Beta-carotene uptake and metabolism in human lung bronchial epithelial cultured cells depending on delivery vehicle

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Abstract

Beta-carotene (BC) could have a protective or pro-carcinogenic role in lung cancer, and cell culture systems are important to evaluate it. Nevertheless, the delivery of the hydrophobic BC to cells is difficult. Different vehicles have been used such as liposomes, tetrahydrofuran and serum lipoproteins, but presenting different problems. Water dispersible beadlets containing BC are a good choice and can produce the greatest BC uptake when compared to the above vehicles, but other beadlet components could alter the results. Dimethylsulfoxide (DMSO) could be a good alternative since it has low toxicity and it enhances the penetration of substances across biologic membranes. We aimed to characterize an appropriate model for delivering all-trans-BC to lung cells in culture and knowing its metabolism. All-trans-BC 5 μM was administered to BEAS-2B cells in beadlets or DMSO, and medium and cell samples were taken at different times. The levels of BC and its main isomers and metabolites were determined by HPLC. All-trans-BC reached the same levels in the medium (about 3.5 μM) either when supplied in beadlets or in DMSO, and, with beadlets, 13-cis-BC was also detected. However the amount of all-trans-BC taken up by the cells was the triple when delivered by DMSO. With both vehicles, intracellular all-trans-BC levels reached its maximum after 24 h of treatment, remaining equal after 72 h. The 9-cis and 13-cis isomers of BC, and oxidized metabolites, were also detected in the cells although in smaller proportion than all-trans-BC, especially with DMSO. An LDH assay did not suggest toxicity of beadlets, DMSO or BC itself. In conclusion, DMSO seems the most appropriate vehicle for delivering BC to lung cells in vitro, and we present a model that allows studying the effects of BC and its metabolism in the lung human BEAS-2B cell line.

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Keywords: Beta-carotene; Lung cancer; BEAS-2B; Dimethylsulfoxide; Beadlet

1. Introduction

Over the last years, beta-carotene (BC) has aroused interest about its possible protective role against lung cancer in risk populations on the basis of epidemiological evidence [1–3]. Nevertheless, when two large-scale human intervention trials assessing this question, the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) trial in Finland [4] and the Beta-Carotene and Retinol Efficacy Trial (CARET) in USA [5], were carried out, the results did not confirm the positive expectations. It was shown that 20–30 mg/person/day doses of supplemental BC increased the risk of lung cancer in risk populations such as smokers and workers exposed to asbestos [4,5]. These unexpected results led to questioning and further investigating the possible protective or pro-carcinogenic effect of BC in cancer, since our understanding of the interactions between tobacco carcinogens and BC is still poor, as well as the actions of BC on gene expression, in target cells as lung epithelial cells. Some interesting in vivo studies have been performed in ferrets [6,7], by using doses comparable to those used in human trials and showing potential mechanisms (alterations in retinoic acid signalling pathways; induction of specific oncogenes) by which β-
carotene supplementation could have enhanced the incidence of lung cancer in the two major intervention trials involving human smokers. In these studies it was shown that, in contrast with a pharmacological dose, a physiological lower dose of BC in smoke-exposed ferrets has no potentially detrimental effects and may afford weak protection against lung damage induced by cigarette smoke [6,7]. These results open new questions about the different protective or harmful roles of BC depending on its dose or concentration.

Having good in vitro models with human cells for studying the effects of high and physiological concentrations of BC on lung cancer development becomes necessary to evaluate the effects of BC on the molecular and cellular processes apparently involved. In this sense, an important problem emerges, because the delivery of BC to cells in culture presents unique challenges due to its high hydrophobicity. Although numerous assays in vitro delivering BC to different cell types have been carried out, some confusion exists, since the BC uptake or its stability have often been poor, or not analyzed or there haven’t been considered the possible effects of the vehicle on the cells or the formation and action of BC metabolites. Thus, references to different vehicles for delivering BC to cultured cells can be found in the literature, such as ethanol, liposomes, mixed micelles, tetrahydrofuran (THF), and human lipoproteins or BC enriched steer serum, but each one with different limitations [8–14]. Water dispersible beadlets containing BC have been proved as a reasonable alternative and they can produce the greatest accumulation of BC in cells when compared to enriched serum, THF and liposomes [11], probably constituting the best vehicle for delivering the BC when compared with all the vehicles given above, both in terms of cellular uptake and minimized degradation [11]. Nevertheless, the beadlets have a complex composition, carrying not only BC, but also other components that could interfere with the experimental results. Dimethylsulfoxide (DMSO) has also been used as a vehicle in different studies, although with less frequency than the others, and its properties for delivering the BC to the cells have not been analyzed in depth. In fact, DMSO is an organic solvent which presents a rapid penetration across biologic membranes [15] and low toxicity properties for cells in vitro [16].

The objective of the present work was to establish and characterize an appropriate model for delivering all-trans-BC (all-t-BC) to human bronchial epithelial lung cells in culture (using BEAS-2B cells), analyzing all-t-BC uptake, its stability and its metabolism in the cells. We compared the two different vehicles that were the major options in our perspective: water dispersible beadlets containing all-t-BC and DMSO.

Our results show that DMSO appears to be the most appropriate vehicle for delivering BC to cells in vitro, and we present an in vitro model for studying the effects of BC in cultured lung cells.

2. Materials and methods

2.1. Chemicals

Immortalized human bronchial epithelial cells (BEAS-2B) were obtained from the American Type Culture Collection (LGC Promochem, Barcelona, Spain). Cell culture medium (BEGM®-Bronchial Epithelial Medium) was from Clonetics (Innogenetics, Barcelona, Spain). Collagen type I-coated 25 cm² flasks were from Becton Dickinson (Madrid, Spain). All-trans-beta-carotene was obtained from Sigma (Sigma-Aldrich Quimica, Madrid, Spain). Water dispersible beadlets with and without 10% BC were a gift from Hoffmann-La Roche (Basel, Switzerland). The composition of the beadlets was approximately the following: fish gelatine–sugar (44%), crystallized sugar (24%), ascorbyl palmitate (5%), corn oil (5.5%), DL-α-tocopherol (1.5%) and fluidized corn starch (20%). HPLC reagents were from Sharlau (Sharlab, Barcelona, Spain), Merk (Barcelona, Spain) and Panreac (Barcelona, Spain); the 13-cis-β-carotene, 9-cis-β-carotene, β-apo-4′-carotenal, β-apo-8′-carotenal, β-apo-12′-carotenal, and 5,6′-epoxy-β-carotene standards were provided as gifts from Hoffmann-La Roche, and the other standards, i.e., all-trans-β-carotene, all-trans-retinal and all-trans-retinol, were purchased from Sigma. Routine chemicals were from Sigma, Merk and Panreac. Filters for producing orange light (778 Millennium Gold) were from Lee Filters (Hampshire, UK), and absorbed all light under 560 nm.

2.2. Cell culture and treatments

We used BEAS-2B, a human bronchial epithelial cell line transformed with the 12-SV40 virus hybrid. The cells were cultured in a serum-free medium (BEGM®-Bronchial Epithelial Medium) following the instructions of the ATCC. The cells were grown in collagen type I-coated 25 cm² culture flasks containing 5 ml of medium and incubated in a humidified atmosphere with 5% CO₂ at 37°C. All the treatments were carried out at day four of culture, when the cells were 70–80% confluent, and under orange light (normal light filtered by 778 Millennium Gold filters) in order to preserve BC integrity. Cells were given a calculated quantity of all-t-BC to obtaining a 5 μM final concentration in the culture medium, dissolved in DMSO (never exceeding the final concentration of 1% DMSO), or by dispersing all-t-BC-containing-beadlets, or with the same quantity of beadlets without BC (placebo), and we also cultured control cells without any treatment (negative controls). Samples of both medium and cells were collected at different times since the beginning of the treatments (0, 6, 12, 24, 48 and 72 h) for carotenoids extraction and HPLC analysis. The treatments and analyses were carried out in three separated experiments performed at least per duplicate. The morphology of the cells was followed by visualizing them with a phase-contrast microscope (Nikon Eclipse TS100-F).
2.3. Cell sample collection

Cell sample collection for HPLC analyses of intracellular BC and its isomers and metabolites was performed as follows. First, the cells were submitted to 6–2–5 min washes with HBS buffer (Hepes-buffered saline [17] plus 2% BSA (fraction IV)), additional washes were performed if any sign of precipitated or crystallized BC was observed. After washing, the cells were detached with trypsin/EDTA/PVP (0.25%/0.02%/0.5%) (1 ml/flask); a 50 μl aliquot was used for cell counting. The rest of the cells were collected by centrifugation (10,000×g, 5 min). The resulting pellets were frozen at −80 °C and used for HPLC analysis.

2.4. Quantitative analysis by HPLC of all-trans-beta-carotene, -retinal and -retinol, 9 and 13-cis-beta-carotene, apo-carotenals and 5,6-epoxy-beta-carotene in cultured cells and cell culture medium

Stock solutions of pure standard compounds, i.e. all-t-BC, its isomers 13-cis-β-carotene, 9-cis-β-carotene, and β-apo-carotenals, were prepared by dissolving 3–5 mg of the compound in n-hexane/dichloromethane 98:2 (v/v) containing 0.025% BHA (v/w). Immediately afterwards, the absorbance was measured (n-hexane was used as a reference) and the exact concentration was determined using the following formula:

\[ \text{Concentration} = \frac{ \text{Absorbance} \times \text{Concentration Standard} }{ \text{Absorbance Standard} } \]

where the absorbance was measured. The cell culture medium (25 ml) was extracted by adding 225 ml of a solvent mixture EtOH/tBME/THF, 9:5:1 (v/v/v), containing 0.025% of BHA (v/w). Immediately afterwards, the mixture was then vigorously vortexed for 1 min and centrifuged for 4 min at 13,000×g. Routinely, 25 μl of the clear supernatant was injected into the HPLC system. The cell pellets were treated with 200 ml acetone containing 0.025% BHA (v/w), vortexed, and dried in speed-vac apparatus. The dried residue was redissolved in 200 ml of the solvent mixture described above, vortexed for 1 min, and centrifuged for 4 min at 13,000×g. Then, 25 ml of the clear supernatant was injected into the HPLC system. Carotenoid levels in the cultured cells and cell culture medium were calculated by determining the peak areas against known amounts of standards.

The HPLC system for all analyses was comprised of a Shimadzu CLASS-LC10 model with diode-array detection. HPLC analyses were performed isocratically on a C18-reversed phase column (Vydac 218TP54, 4×250 mm; The Separations Group, Hesperia, CA, USA) with acetonitrile/tBME/aqueous ammonium acetate 80 mM/triethylamine, 730:200:70:0.5 (v/v/v/v), as eluent. The flow rate was 1.5 ml/min and the peak detection was set at 450 nm. Additionally a photodiode array detector was used to measure the absorption spectra of each detected peak.

The BC isomers and BC oxidation products were characterized on the basis of their UV/vis spectra and retention times (see Table 1). The isomers of β-carotene were identified as 13-cis-β-carotene, 9-cis-β-carotene and all-trans-β-carotene. The principal metabolites were identified as β-apo-4’-carotenal, β-apo-8’-carotenal, β-apo-12’-carotenal, all-trans-retinal (β-apo-15’-carotenal), all-trans-retinol and 5,6-epoxy-β-carotene.

2.5. Lactate dehydrogenase (LDH) assay

In order to determine any possible toxic effect of the vehicles used or the beta-carotene itself, the LHD assay was performed. LDH in the culture medium was quantified using a LD-L 10 kit (Sigma). Total cellular LDH was quantified from the culture medium of cells treated with 1% Triton X-100.

2.6. Statistical analysis

All data are presented as mean value ± S.E.M. The effects of the different treatments on the studied parameters were tested by one- and two-way analysis of variance (ANOVA), and contrasts between means were assessed by least-significant difference (LSD) and t-test post hoc comparisons, using the program SPSS® for Windows. Results were considered statistically significant at the P<0.05 level.

3. Results and discussion

In the present work, our aim was to find a good vehicle for delivering the hydrophobic BC to human lung cells in culture and knowing its metabolism in these cells, in order to establish an appropriate model for studying the effects of BC in the cells of the human lung in vitro. Here it is shown that, in spite of previous reports, dissolving BC in DMSO seems the best way to deliver it to cultured cells, as it is taken up in bigger amounts than with all the other vehicles used in the literature and it keeps its integrity in the cells for a prolonged period of time (at least up to 72 h as studied here).
We have compared the delivery and the integrity of BC dissolved in DMSO with BC as a component of water dispersible beadlets since it has been demonstrated by Williams et al. [11] that the beadlets are a more appropriate vehicle when compared with common vehicles such as THF and liposomes (both in terms of BC integrity and uptake). Moreover, the beadlets allow, in fact, greater accumulation of BC in cultured prostate cancer cells than BC enriched steer serum, which perhaps would be a more physiological approach but not useful for most of scientists due to the fact that BC must be incorporated in vivo into the lipoprotein particles (as it happens with the use of human liproteins) [9,11]. Nevertheless, the beadlets have other components that could alter the experimental results, such as alphatocopherol, corn oil, ascorbyl palmitate, etc.; thus, if we deliver the BC to the cells in beadlets, although using control experiments with beadlets without BC, we would be analyzing the effects of BC in the presence of other antioxidants and components and not the effects of BC per se. With respect to the other vehicles used in the literature, solvents such as ethanol or the above mentioned THF show important problems. Ethanol has a poor solvent capacity for BC [12]. THF has been widely used (alone or in combination with ethanol or others) for the in vitro delivery of BC but, as Cooney et al. observed [8], it can be toxic to certain cell lines, and it is also associated with the enhancement of tumour formation [10,14]. THF has also been shown to inhibit a number of cytochrome P450 dependent mixed function oxidase activities [18], and the cytochrome P450 is one of the affected systems when assessing the co-carcinogenic effect of BC in rats [19,20]. These facts indicate that the use of THF as a solvent for BC can interfere in the studies related to cancer. Other methods such as liposomes or mixed micelles are not useful due to problems of instability of carotenoids and poor uptake by the cells [9,11].

In this scenario, the comparison of the also used but less studied DMSO as a vehicle with water dispersible beadlets seems a reasonable approach. In fact, as we see in Fig. 1, when all-t-BC is given to the lung BEAS-2B cells in culture in order to reach a final concentration of 5 μM, the actual concentration reached is quite high, about 3–3.5 μM, both with DMSO and beadlets. The time-course all-t-BC degradation curve in the medium presented no differences when using either DMSO or beadlets, and we can still find about 10–15% of the initial BC amount at the final of the experiment (72 h from the beginning of treatment). With the beadlets vehicle, the isomer 13-cis-BC is also found in significant quantities in the medium, although at much lower levels when compared with all-t-BC. Nevertheless, DMSO allows having a more pure all-t-BC containing medium, since this 13-cis isomer does not appear in the medium of the cells treated with BC given in DMSO. The 9-cis-BC isomer and the oxidized metabolites of BC were not detected in the culture medium (data not shown).

Although the all-t-BC levels in the culture medium are essentially equal throughout the experiment when we compare both vehicles, the cellular uptake is tripled when given in DMSO than in beadlets (see Fig. 2). In the time-course uptake curves it is shown that almost since the beginning of the treatment (at 6 h) the DMSO-BC treated cells take up more all-t-BC. With both vehicles, the maximum intracellular amount of BC is reached at 24 h and these levels are maintained until the end of the experiment (72 h). Thus, the dynamics of the all-t-BC uptake is practically the same when delivered in DMSO than with beadlets, but allowing a significantly higher uptake with the first one. On the other hand, the maximum quantity of all-t-BC taken up (638 pmol with DMSO and 268 pmol with beadlets) cannot account for the all-t-BC lost in the culture medium, not only because of the different time curves of cellular uptake and disappearance in the medium.

![Fig. 1. Time-course curves of all-t-BC and 13-cis-BC—13(Z)-BC—levels in the culture medium after the beginning of treatment (time 0). Two-way ANOVA: T, time effect; V, vehicle effect; and TxV, interactive effect. Post-hoc comparisons (P<0.05): for LSD, within the same group of vehicle, values not sharing a common letter are statistically different; for Student’s t-test, * indicates significant differences between BC-DMSO and BC-beadlets-treated cells.](image1)

![Fig. 2. Time-course curves of intracellular all-t-BC levels after the beginning of treatment (time 0). Two-way ANOVA: T, time effect; V, vehicle effect; and TxV, interactive effect. Post-hoc comparisons (P<0.05): for LSD, within the same group of vehicle, values not sharing a common letter are statistically different; for Student’s t-test, * indicates significant differences between BC-DMSO and BC-beadlets-treated cells.](image2)
(see Figs. 1 and 2), but also due to the fact that these quantities are much lower than the total quantity of all-t-BC in the medium, where it is in excess, at 24 h and still at 72 h. At the sight of all these results, it is shown that DMSO allows an enhanced uptake of the all-t-BC by the cells; in fact, DMSO is an organic solvent which has a very strong affinity for water and rapid penetration across biologic membranes, as well as it enhances the penetration of substances dissolved in it [15], being an interesting membrane penetrating-carrier.

Despite in the culture medium we can basically find pure all-t-BC, different isomers and oxidation products are found within the cells (see Figs. 3 and 4 and Table 2). The 9-cis-beta-carotene—9(Z)-BC—and 13-cis-beta-carotene—13(Z)-BC—increase their intracellular levels in a similar way when the all-t-BC is given with both DMSO and beadlets, although there is a little different behaviour of the 13-cis-BC intracellular levels when comparing both vehicles. The initial slightly higher 13-cis-BC levels in the cells treated with beadlets (at 24 h) could be due to the above reported presence of this isomer in this vehicle (Fig. 1). Nevertheless, considering the appearance of the 13-cis isomer also in the BC-DMSO treated cells, it can be deduced that the 13-cis-BC isomer is formed inside the cells, as well as the 9-cis-BC isomer. Although the total amounts of these isomers are small when compared with the total intracellular all-t-BC, their possible effects in the cells can be of importance and need to be considered in future studies of BC actions in lung cells. For instance, the 9-cis-BC isomer is known to be the precursor of 9-cis-retinoic acid [21,22], which is active in gene regulation [23,24] and has been postulated to be a potential chemopreventive agent for former smokers [25].

Table 2
Intracellular levels (pmol/10^6 cells) of the studied BC metabolites depending on time exposure to all-t-BC given in DMSO or beadlets

<table>
<thead>
<tr>
<th>Compound</th>
<th>Vehicle</th>
<th>Time</th>
<th>0 h</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-apo-4’-carotenal</td>
<td>Beadlets</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.79±0.39^a</td>
<td>4.57±1.58^b</td>
<td>8.93±2.59^c</td>
<td>T, V, TxV</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>ND</td>
<td>ND</td>
<td>0.56±0.43^b</td>
<td>0.11±0.07^a</td>
<td>1.10±0.53^b</td>
<td>2.21±0.89^c</td>
<td>T, V</td>
<td></td>
</tr>
<tr>
<td>β-apo-8’-carotenal</td>
<td>Beadlets</td>
<td>ND</td>
<td>1.26±0.73^b</td>
<td>ND</td>
<td>ND</td>
<td>0.04±0.04^a</td>
<td>2.13±1.30^b</td>
<td>ND</td>
<td>T, V</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>ND</td>
<td>2.38±0.80^ab</td>
<td>3.53±0.65^b</td>
<td>2.63±1.04^b</td>
<td>1.72±0.23^ab</td>
<td>5.52±1.59^b</td>
<td>ND</td>
<td>T, V</td>
</tr>
<tr>
<td>β-apo-12’-carotenal</td>
<td>Beadlets</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.50±0.50</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.52±0.22</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>ND</td>
<td>1.15±1.15</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.97±0.50^a</td>
</tr>
<tr>
<td>5’,6’-epoxy-β-carotene</td>
<td>Beadlets</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1.74±0.68^a</td>
<td>3.17±0.75^a</td>
<td>5.86±1.58^b</td>
<td>11.97±0.48^a</td>
<td>T</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND: non-detected levels. Significant differences were tested by ANOVA and least-significant differences (LSD) or Student’s t-test post-hoc comparisons (P<0.05): T, time-course effect; V, vehicle effect; TxV, interactive effect. Values within a row not sharing a common letter are statistically different. *indicates significant differences of BC-DMSO versus BC-beadlets-treated cells. Retinol and retinal were also analyzed, but there were non-detectable levels in all the samples.
With respect to the oxidized metabolites of the BC, some of them have also been detected within the cells, but in small amounts (see Table 2). There is an excentric cleavage pathway in mammals for the oxidative conversion of all-t-BC to retinal (and thereafter to retinol or to retinoic acid, which is of great importance in the gene regulation of the epithelial lung cells) [26,27], and the metabolites of this pathway studied here (the \(-\text{apo}4', -\text{apo}-8'\) and \(-\text{apo}12'\) carotenals) appear in very small amounts, suggesting that this pathway is not very active in this culture model, although the first of this metabolites that appears in the pathway, the \(-\text{apo}4'\)-carotenal, seems to reach slightly but significantly higher levels after 72 h of treatment with the beadlets vehicle, suggesting that this pathway would be a little more active in the BC-beadlets-treated cells. Other pathway which can produce retinal is the central cleavage of BC [26,27], but it also does not seem very active here since neither retinal nor retinol was detected in the cells, analyzed both with the DMSO or the beadlets vehicles. Thus, the possible effects of the treatment of all-t-BC in these lung epithelial cells would be mainly due to the high presence of all-t-BC and not to its main metabolites. Nevertheless, with respect to the \(5',6'\)-epoxy-\(\beta\)-carotene, although it also appears at low levels when compared with all-t-BC, its slightly but significant increase with time after the treatment could be of some significance, since it has been described that it shows higher activity in cell differentiation than BC and other BC-epoxides in NB4 cells (a cell line that contains the chromosomal transposition characteristic of acute promyelocytic leukaemia) [28].

Finally, it is important to note that any cytotoxic effect was reported with any treatment (as assessed by the LDH assay), as well as neither the number nor the morphology of the cells were different between treatments (data not shown).

Given all the results discussed here, DMSO seems a better vehicle than water dispersible beadlets for delivering BC to cultured cells. Moreover, DMSO also seems to have low toxic properties for cells in vitro at the generally used final concentration of 1% DMSO, and it could also be used at higher concentrations (up to 10%) without any significant cell damage, as shown in Caco2/TC7 cells [16]. In conclusion, DMSO appears to be the most appropriate vehicle for delivering BC to cells in vitro, in terms of BC uptake, stability and low formation of by-products. Furthermore, here we present an in vitro model that allows studying the possible effects of BC per se in human lung cells and to reach pharmacological concentrations of the molecule, as well as knowing the possible isomers and metabolites that can be formed in the lung human epithelial cells BEAS-2B.

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