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Decreased Retinyl Ester Concentrations in UV-induced Murine Squamous Cell Carcinomas

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Squamous cell carcinomas were induced in hairless mice by repeated irradiations with UVB (280–320 nm, total dose 30 J/cm^2) plus UVA (320–400 nm, total dose 168 J/cm^2). The irradiated animals and non-irradiated controls were fed on diets with or without vitamin A supplementation (20,000 IU/kg). At the appearance of tumours, 30 to 43 weeks after the last irradiation, the vitamin A (retinol plus retinyl ester) concentrations in the serum, liver, epidermis and tumours and the retinol esterifying activities in microsomes from epidermis and tumours were measured.

The liver and epidermal vitamin A concentrations were 2–3 times higher in vitamin A supplemented than in unsupplemented animals, but did not differ between tumour-bearing animals and non-irradiated controls receiving identical diets. The vitamin A concentration in the tumours was significantly lower than in perilesional epidermis. The largest difference ($p < 0.001$) between the tumour and epidermal values was observed in the vitamin A supplemented group.

The low vitamin A content of the tumours was entirely due to a marked (2 to 6-fold) reduction in the retinyl ester fraction. In contrast, the retinol content of the tumours was increased to twice that of normal epidermis. The activity of the esterifying enzyme, acyl-CoA:retinol acyltransferase (EC 2.3.1.76), was unchanged. The reason for the reduced retinyl ester concentration thus remains unclear. Still, it is possible that a disturbed interconversion of retinol to retinyl esters plays a role in murine photo-carcinogenesis. ***Key words: Vitamin A; Acyl-CoA:retinol acyltransferase; Hairless mice; Ultraviolet radiation; Murine skin tumours.***

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Non-melanoma skin cancers are considered to be primarily due to solar radiation (for review see Ref. 1). It has been well established that UVB (280–320 nm) is the most carcinogenic wavelength range (2). Recent studies indicate, however, that UVA (320–400 nm) can augment the cancer-producing effect of UVB (3), and tumours have been induced by UVA alone when administered in very large doses (4). The type of cancer produced, when a combination of UVA and UVB is used, is usually squamous cell carcinoma (3, 5).

The relationship between epithelial cancer and vitamin A* has attracted considerable interest over the years. Vitamin A deficient animals respond to carcinogens more readily than those sufficient in vitamin A. The ability of synthetic derivatives of vitamin A (retinoids) to prevent chemical carcinogenesis in mouse skin has been convincingly demonstrated by Bollag & Matter (6) but the effect of retinoids on UV-induced carcinogenesis is more obscure (for review see Ref. 7).

Vitamin A is sensitive to UV radiation and some years ago, we reported that experimental UV irradiation lowers the concentration of vitamin A in rabbit and human skin in a dose- and wavelength-dependent manner (8, 9). We also found decreased vitamin A level in solar-induced squamous cell dysplasia of human skin (10). These and other data (11) suggested that UV-induced vitamin A deficiency in the skin might play a role in photocarcinogenesis.

Although vitamin A is normally supplied to the keratinocytes as retinol, substantial amounts of the vitamin are converted to retinyl esters. These compounds are biologically less active than retinol and presumably function as a local reservoir from which the vitamin can be rapidly released (12). The esterification of retinol is catalyzed by a microsomal enzyme, acyl-CoA:retinol acyltransferase (ARAT; EC 2.3.1.76), which has been demonstrated in both human and murine epidermis (13, 14).

*Vitamin A is used here generically to denote both retinol and its ester derivatives.

Table I. *Intervals at which the animals were sacrificed*

Group	Vit. A suppl.	Time (weeks) after start of irradiations: No. of animals killed 48	51	55	57	58	60	61	Total
1 Irradiated	–	3	2	1	3	2	4	7	22[a]
2 Irradiated	+	3	2	6	3	1	2	2	19[a]
3 Control	–	6	3	3	3	3	1	2	21
4 Control	+	6	3	3	2	3	1	3	21

[a] The animals had one or several tumours each.

It was observed in a previous study that within a few days after a single UVB irradiation the ARAT activity in hairless mouse epidermis was altered probably due to changes in the endogenous amounts of the co-substrate, acyl-CoA (15). In the present investigation we have examined the long-term effects of UV irradiation on the vitamin A concentrations and retinol esterifying enzyme in murine epidermis, with special reference to the situation in UV-induced tumours and to the effects of variation of the vitamin A content of the diet.

MATERIALS AND METHODS

Chemicals

[11,12 (n)-^{3}H] All-trans retinol (TRK 646:60 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, UK. All-trans retinol, dithiothreitol, palmitoyl-CoA, bovine serum albumin and butylated hydroxytoluene were purchased from Sigma (St Louis, Mo, USA). Ro 12-0586 was a generous gift from Hoffmann-LaRoche (Basle, Switzerland). All solvents were spectrograde reagents from Merck AG, Darmstadt, FRG or Rathburn Chemicals, Peeblesshire, Scotland.

Animals and irradiations

Female hairless mice (hr/hr) were purchased from Bomholtgård Breeding and Research Centre (Ry, Denmark) and allowed six weeks of acclimatisation before the experiments were commenced. The animals (n=156) were divided into four groups:

Group 1 received an unsupplemented diet and UVB + UVA irradiation.

Group 2 received a vitamin A supplemented diet and UVB + UVA irradiation.

Group 3 received an unsupplemented diet and no irradiation.

Group 4 received a vitamin A supplemented diet and no irradiation.

The animals had free access to water and a specially designed diet. The pellets were obtained from Chemovit (Hellerup, Denmark) and contained 800 IU/kg (unsupplemented) or 20,000 IU/kg (vitamin A supplemented) of retinol equivalents.

A combination of UVB and UVA irradiation was given from one sunlamp (Westinghouse 40W FS 40) and two blacklights (Philips TL 40 W/09) (3). The intensities from these sources were 0.22 mW/cm2 in the UVB range and 1.23 mW/cm^2 in the UVA range. The distance from the tubes to the backs of the animals was 18 cm. Irradiations were given for a total of 18 weeks with gradually increasing exposure times. In order to ensure equal exposure, the cages were rotated under the tubes at fixed intervals. The cumulated dose was 30 J/cm^2 UVB plus 168 J/cm^2 UVA.

The animals were observed for up to 61 weeks after the start of irradiation. They were killed within 3–5 weeks after the appearance of tumours (>1 mm in diameter), which previously had been identified as squamous cell carcinomas (3). The possible existence of tumours other than squamous cell carcinomas can not be totally excluded since each tumour was not examined histologically. The above procedure took place at the Finsen Institute, Copenhagen. Table I shows the outcome in terms of numbers of tumour-bearing animals and matched controls that were killed at each of seven points of time.

Sampling

Serum samples were taken from the tail veins and the animals were then decapitated and stored at –70°C for less than a week. After being transported to Uppsala by direct flight, the animals were semi-thawed and samples of dorsal and abdominal skin and of the liver were taken. The samples were stored at –70°C for up to 8 months until processed.

High performance liquid chromatography (HPLC)

The equipment for analysis of endogenous vitamin A consisted of a Waters M-45 pump (Waters Inc, Milford, MA, USA) used in combination with an LDC UV monitor D (360 nm) (LDC, Milton Roy, Riviera Beach, FL, USA). Reversed-phase HPLC was performed on a Nucleosil 5 µ PEAB-ODS column (4.6×200 mm) eluted with 14 % H_2O in acetonitrile at a flow rate of 1.2 ml/min.

The equipment for analysis of radioactive samples consisted of an Altex Model 110 pump in combination with an LDC 1203 UV monitor (360 nm). Reversed-phase HPLC was accomplished on a Nucleosil 5 µ PEAB-ODS column (4.6×200 mm) eluted with 15 % ethyl acetate in methanol at a flow rate of 1.6 ml/min. The eluate was collected in fractions (FRAC-100, Pharmacia Fine Chemicals, Uppsala, Sweden) corresponding to retinol and retinyl palmitate.

Table II. *Vitamin A concentrations in the serum and liver (x ± SD)*

	Unsupplemented animals		Vitamin A supplemented animals	
	Serum (ng/ml)	Liver (μg/g ww)	Serum (ng/ml)	Liver (μg/g ww)
Irradiated (groups 1 & 2)	177 ± 44	1 069 ± 259	224 ± 108[a]	2 668 ± 333[b]
Controls (groups 3 & 4)	191 ± 41	1 044 ± 255	202 ± 90	2 392 ± 690[c]

[a] $p < 0.05$ (significance of differences vs group 1).
[b] $p < 0.001$ (significance of differences vs group 1).
[c] $p < 0.001$ (significance of differences vs group 3).

Vitamin A analysis

Quantitative determinations of the vitamin A content (free plus esterified) in the serum, liver, heat-separated epidermis, and central parts of the tumours were performed by reversed-phase HPLC after alkaline hydrolysis (15). The values were correlated to the wet weight of the tissue samples.

Semiquantitative determinations of unesterified and esterified retinol were made by HPLC as previously described (16). Briefly, the tissue was mechanically homogenized in 2 ml of a 0.2 M phosphate buffer, pH 7.4, and extracted with 6.6 ml hexane after addition of 1.7 ml ethanol and internal standard (Ro 12-0586). The hexane layer was divided into two portions, each of which was evaporated. The first portion (A) was redissolved in methanol and analysed for unesterified retinol by injecting it directly into the HPLC system. The second portion (B) was redissolved in ethanolic-KOH (10 %) and heated (80°C) for 10 minutes in order to saponify the retinyl esters. After extraction, retinol was measured as described above. The amount of retinyl esters was calculated from the difference between the values for unesterified (A) and total (B) retinol. Although this method does not distinguish fatty acyl esters from other retinyl conjugates, the former conjugate usually constitute > 90 % of the tissue retinol released after saponification (17).

Determination of ARAT activity

The uninvolved epidermis was curettaged and epidermal microsomes were prepared as described before (14). The tumours were dissected macroscopically and microsomes were prepared. The microsomal ARAT activity was determined as before (14). Briefly, [^{3}H]retinol was added to a test tube which contained microsomes, palmitoyl-CoA, DTT and BSA in phosphate-buffer, total volume 0.5 ml. Incubations were terminated after 20 minutes by adding 0.5 ml ice-cold ethanol. Retinoids were extracted and subjected to HPLC after evaporation. Calculations were performed as described before (14).

Statistics

The results are presented as means and standard deviations. The statistical significance of differences was assessed by Student's *t*-test.

RESULTS

Serum and liver

Table II shows the vitamin A concentrations in serum and liver in the different groups of animals. Except for a slight increase in serum vitamin A in the irradiated animals receiving vitamin A supplements ($p < 0.05$), the serum values were similar in the four groups. The liver values were markedly elevated ($p < 0.001$) in animals receiving vitamin A supplements (groups 2 and 4), indicating that the vitamin

Table III. *Vitamin A concentrations (ng/g ww) in epidermis and tumours (x ± SD)*

	Unsupplemented animals		Vitamin A supplemented animals
Irradiated (groups 1 & 2)			
Epidermis	786 ± 403[b]	$p < 0.001$	1 344 ± 476
	$p < 0.05$		$p < 0.001$
Tumours[a]	485 ± 445		407 ± 319
			$p < 0.01$
Controls (groups 3 & 4)			
Epidermis	497 ± 100	$p < 0.001$	1 128 ± 570

[a] Eight tumours from different animals in each group were analysed.
[b] $p < 0.01$ (significance of differences vs group 3).

Table IV. *Acyl-CoA:retinol acyltransferase (ARAT) activity (pmol/mg protein/min) in epidermis and tumours (x±SD)*

	Unsupplemented animals	Vitamin A supplemented animals
Irradiated (groups 1 & 2)		
Epidermis[a]	15.7±4.3	19.6±3.9
Tumours[b]	20.0±7.8	18.3±6.0
Controls (groups 3 & 4)		
Epidermis[a]	23.5±7.1	16.3±5.4

[a] Each value denotes 3 analyses of pooled samples from 2–3 animals.
[b] Six tumours from different animals in each group were analysed.

was readily assimilated by the animals. Irradiation did not affect the vitamin A concentrations in either serum or liver.

Epidermis and tumours

The concentrations of vitamin A (free plus esterified) in perilesional and control epidermis and in tumours are shown in Table III. The epidermal values were significantly ($p<0.001$) higher in the vitamin A supplemented animals (groups 2 and 4) than in the unsupplemented ones (groups 1 and 3). The values were also higher in irradiated, unsupplemented animals than in the matched controls ($p<0.01$).

The vitamin A concentrations were lower in the tumours than in perilesional epidermis in both unsupplemented (485 vs 786 ng/g; $p<0.05$) and vitamin A supplemented (407 vs 1344 ng/g; $p<0.001$) animals. Similar differences were found when the values were related to the protein content of the samples (results not shown).

The proportion of retinyl esters in the epidermis was almost identical (89–94 %) in the four groups (Fig. 1). In tumour tissue, however, this proportion was significantly decreased; the difference compared with uninvolved epidermis was most pronounced in the vitamin A supplemented group (45 vs 94 %).

Retinol esterification

Table IV shows the ARAT activity in microsomes from the epidermis and tumours. No significant differences in the ARAT activities were found.

Since the epidermal ARAT activity is critically dependent on the palmitoyl CoA concentration in the reaction mixture (14) and the requirement is altered after a single UVB irradiation (15), the palmitoyl CoA dependency of retinol esterification was examined in irradiated epidermis and tumours. Whereas control microsomes show maximal stimulation of ARAT activity at palmitoyl-CoA concentrations above 50 μM, the microsomes from irradiated epidermis and tumours exhibited maximal activity at a concentration as low as 10 μM (data not shown).

DISCUSSION

The most significant finding in this study is the decreased vitamin A concentrations in tumour tissue from both vitamin A supplemented and unsupplemented animals. Semiquantitative measurements of retinol and retinyl esters showed that the decrease in tumour vitamin A was due to a marked reduction in the retinyl ester content. Table V shows the estimated concentrations of retinol and retinyl esters in the tu-

Table V. *Estimated contents (ng/g ww) of retinol and retinyl esters in epidermis and tumours*[a]

	Unsupplemented animals		Vitamin A supplemented animals	
	Retinol	Retinyl esters	Retinol	Retinyl esters
Irradiated (groups 1 & 2)				
Epidermis	71	715	81	1 263
Tumours	136	349	224	183
Controls (groups 3 & 4)				
Epidermis	55	442	113	1 015

[a] Values derived by multiplying the vitamin A concentration (Table III) by the relative figures for retinol and retinyl esters in the same tissue (Fig. 1). The retinyl ester values are expressed in equivalents of retinol.

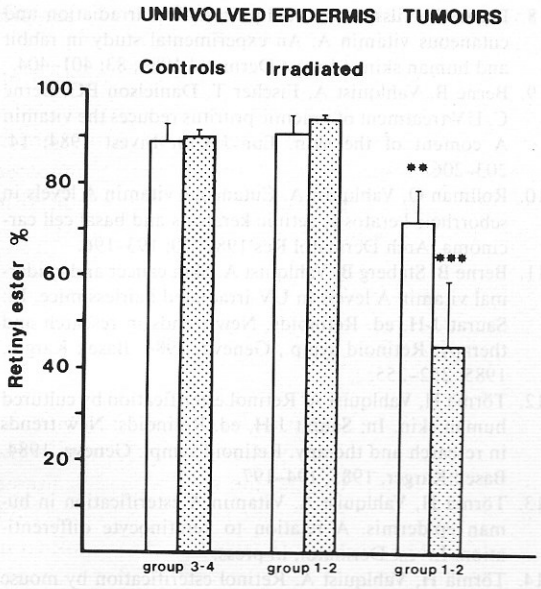

Fig. 1. Retinyl esters expressed in per cent of the total vitamin A concentration in epidermis and tumours. Dotted columns represent values from vitamin A supplemented and open columns values from unsupplemented animals. ** denotes $p<0.01$, *** denotes $p<0.001$ (significance of differences compared with irradiated, uninvolved epidermis).

mours and in control skin (obtained by multiplying the mean vitamin A concentration (Table III) by the percentages of retinol and retinyl esters in the same tissue (Fig.1)). It can be seen that the retinyl ester concentrations in the tumours are only half to one-sixth of those in perilesional epidermis. Vitamin A supplementation increased the retinyl ester concentration in both irradiated and control epidermis, but had no effect on the tumour values. This result, and the similar vitamin A concentrations in the serum and liver of tumour-bearing and control animals (Table II), strongly argue against nutritional vitamin A deficiency as a cause of the low retinyl ester levels in the tumours. In sharp contrast to the retinyl ester concentration, the retinol concentration in the tumours was two to three times higher than in normal epidermis (Table V). This prompted us to look for defective retinol esterification in the tumours.

In most tissues, the equilibrium between retinol and retinyl esters is regulated by two opposing enzymes, retinol acyltransferase (ARAT); and retinyl palmitate hydrolase (18). The relative importance of these enzymes for retinyl ester homeostasis in mouse epidermis is not known. Previous studies have shown reduced ARAT activity in several types of human skin tumours (13), and the concentrations of retinol and retinyl esters are also abnormal in these tumours. In murine hepatomas there are reports on low retinyl ester levels (19) and in a recent report, Ball et al. (20) described a transplantable rat mammary tumour which exhibited low ARAT activity. In the present study the activity of the esterifying enzyme, ARAT, was unchanged in the tumours when assayed under standard conditions. A shift was noted, however, in the palmitoyl CoA dependency of the enzyme, so that if the ARAT assay had been conducted at a palmitoyl CoA concentration of 10 μM (instead of ordinary 50 μM), *increased* activities (20-45 %) would have been recorded in the irradiated and the tumour samples. But in no way could the shift in palmitoyl CoA dependency have obscured a reduction in the ARAT activity in the tumours. The importance *in vivo* of the altered sensitivity to palmitoyl-CoA *in vitro* is not known at the present.

There are several other possible explanations for a tumour-associated disturbance in retinol esterification: 1) retinol may be incorrectly presented to ARAT, e.g. because of abnormal compartmentalization of the substrate or the enzyme; 2) the tumour cells may be deficient in co-factors essential for retinol esterification; and 3) the retinyl palmitate hydrolase activity (not examined) may be increased in the tumours. Whatever the origin, an increased proportion of unesterified retinol may play an important role in epidermal carcinogenesis. For instance Fuchs and co-workers (21) recently showed that the terminal differentiation of cultured squamous carcinoma cells is suppressed and the proliferation enhanced if a surplus of retinol is added to the medium. The higher retinol concentration in tumours from animals supplemented with vitamin A (see Table III) might then also be consistent with the somewhat higher incidence of tumours among these animals as compared to unsupplemented, irradiated controls (Staberg et al, to be published).

We do not know whether combined irradiation with UVA and UVB, which increases the yield of tumours compared with UVB alone (3), is a prerequisite for induction of defective retinol esterification in the tumours. The acute effects of UVB and UVA on epidermal vitamin A are similar, but the latter type of radiation results in a more persistent (weeks) deficiency of vitamin A in the skin (15). However, any direct relation in the present study between the UVA irradiation and the low vitamin A level in the tumours is precluded by the long interval (7 to 10

months) between the end of the irradiation period and the sampling. Furthermore, there was no such reduction in irradiated uninvolved epidermis. At large, our study failed to demonstrate any long-lasting effects of tumourigenic UV doses on the vitamin A status of animals with small to medium-sized tumours. Retrospectively, our previous finding of low vitamin A levels in tumour-adjacent epidermis from UV-irradiated hairless mice (11) is probably explained by cachexia and vitamin A malnutrition in association with massive tumour growth.

In conclusion, we have found evidence for disturbed vitamin A homeostasis in UV-induced murine squamous cell carcinomas. The disturbance appears to be independent of the vitamin A status of the animals and does not extend to the tumour-free regions of the epidermis. The pathogenetic and biological implications of these findings remain to be elucidated.

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