Vitamin A Status and Metabolism of Cutaneous Polyamines in the Hairless Mouse After UV Irradiation: Action of ß-Carotene and Astaxanthin

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Summary: Solar radiations (UV A and B) can cause epidermis photoaging and skin cancers. These frequently irreversible effects result from the in situ generation of free radicals. However, it has been noted that nutritional factors can modulate photochemical damage, in particular the common carotenoids present in food, which can be considered as potential prophylactic agents against carcinogenesis. We investigated the effect of UV A and B radiations on the skin of the SKH1 hairless mouse fed a diet either lacking in vitamin A or supplemented with retinol, ß-carotene or astaxanthin. The latter is an oxygenated carotenoid (like canthaxanthin) without provitamin A activity and with strong singlet oxygen quenching ability.

After analyzing of vitamin status of each group (plasma retinol concentrations and hepatic reserves), we searched for UV-induced modifications of polyamine metabolism by measuring epidermal ornithine decarboxylase (ODC) activity and free polyamines concentrations (putrescine, spermidine and spermine).

In the basal state without irradiation, differences in ODC activity between groups were non-significant; but after UV stimulation, ODC increased markedly in the skin of vitamin A-deficient animals, much more than in other groups. Curiously, the addition of astaxanthin or ß-carotene to the regimen containing retinol reduced the protective effect of retinol alone.

Regarding polyamines after irradiation, putrescine was significantly increased in the skin of deficient animals, in parallel with ODC activity.

However, astaxanthin had a stronger inhibitory effect on putrescine accumulation than retinol, and decreased spermidine and spermine concentrations: this suggests a specific action on transglutaminases.

Introduction

The skin is the tissue most directly exposed to mechanical, chemical or physical damage. In particular, solar radiation (UV A and B) can cause premature aging of the epidermis and skin cancers. The harmful effect of these radiations is partly due to the in situ generation of free radicals from excited molecules [1]: light converts sensitizer molecules into electronically excited forms, usually singlet oxygen, which may be
converted to a triplet sensitizer state and lead to DNA damage [2].

There is increasing interest in the nutritional factors capable of modulating photochemical damage, particularly β-carotene which has been considered as a key protective agent in green and yellow vegetables. But other types of carotenoids coexist with β-carotene in foods (α-carotene, lycopene, lutein, zeaxanthin and canthaxanthin) and may also have anticarcinogenic effects [1]. In particular, α-carotene has a better protective action than β-carotene on spontaneous liver carcinogenesis in mice, and a higher potency of anti-tumor-promoting activity in several experimental systems of skin carcinogenesis [3]. Carotenoids are remarkably effective in neutralizing not only singlet oxygen but also triplet sensitizers, thus preventing phototoxic reactions [1, 4]. β-carotene, one of the most important carotenoids identified in plasma, is found in many tissues, particularly the skin where it is partially converted to vitamin A (retinol, retinyl ester). Interestingly, it may have an anticarcinogenic effect through a mechanism unrelated to its role as a vitamin A precursor [5, 6], and is an effective singlet oxygen quencher [7]. However, current data are not sufficient to ascertain the beneficial effect of β-carotene in human cancers: an extended clinical trial on the prevention of basal-cell and squamous-cell cancers of the skin did not show any reduction in the occurrence of skin cancers during a five-year treatment with beta-carotene [8].

Astaxanthin for its part is not an ordinary plasma carotenoid. Widespread in crustaceans and fish, it may occasionally contribute to the dietary supplementation of carotenoids in man. Closely related to oxygenated carotenoids such as canthaxanthin, it is not generally considered as a provitamin A: in particular, it has no action on the growth or survival rate of deficient rats. However, its dramatic action on xerophthalmia and its possible role as a vitamin A substitute in the retina of deficient rats has already been clearly described by Grangaud [9] who considered it as an original carotenoid with partial vitamin A activity. In other respects, it has a strong singlet oxygen quenching ability [10, 11] which is most potent after lycopene and considerably more effective than β-carotene.

In this context, we assessed the effect of UV A and B on the skin of the SKH1 hairless mice (where skin tumors can be induced by UV irradiations) under different nutritional conditions involving changes in vitamin A status: a normal diet with sufficient physiological content of vitamin A; a diet without vitamin A activity; both types of diets supplemented either with β-carotene (provitamin A) or astaxanthin (non provitaminic).

At the end of the experiment, after a careful analysis of the variations in vitamin status between the different groups studied, we investigated the modifications of polyamine metabolism induced by skin irradiation in these various situations. We determined the activity of cationic ornithine decarboxylase (ODC) [12], a key enzyme for polyamine metabolism, and the epidermal content of free polyamines, namely putrescine on the one hand and spermidine and spermine on the other. ODC is the first enzyme in the biosynthetic pathway for putrescine, spermidine and spermine. ODC and polyamines are central to normal and abnormal growth, and an activation of the polyamine metabolism is involved in tumor promotion [13].

The effects exposing hairless mice to ultraviolet radiations on induction of epidermal ODC has already been studied [14, 15]; ODC may be an useful marker for skin photodamage. Acute ODC assay had even been proposed as a short-term marker for predicting the effectiveness of certain molecules against chronic skin photo-damages [16].

Retinoic acid (RA), antiproliferative in skin cells, decreases the activity of ODC, whose levels correlate with the growth status of the cell. It regulates the enzyme gene expression at the transcriptional level: ODC mRNA levels are suppressed by RA in human skin cells [17].

The aim of our study was thus to investigate the biological effect of vitamin A status and of photoprotective agents on the first steps of carcinogenesis under UV irradiation.

Materials and Methods

Products. L-(1-14C) ornithine hydrochloride (59 mCi/mmol) was obtained from Amersham. β-carotene and astaxanthin were kindly provided by Roche (Basel, Switzerland). All eth-
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(59 mCi/mmol) and astaxanthin (veratral). All other products were supplied by Sigma Chemical Co. (St Louis, MO).

Animals: Female SKH-1 hairless mice (Charles River) weaned at 4 weeks were housed 8 per cage in a controlled atmosphere: 21°C; humidity 50±10%; 12h/12h light cycle.

Diet: A basic semi-synthetic diet (BD) without vitamin A activity, prepared in the laboratory, contained devitamized casein 20%, peanut oil 2.5%, rapeseed oil 2.5%, wheat flour 20%, sucrose 15%, brewers' yeast 6% and mineral salts 4%. Diet was supplemented with liposoluble vitamins (except retinol) in an oil solution, and with ascorbic acid.

Experimental groups: After weaning, the mice were randomly assigned to groups of 16 subjects:

- Group A: BD + retinol (850 μg of retinyl acetate per kg of diet).
- Group B: BD alone (the group without vitamin A).
- Group C: BD + retinol + B-carotene (5 mg/kg of diet).
- Group D: BD + B-carotene (5 mg/kg of diet).
- Group E: BD + retinol + astaxanthin (10 mg/kg of diet).
- Group F: BD + astaxanthin (10 mg/kg of diet).

Experimental assays: The animals were weighed each week throughout the experiment. At the time of sacrifice, 4 months after weaning, the blood and liver of 8 to 10 subjects per group were used to assay retinol, α-tocopherol and (in liver only) retinyl palmitate. After hexane extraction of plasma and liver homogenates, HPLC assays were performed (methanol being used in the mobile phase as a RP 18 column, with retinyl acetate as internal standard). Vitamin A (retinol as well as retinyl acetate for liver) was detected at 325 nm, and vitamin E at 290 nm. Results were expressed in μg/ml for plasma and mg/ g of liver for homogenates.

The animals had then attained a steady weight, and there were no significant variations in the mean weight of the different groups. Group B showed no clinical signs of vitamin A deficiency, and plasma retinol concentrations were close to normal. However, liver retinol reserves had decreased by 80%.

Eighteen hours before sacrifice, half of the mice in each group were subjected to UV A + B irradiation (3 J/cm²), i.e., equivalent to a threefold minimal erythema dose (MED). A Heraeus xenon lamp was used for irradiation, and doses were evaluated with an Osram dosimeter for both UV A and B.

The mice were sacrificed by retro-orbital bleeding after mild ether anesthesia. Two-centimeter-square biopsy specimens of irradiated dorsolateral skin were obtained, and identical specimens were taken from non-irradiated controls. Each specimen was cut into two equal parts, one for polyamine assays and the other for measuring ODC activity. All samples were stored at -80°C until extraction [14, 18].

ODC activity: ODC activity was measured for all groups using a modified Russell and Snyder method [19, 20] with L-[1-14C] ornithine as a substrate. All preparatory procedures were performed at 4°C. Biopsy specimens were weighed, and epidermis was scraped free on a microscope slide and homogenized in a phosphate buffer (0.05 M NaH₂PO₄, pH 7.4) containing EDTA (0.125 mM), dithiothreitol (1 mM), pyridoxal phosphate (0.27 M) and sucrose. After centrifugation at 70,000 × g for 20 min at 4°C, the supernatant was used for the assay. Results were expressed in pmol of CO₂ released in 60 min per mg of protein (Bradford's method, with Coomassie brilliant blue staining). Each assay series performed in triplicate simultaneously evaluated the enzymatic activity in the 6 nutritional situations.

Statistics: Student's t-test was used for data comparison analysis.

Polyamine determinations: After epidermis preparation as above, polyamine concentrations (putrescine, spermidine and spermine) were determined in biopsy specimens by dansylation of perchloric-extracted polyamines followed by reversed high performance liquid chromatography (HPLC) [21].

Extraction: Epidermal samples were homogenized in cold perchloric acid (10% HClO₄). After centrifugation at 25,000 × g for 20 min at 4°C, supernatants were stored at -20°C until dansylation.

Dansylation: 0.5 ml of perchloric extract was added to 0.25 ml of a saturated aqueous solution of Na₂CO₃, to which 0.5 ml of dansylchloride solution (5 mg/ml acetone) was added after shaking. Tubes were left open overnight under a dark fume hood to allow selective evaporation of acetone. The dansylated derivatives were extracted twice with 0.5 ml of benzene and the extracts evaporated to dryness at 60°C. Residues were dissolved in 0.5 ml acetonitrile and subjected to HPLC [22].

HPLC analysis was carried out on dansylated derivatives of polyamines according to Saeki's techniques [23], using a binary solvent system starting with 35% acetonitrile in water and going to pure acetonitrile after 10 min. A flow rate of 2 ml/min was used on a 250 x 5 mm reversed phase Hichrom C18 column using a Varian HPLC chromatograph. Fluorescence intensity was detected on a LDC fluorometer at an excitation wavelength of 340 to 380 nm with an emission filter of 418 to 700 nm.

Total proteins were determined by the method of Bradford. Polyamine levels were expressed in nmol/mg of protein.

Statistics: Data were compared by statistical analysis using Student's t-test and the Mann-Whitney test.

Results

Nutritional status of subjects: Mean weight at the time of weaning was 12 ± 0.5 g. All groups reached stable weight at the end of the second month and maintained it until the end of the fourth month (mean weight 30 g). At that time, there were no significant weight differences between groups, and group B (BD alone) was not apparently affected by denutrition.

Tables I and II show the vitamin concentrations in plasma and liver.

Plasma retinol concentrations did not differ according to group, except for an increase in group E subjects which received both retinol and...
astaxanthin (p < 0.001). α-tocopherol concentrations were lower in groups C and F (p < 0.001) but tended to increase slightly in group B (p < 0.1).

Vitamin A liver reserves (retinol + retinyl palmitate) were lower (p < 0.001) in deficient subjects (group B), representing only 22% of control concentrations. B-carotene raised this level to 43.5%, and astaxanthin (non-provitaminic) to 28%.

ODC activity: Table III shows cutaneous enzyme activity in different nutritional conditions, either with or without UV irradiation. In the basal state without irradiation, inter-group differences in ODC activity were not significant, although all experimental groups exhibited lower activity relative to controls (group A).

After irradiation, enzyme stimulation was general and intense for all groups, and particularly marked in both relative and absolute terms for group B (p < 0.001). B-carotene alone (group D) provided considerable protection, whereas astaxanthin in the same conditions (group F) was not significantly effective in reducing the reaction. Paradoxically, the association of retinol with B-carotene or astaxanthin was less effective than retinol alone.

Polymamines: Polymamines were assayed only in groups A, B, E, and F. Table IV shows the epidermal free putrescine concentrations for these

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<th>Table I: Vitamin plasma concentrations (μmol/l ± SEM)</th>
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<th>Table II: Liver vitamin concentration (nmol/g ± SEM)</th>
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<td>α-tocopherol</td>
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<th>Table III: Skin ODC activity (pmol CO2/60 min/mg proteins ± SEM)</th>
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II – irradiated skin

| Group | A | B | C | D | E | F |
| ODC | 17.4 ± 5.4 | 33.8 ± 6.9 | 29.6 ± 5.3 | 19.6 ± 5.2 | 27.9 ± 9.8 | 30 ± 9.7 |

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<th>Table IV: Polyamine concentrations (nmol/mg proteins), PUT: putrescine, SPD: Sperridine, SPM: Sperrmine</th>
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Discussion

Nutritional study, first of subclinical nutrition or marginal deficiency body's physical appearance of precarious vitaminosis, low liver retinoid abnormal bi radiations, metabolic cellular interest human nutri
4 groups. Without irradiation, the putrescine baseline level differed significantly between deficient subjects (group B), where it was markedly lower, and the other nutritional groups (p < 0.05). However, variations in ODC activity between the different groups were not very significant.

After irradiation, putrescine concentrations increased in all groups. The increase was most marked in group B, exceeding that of controls in absolute terms, as for ODC activity. Astaxanthin alone or in association with retinol clearly tempered the putrescine response to irradiation, in contrast to its effect on ODC.

Table IV gives the results for spermidine (SPD) and spermine (SPM).

In basal state, the putrescine SPD transformation was relatively more active in deficient subjects (group B) than in the other groups. However, absolute values were slightly lower in deficient subjects and tended to increase under the effect of astaxanthin (p < 0.05).

After irradiation, SPD and SPM concentrations remain stable in deficient subjects and decrease in the other groups whose behavior was contrary to that observed for putrescine.

Thus, polyamine biosynthesis seemed particularly disturbed in deficient subjects when damage was induced.

Discussion

Nutritional status: Our protocol enabled us to study, first of all, the reactions of mice in a state of subclinical vitamin A deficiency, without demineralization or any clinical signs of avitaminosis, by comparison with normally fed controls. A marginal deficiency is characterized by inadequate body stores of vitamin A to maintain normal physiological functions but without the appearance of clinical signs of deficiency [24]. The precarious vitamin status of mice with latent avitaminosis was here indicated merely by very low liver retinol concentrations, but it permitted abnormal biological reactions of the skin under radiations, without interference from associated metabolic disturbances. This state is of particular interest since similar situations may occur in human nutrition.

Plasma retinol was not reduced under these conditions, such a decrease occurring only as a late sign of avitaminosis.

Our experiment clearly showed that normal plasma retinol concentration is a safeguard and can in fact mask precarious reserve conditions and hence the impossibility to respond to an increased need resulting from stress or damage.

In addition, the protocol permitted assessing the effect of provitamin (β-carotene) or non-provitamin (astaxanthin) carotenoids in various vitamin A conditions (sufficiency or deficiency).

The addition of β-carotene to the diet without vitamin A activity (group D) had only a moderate influence on liver vitamin A reserves which were twice as high as in subjects with latent avitaminosis but less than half those of normally fed controls. However, the quantity of β-carotene was computed in consideration of the classic equivalence for retinol in human nutrition (1 Eq retinol = 6 μg of carotene). In mice, the retinol yield of carotene conversion at these doses appears to be appreciably lower.

Astaxanthin was chosen (Fig. 1) as an oxygenated carotenoid because, classically, it has no vitamin A activity and appears to have some original properties [9]. Like canthaxanthin, it is also more effective than β-carotene in neutralizing free radicals and also has better singlet oxygen quenching ability. Moreover, astaxanthin has immunoenhancing properties and has an inhibiting effect on tumor growth.
The addition of astaxanthin to the diet containing retinol (group E) increased plasma retinol and led to a slight decrease in liver reserves, suggesting a mobilization of hepatic retinol and a modification in the regulation of vitaminic homeostasis under the influence of a carotenoid deprived of vitaminic activity. This unexpected relationship has not been reported before and will be investigated in future research.

In subjects with latent avitaminosis, astaxanthin did not have the same effect on plasma retinol concentrations, apparently because retinol reserves were too low to be mobilized. Moreover, it had no significant sparing effect on liver vitamin A concentration in group D subjects.

Increased concentration of α-tocopherol in plasma and especially in the liver are typical during vitamin A deficiency and were noted in our subjects in a state of latent avitaminosis. This tocopherol variation would thus appear to be a sensitive marker of decline in vitamin status.

β-carotene, given as a supplement to the basic diet, restored normal tocopherol values in the liver and even tended to reduce them below normal, which was surprising in our experimental conditions since β-carotene only partially replaced the retinol supply. This result suggests that β-carotene itself has a particular effect, comparable to the remarkable powers of astaxanthin which, without vitaminic properties, can lower liver tocopherol concentrations below the usual values in normal (E) or even deficient subjects (F), thereby limiting the tissular accumulation noted during pure vitamin A deficiency.

These observations have shed some light on the relationships between retinol and α-tocopherol whose respective variations are often opposite. One of the factors involved may not concern vitamin A properties themselves but other biochemical properties shared by retinol with carotenoids and possibly implicated in effects on active forms of oxygen.

ODC: In the absence of stimulation, no noticeable variations in basal ODC activity were observed in the different groups, other than a tendency toward lower activity in all experimental groups relative to controls. After UV stimulation, activity increased markedly in all groups, especially in subjects with latent avitaminosis.

In skin, we noted the remarkable ODC reactivity previously obtained with promoters in various tissues (esophagus, colon, lung) of animals with vitamin A deficiency [20].

The pronounced cutaneous ODC response after UV irradiation, even in a relatively moderate state of latent avitaminosis, clearly indicates the degree of fragility that this state could induce. It would be worth investigating whether such a phenomenon could be observed in man under similar nutritional conditions.

β-carotene alone (group D), practically restored normal ODC response, but paradoxically increased reactivity in subjects receiving retinol (group C), as if there was an overload effect with inverted action.

Astaxanthin, like β-carotene, reduced the protective effect of retinol (group E) but alone, provided very slight protection to subjects with latent avitaminosis (group F) compared to deficient subjects (group B).

This analogy between the behaviours of the two carotenoids which, when associated with retinol, either moderate or roughly abrogate the slowing effect on ODC after irradiation, deserves further investigation. It is noteworthy that the present study showed rather important variations in individual responses for groups C, E and F.

Polyamines: Our measurements revealed the main differences in the behaviour of putrescine on the one hand, and spermidine and spermine on the other, with respect to nutritional situations and response to damage. Free putrescine increased after irradiation, particularly in subjects with latent avitaminosis, demonstrating good correlation with the strong stimulation of ODC, an enzyme directly involved in the formation of this polyamine. The addition of astaxanthin to normal diets or to those without vitamin A (groups E and F) led to an appreciable reduction in putrescine concentration after irradiation, relative to controls. This strictly inhibitory activity on putrescine accumulation contrasted with the strong ODC stimulation observed in these groups. The difference may have been due to the involvement of putrescine formed in an active consumption way, most like...
vitamin A status controls the metabolic reactions of skin polamynes in hairless mice after UV irradiation. In particular, as noted for other tissues, a mild deficiency led to much more intense activation of ODC than in normally fed subjects and greater accumulation of free putrescine. These effects suggest that even a moderate vitamin A deficiency may induce skin in a state of particular sensitivity to ageing and carcinomatosus degeneration. The action of carotenoids differed according to the vitamin A status of subjects. In the case of retinol deficiency, it generally led to attenuation of ODC reactivity, which was quite marked for β-carotene. Astaxanthin was remarkably effective in preventing increases of free putrescine in groups E and F after damage was induced. The biosynthesis of polamynes was apparently well controlled in groups A, E and F during irradiation. The interpretation of these results requires more extensive exploration of polamine metabolism, in particular of downstream enzymes, and especially transglutaminases.

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References


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Summary. The influence of vitamin A (VA) treatment on liver steatosis, a common complication of obesity, has not been fully evaluated. In this context, the present study demonstrates that VA treatment is effective in reducing liver steatosis and, in contrast, increases in hepatic fat content are observed in livers of mice treated with the highest doses of VA. Therefore, further investigations are needed to determine the mechanisms by which VA influences hepatic fat content.

Abbreviations used in this study. MLT, melittin-like transferase; MT, melittin-like transferase; VA, vitamin A; VA, vitamin A.