to that of Naves *et al* (2001) who found β -carotene and vitamin A modulated *connexin43* gene expression on hepatocytes. The study of Novo *et al.* (2013) showed that β -carotene increased cardiac *connexin43 in vivo*. According to Haddad *et al* (2013), β -carotene is involved in regulating *connexin 43* gene expression.

Our previous study (Roosita *et al.*, 2013) revealed that β -carotene treatment exhibited aldehyde dehydrogenase (*ALDH1A2*) gene expression and changed morphological structure of CMT-93 epithelial cell lines. According to Jackson *et al.* (2011) and Duester (2000), *ALDHs* play an important role in the metabolism of β -carotene. After conversion of β -carotene to retinal that is catalysed by β -carotene-15,15-dioxygenase, *ALDH* catalyses retinal oxidation to retinoic acid. The 9-cis-retinoic acid functions as ligands for nuclear retinoic acid receptors (RAR) that regulate gene expression. According to Yeh & Hu (2003), high

concentrations of β -carotene treatment increase gap junction intracellular communication (GJIC) and *connexin 43* synthesis

Figure 4 also shows that *connexin (Cx43)* gene expression is correlated with β -casein (*Csn2*) gene expression. According to Monaghan & Moss (1996) in the normal human breast, the basal cells express *connexin43*, although human mammary epithelial cells *in vitro* have been reported to express both *connexin26* and *connexin43*.

According to Nagamatsu & Oka (1983), casein-gene expression is an important marker of lactogenesis. The regulation of the synthesis of casein, during the development of the mammary gland is of particular interest. As casein is a major milk protein beside lactalbumin, this protein can serve as a specific marker for the differentiated function of mammary epithelium.

Talhok *et al.* (2005) proved that *connexin* is a protein that develops gap junction structure in neighbouring mammary epithelial. Furthermore, according to O'day (2010), *Cx3* is an important protein during lactogenesis, especially for milk secretion and in preventing breast cancer.

In conclusion, due to EGF application, β -carotene does not inhibit proliferation of mammary gland cell lines (HC11). Meanwhile, differentiation of mammary gland cells lines, that are represented by mammosphere development, expression of *connexin (Cx43)* and β -casein (*Csn2*) genes are induced by β -carotene.

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