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THE ROLE OF ULTRAVIOLET RADIATION AND TYROSINE STIMULATED MELANOGENESIS IN THE INDUCTION OF OXIDATIVE STRESS ALTERATIONS IN FAIR SKIN MELANOCYTES

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Background: **Melanocytes are producing melanin after UV irradiation as a defense mechanism. However, UV-induced damage is involved in melanoma initiation, depending on skin phototype. Melanocytes seem to be extremely susceptible to free radicals. Their main enzymatic antioxidants are superoxide dismutase and catalase.** ***Aim:*** **To study how melanin synthesis modulates the activity of the oxidative stress defense enzymes and cell proliferation after UV induced cell damage.** ***Methods:*** **Normal human melanocyte cultures from fair skin individuals were exposed to high levels of L-tyrosine and irradiated, with 20, 30, 40 mJ/cm^2 UVA, and respective UVB. Proliferation was measured using a MTS assay; viability was assessed by trypan blue exclusion dye method. Spectrophotometrical methods were used to determine total melanin content, the enzymatic activity of tyrosinase, superoxide dismutase and catalase.** ***Results:*** **Tyrosine had a negative effect on proliferation, enhanced with time elapsed. Overall, UV irradiation decreased proliferation. UVA increased proliferation relative to UVB in the cultures exposed for a longer time to high (2 mM) tyrosine concentration. There were no proliferation differences between UVA and UVB irradiation in lower tyrosine concentration exposed melanocytes. Both, UV irradiation and tyrosine increased melanogenesis. Exposure of the melanocytes to increased levels of tyrosine in medium (0.5 mM and 1 mM) and UV irradiation enhanced the activity of superoxide dismutase and catalase. The enzymes showed a high activity rate in melanocytes while exposed for a short time to 2 mM tyrosine, but their activity was dramatically decreased with longer tyrosine exposure and UV irradiation.** ***Conclusion:*** **Our data indicate that in low phototype melanocytes, melanogenesis, either following UV irradiation, or tyrosine exposure, especially in high concentrations, was detrimental for the cells by reducing the activity of catalase and superoxidedismutase, the natural antioxidants. UVA was more efficient in stimulating the activity of superoxide dismutase and catalase but also in depleting the reserves of the enzymatic defense against oxidative stress, especially catalase, than UVB. This physiologic response to UV light can be considered as an adjunctive risk factor for people with low phototype for developing a melanoma, when exposed to UV irradiation.**

Key Words: **melanocyte, tyrosine, ultraviolet radiation, oxidative stress, melanogenesis.**

Melanocytes play a central role in the response of skin to sunlight exposure. They are directly involved in ultraviolet (UV)-induced pigmentation as a defense mechanism [1]. However, their alteration can lead to melanoma, a tumor that has become one of the most rapidly increasing malignancies in the Caucasian population [2]. Melanoma seems to be the result of complex interactions between environmental, constitutive and genetic factors [3]. Although direct evidence is lacking, it is assumed that solar ultraviolet A (UVA) radiation (320–400 nm) may play a significant role relative to ultraviolet B (UVB) radiation (290–320 nm) in melanoma etiology [2, 4]. The transformation process whereby UV damage may result in melanoma initiation is poorly understood, especially in terms of UV-induced genotoxicity in pigmented cells, where melanin can act either as a sunscreen or as a photosensitizer [1, 5, 6]. People with different skin color possess varied sensitivity to ultraviolet (UV) exposure, with darker skinned individuals being less susceptible to sun-induced skin alterations, including cancer, than fair skinned ones [7]. Such a difference can be explained in terms of UV transmission of the epidermis, because the skin color is also related to the type of melanin, the number, size, type, distribution and degradation of melanosomes, and the tyrosinase activity in melanocytes [8, 9]. Three enzymes, phenylalanine hydroxylase, tyrosine hydroxylase isoform I and tyrosinase are crucial for the initiation of melanogenesis. Intracellular phenylalanine hydroxylase is providing L-tyrosine through the conversion of L-phenylalanine while the last two enzymes are using L-tyrosine as a substrate [10, 11]. Tyrosinase catalyzes the hydroxilation of L-tyrosine [12] and the production of *ortho*-quinones from both monohydric and dihydric phenols [13]. Tyrosine hydroxylase isoform I uses L-tyrosine to form L-DOPA in melanosomes [14].

High levels of tyrosine are known to reduce the proliferative effect of alpha-MSH and forskolin and also alter melanocytes morphology [15]; tyrosine also stimulates the activity of tyrosinase and melanogenesis [4, 15–18].

In the melanocytes, the dominant skin pigment melanin and its precursors are complex redox systems, the resultant properties of which are modified by pH, temperature, illumination with ultraviolet and visible light [19]. There are conflicting reports on the role of melanin or melanin precursors in modulating the biologic effects of UV radiation [20]. It is conceivable,

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Abreviations used: AUC – area under curve; Cat – catalase; DMEM – Dulbecco's modified Eagle medium; FCS – fetal calf serum; KGM – keratinocyte growth medium; MGM – melanocyte growth medium; MTS – (3-(4,5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; PMS – phenazine methosulphate; ROS – reactive oxygen species; SOD – superoxide dismutase; UV – ultraviolet; UVA – ultraviolet A; UVB – ultraviolet B.

however, that melanin, which may be present in large concentration in melanocytes, may be the most important antioxidant [4]. Melanocytes, in particular, seem to be extremely susceptible to free radicals, either in the activation of their physiologic role or in deleterious effects [1, 21, 22]. The main enzymatic antioxidants in the melanocytes are superoxide dismutase (SOD) and catalase (Cat), considering the low glutathione peroxidase activity (GSH-Px) in melanocytesn [23], so that the SOD/Cat ratio is considered as a parameter of the cells susceptibility to external oxidative stress [1].

The different pattern of antioxidants in melanocytes from people with low phototype and their physiologic response to UV light could be an adjunctive risk factor for developing a melanoma, when exposed to UV irradiation [1, 24].

We studied the effects of UV induced oxidative stress, using normal human melanocyte cultures from caucasian individuals, phototype II, III, according to Fizpatrick classification, as cellular models [25]. L-tyrosine was used to selectively modulate melanin synthesis in the melanocytes. The aim of this study was assessment of melanogenesis, cell survival, proliferation and defense against oxidative stress in fair skin melanocytes when exposed to high levels of tyrosine and UVA, respective UVB radiation. In view of the potential role of UV irradiation in skin carcinogenesis and especially melanoma it is important to understand how melanin synthesis modulates the activity of the oxidative stress defense enzymes, such as SOD and Cat after UV induced cell damage.

MATERIALS AND METHODS

Melanocyte cultures. Adult human melanocytes were grown as previously described [26, 27]. Skin biopsies were taken from healthy skin, trimmed of excess subcutaneous tissue and dermis. Separation of epidermis from dermis was done after overnight incubation in 2000 UI/ml colagenase (Cellsystems, Germany). The epidermal cells were separated by trypsinisation and the cells of the stratum basalis were collected by gentle scraping. Recovered cells were resuspended and seeded onto a 25 cm^2 culture plates in serum free, keratinocyte growth medium (KGM) (Promocell, Germany). All cultures were fed twice weekly and incubated in a 37 °C and 5% CO_2, humidified environment. At first passage, the melanocytes were separated from the keratinocytes by differential trypsinization and resuspended in complete melanocyte growth medium (MGM) (Cellsystems). We used 3 primary epidermal human adult melanocytes cultures from individuals with phototype II and III and one epidermal human melanocyte culture from Caucasian newborn foreskin (Promocell).

L-tyrosine media. L-tyrosine (Sigma Chemical Co., St. Louis, USA) was dissolved in DMEM, supplemented with 5% FCS to prepare the media with concentrations of 0.5 mM, 1 mM, 2 mM and respective 3.4 mM, including the tyrosine already present in the medium. Although this medium is not optimal for melanocyte growth; it readily maintains cell survival over a short period of time (24 h, respective 72 h) and thus allowed for the experiments to be performed on melanocytes without exposure to potent non-physiologic stimulants such as phorbol esters or cholera toxin [26].

Melanocyte bioassay. All the experiments were conducted in subdued light, in triplicate. Melanocytes in the 3rd and 4th passage were used. In proliferation assays the melanocytes were seeded at 10^4 per well in ELISA 96 wells micro titration flat bottom plaques (TPP, Switzerland). For the enzymatic bioassays and melanin assessment, the cells were seeded at 2×10^4 cells per 35 mm Petri dish. After 24 h accommodation in complete MGM at 37 °C, 5% CO_2, humidified environment, the cells were washed and exposed to the L-tyrosine media for 24, respective 72 h. Untreated controls were exposed to DMEM, supplemented with 5% FCS, containing 0.397 mM tyrosine.

UV irradiation. Irradiation was conducted using a 6 W power UV lamp (Fisher Bioblock Scientific, Belgium) with filters for UVB (312 nm), respective UVA (365 nm). Melanocytes were washed twice in PBS and irradiated, in PBS, with 20, 30, 40 mJ/cm^2 UVA, respective UVB. The light intensity at the position of the irradiated cell plates with UVA was 700 μW/cm^2 and with UVB was 680 μW/cm^2. Then, the cells were incubated for 24 h in basal medium for melanocytes (Promocell).

Melanocyte proliferation/cytotoxicity assay. It was done using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega Corporation, U.S.A). The cells in 100 μl medium were exposed to 20 μl of MTS/PMS mixture (2 ml/100 μl), for 1–4 h. Absorbance at 490 nm was recorded using an ELISA plate reader (Tecan, Austria).

Morphology. Morphological aspect of the melanocytes was observed by microscopic examination (Nikon Eclipse T 100, Japan) and documented photographically. Melanocytes were released from the culture dishes with a soft rubber cell scraper and pelleted.

Cell viability. It was assessed by trypan blue exclusion dye method (Biochrom AG).

Cell lysis. Cells were lysed on ice, in Nonidet 1% (Sigma) in PBS solution for one hour, in the presence of 1% complex of protease inhibitors (Sigma). Cell extracts were spun at 14 000 g for 30 min at 4 °C. Supernatant was removed and a fixed volume (50 μl) was used for determining the protein content by the Bradford method (Biorad, USA).

Total melanin content. It was determined as previously described [28]. Remaining pellet was dissolved in 0.1 ml 1 M NaOH, and diluted with 0.4 ml water. Melanin content was assessed by spectrophotometric determination (DU 730 UV VIS Beckman Coulter, USA) of absorption at 475 nm against a standard curve of synthetic melanin (Sigma).

Tyrosinase enzymatic activity as DOPA oxidase. 100 μl cell lysate were incubated for 30 min at 37 °C with 1000 μl DOPA (2.5 mg/ml) in 10 mM phosphate buffer, pH 7.2. The recording L-dopacrom formation at 475 nm was measured by spectrophotometry (DU 730 UV VIS Beckman Coulter); absorbance was compared with a standard curve using mushroom tyrosinase (Sigma) [29, 30].

SOD enzymatic activity. 50 µl cell lysate were added to 2.9 ml cytochrome c (2 µmol) from horse heart (Sigma) in 50 mM phosphate buffer, ph 7.8, containing 0.1 mM EDTA solution and the reaction was started with 50 µl of freshly prepared solution of 0.2 U/ml xanthine oxidase in 0.1 mM EDTA. Absorbance at 550 nm was recorded by spectrophotometry (DU 730 UV VIS Beckman Coulter), against a standard curve using pure bovine liver SOD (Sigma) [31, 32].

Cat enzymatic activity. 20 µl of cell lysate were mixed with 3 ml solution of 10 mM H_2O_2 in 50 ml potassium phosphate buffer; absorbance at 240 nm was continuously measured by spectrophotometry (DU 730 UV VIS Beckman Coulter) at 240 nm, for 3 min. For calculation we considered one unit of catalase as the amount of enzyme which induces a change of 0.43 in the absorption (240 nm) during the 3 min incubation period [31, 32].

Statistics. Data were analyzed using non-parametric methods: Mann — Whitney U Test, Kruskall — Wallis test, Spearman *r* calculus. Dynamic evaluations were assessed by means of area under curve (AUC) calculations. Specific tests (Mann — Whitney U Test, Kruskall — Wallis test) were used to evaluate differences between 2 or three dynamic patterns. Although chars are presented with equal interval among moments in time, real time scale was used when determining AUC values and for testing AUC differences. Results were considered significant for $p \leqslant 0.05$. Statistical packages SPSS 13.0 — Statistical Software Package (SPSS Inc, Chicago, Illinois, USA) and MedCalc 8.1.0.0 were used for data analysis.

RESULTS

Melanocyte proliferation and cytotoxicity. Overall, tyrosine had a negative, statistically significant dynamic effect on proliferation, as seen in Fig. 1 (overall AUC comparison among all tyrosine concentrations, $p = 0.000$). Tyrosine diminished cell proliferation compared to controls, when used in lower concentrations (AUC control versus tyrosine 0.5 mM, $p = 0.000$; respective AUC control *vs* tyrosine 1 mM, $p = 0.000$). However, no significance was obtained between tyrosine 0.5 mM *vs* 1 mM dynamic effect (AUC comparison, $p = 0.987$).

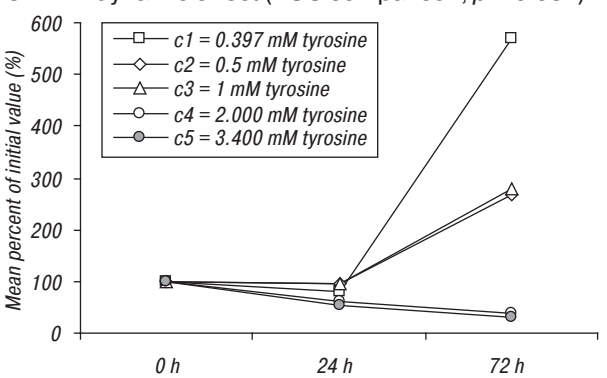

Fig. 1. Melanocyte proliferation after tyrosine exposure for different time periods

High tyrosine concentrations had a significant negative effect on cell proliferation (AUC control *vs* tyrosine 2 mM, $p = 0.000$, respective control *vs* tyrosine 3.4 mM, $p = 0.000$). However, no significance was obtained and between tyrosine 2 mM *vs.* 3.4 mM dynamic effect (AUC comparison, $p = 0.897$).

UV irradiation of the tyrosine exposed melanocytes reduced cell proliferation. There were no significant differences in the proliferation rates after exposure to low concentrations of tyrosine (0.5 mM, 1 mM) according to tyrosine concentration and UV irradiation, compared with controls (Fig. 2).

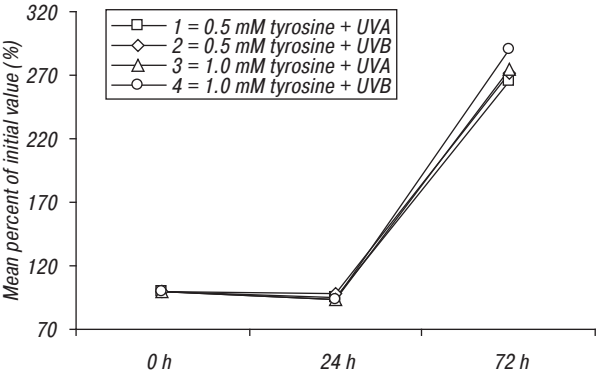

Fig. 2. Melanocyte proliferation after UV irradiation of previously low tyrosine concentrations exposed cultures for different periods of time

However, 24 h exposure to high tyrosine concentrations (2 mM, 3.4 mM) induced an increased cell proliferation rate after UVB irradiation compared to UVA, while the 72 h tyrosine exposed cultures showed an increased proliferation rate after UVA irradiation, compared to UVB radiation and non irradiated cultures (Fig. 3).

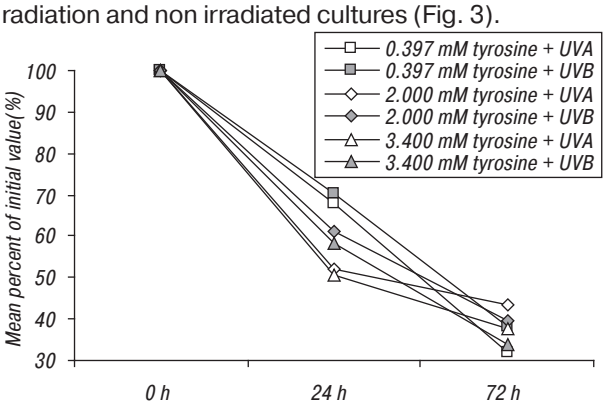

Fig. 3. Melanocyte cytotoxicity assay after UV irradiation (30 mJ/cm^2) of previously high tyrosine concentrations exposed cultures for different periods of time

The differences between the proliferation rates of UVA and UVB irradiated cultures previously exposed to tyrosine were not statistically significant (AUC control UVA, *vs* control UVB, $p = 0.769$; tyrosine 2 mM UVA *vs* UVB, $p = 0.825$; tyrosine 3.4 mM UVA *vs* UVB, $p = 0.854$). High tyrosine concentration strongly inhibited cell proliferation in the UVA irradiated cultures (AUC control *vs* tyrosine 2 mM, $p = 0.026$, AUC control *vs* tyrosine 3.4 mM, $p = 0.019$). However, no statistically significance was obtained between AUCs of tyrosine 2 mM *vs* 3.4 mM, $p = 0.897$. Same results were recorded after UVB irradiation (AUC control *vs.* tyrosine 2 mM, $p = 0.036$, AUC control *vs* tyrosine 3.4 mM, $p = 0.029$, but AUC tyrosine 2 mM *vs* 3.4 mM, $p = 0,657$). There is no statistical significance between AUCs of tyrosine 2 mM unirradiated *vs* UVA, $p = 0.843$ respective no irradiated *vs* UVB, $p = 0.954$ (see Fig. 3).

Microscopic examination. The short period (24 h) of tyrosine exposure did not produce visible alterations in the melanocyte morphology; their aspect was similar with untreated controls (Fig. 4, *a*). Melanocytes exposed to tyrosine for the longer period (72 h) were deeply modified. 2 mM tyrosine exposed cells were heavily pigmented, with the cell body occupied with large melanosomal complexes (Fig. 4, *b*). Higher tyrosine concentration (3.4 mM) severely altered the microscopic aspect; the cells were showing mosaicism, polymorphism, with heterogeneity of pigmentation, vacuolar degeneration, and loss of surface adherence (Fig. 4, *c*) — characteristics of a late-passage, senescent melanocyte culture *in vitro* [33].

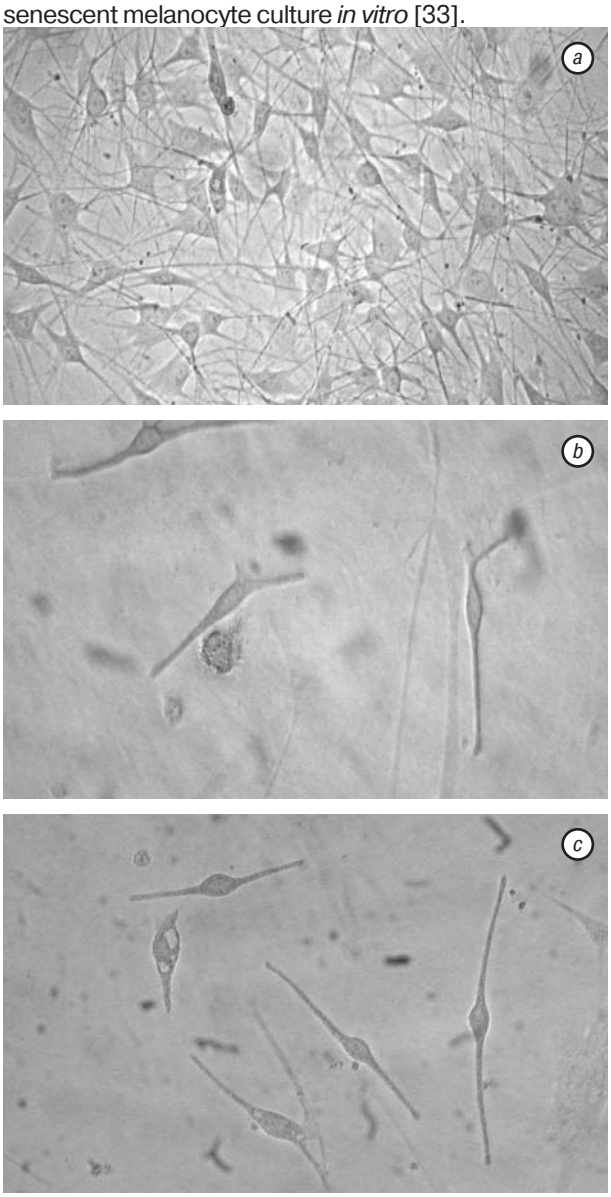

Fig. 4. Microscopic examination of the melanocyte cultures. *a*, normal microscopic aspect of the epidermal human melanocytes in culture (untreated controls, photo taken through Nikon Eclipse T 100 microscope, 40 x). *b*, heavily pigmented cells, with a few, short, plump dendrites; the cell body is occupied with large melanosomes, except the central area, where the nucleus is still visible (melanocyte cultures exposed for 72 h to 2 mM tyrosine, photo taken through Nikon Eclipse T 100 microscope, 40 x). *c*, polymorphism exhibiting large, monstrous cells, small cells, bipolar and round cells, uneven distribution of the melanin pigment, vacuolar degeneration, loss of surface adherence (melanocyte cultures exposed for 72 h to 3.4 mM tyrosine, photo taken through Nikon Eclipse T 100 microscope, 40 x)

Melanocyte viability was assessed for the untreated controls and the higher (2 mM) tyrosine exposed cultures (Table 1). It showed a high viability ratio. Viability was significantly decreased with tyrosine exposure (Table 2). Interestingly, time exposure to tyrosine had no significant influence on melanocyte viability. We noticed a slight viability decrease when melanocytes were exposed to tyrosine and UVB, compared with the tyrosine and UVA combination, but the difference was not significant (Table 3).

Total melanin content. Tyrosine exposure had a different effect on melanin synthesis according to concentration. Lower concentrations (0.5 mM, 1 mM) significantly increased melanin content, while the high concentration (2 mM) decreased it (not significant), compared to controls (see Table 1). Melanin content was significantly increased with time exposure to tyrosine in 1 mM tyrosine treated melanocytes (see Table 2). Pigment production was increased with higher UV energy irradiation; significantly after UVA irradiation 30 and 40 mJ/cm^2, respective after UVB irradiation 40 mJ/cm^2, compared to unirradiated controls (see Table 3). There was a higher melanin content in the cultures exposed to the combination of UVA and tyrosine than the combination of UVB and tyrosine (Table 4).

Tyrosinase enzymatic activity significantly increased with high tyrosine concentration (2 mM) (see Table 1). Time exposure to tyrosine increased tyrosinase activity (see Table 2).

Both UVA and UVB stimulated tyrosinase activity. UVA irradiation determined a higher stimulation of tyrosinase activity than UVB; the difference was significant with lower concentrations (0.5 mM, 1 mM) (see Table 3). Tyrosinase enzymatic activity increased with irradiation energies, not significant (see Table 4).

SOD and Cat enzymatic activity were significantly increased in the melanocyte cultures exposed to lower tyrosine concentrations (0.5 mM, 1 mM) compared to controls (see Table 1). Time exposure to tyrosine significantly altered the activity of SOD. Cat activity differed significantly when melanocytes were exposed to 0.5 mM and 2 mM tyrosine concentration. Interestingly, after high tyrosine concentration exposure, SOD and Cat showed increased activity rates after a short treatment, compared to untreated controls, but their activity was dramatically decreased with longer tyrosine exposure. However, this situation was completely different with low tyrosine concentrations (see Table 2).

UVA irradiation stimulated SOD and Cat activity at lower tyrosine concentrations (0.5 mM) than UVB (1 mM). UVA increased Cat activity after low tyrosine concentration (0.5 mM) exposure, relative to UVB. However, higher concentrations of tyrosine exposure prior to UV irradiation changed this effect. After 1 mM tyrosine concentration exposure, the effects of UVA and UVB on Cat activity were similar, while at high tyrosine concentration (2 mM) UVA decreased Cat activity compared to UVB and controls (see Table 3). The enzymatic activity of both SOD and Cat was increased with the energy of irradiation (see Table 4).

Table 1. Melanocyte bioassay: effects of different tyrosine concentrations on cell viability, total melanin content and the enzymatic activity of tyrosinase, SOD and Cat

Tyrosine concentration (mM)	Total melanin content (μg/culture)	Enzymatic activity of tyrosinase (U)	Superoxide dismutase (U/mg protein)	Catalase (U/mg protein)	Viability (%)
	Median/range	Median/range	Median/range	Median/range	Median/range
0.397	3.39/4.7	18.44/57.52	1363.91/3618.66	27.335/43.04	88.965/15.71[a]
0.5	2.64/3.1[b]	20.08/50.16[a]	2022.13/2355.80[b]	37.086/231.54[b]	
1	2.38/2.1[b]	19.86/32.44[a]	2247.57/2724.52[b]	69.15/190.54[b]	
2	3.4/5.1[a]	73.54/91.44[b]	1649.58/4421.74[c]	37.165/81.46[a]	91.86/8.93[b]

[a]$p > 0.05$, not statistically significant, [b]$p < 0.05$, statistically significant, [c]$p = 0.166$, marginally significant, compared with the untreated control, represented by the melanocyte cultures treated with DMEM with 5% FCS, tyrosine concentration 0.397 mM.

Table 2. Melanocyte bioassay: effects of different periods of time exposures to tyrosine on cell viability, melanin content and the enzymatic activity of tyrosinase, SOD, Cat

Tyrosine concentration mM	Exposure time to tyrosine (h)	Total melanin content (μg/culture)	Enzymatic activity of tyrosinase (U)	Superoxide dismutase (U/mg protein)	Catalase (U/mg protein)	Viability (%)
		Median/range	Median/range	Median/range	Median/range	Median/range
0.397	24	3.3/3.5	13.46/29.52	1855.47/3367.49	30.16/20.18	88.825/14.59
	72	3.79/4.5[a]	27.44/56.80[a]	939.43/1840.54[b]	20.285/43.04[a]	91.475/10.78[a]
0.5	24	2.76/3.1	18.56/50.16	1698.97/2282.54	34.10/28.54	
	72	2.42/3.6[a]	25.04/35.08[c]	2580.84/2105.83[b]	87.37/230.32[b]	
1	24	1.96/2	19.36/29	1982.14/2724.52	56.43/170.68	
	72	2.4/1.6[b]	24.84/27.84[a]	2620.61/1943.48[b]	81.61/190.54[a]	
2	24	3.45/2.4	15.50/45.04	2695.47/2861.13	70.85/50	41.94/61.31
	72	3.2/3.9[a]	87.12/41.28[b]	941.68/2779.75[b]	20.55/62.62[b]	20.285/45.73[b]

[a]$p > 0.05$, not statistically significant, [b]$p < 0.05$, statistically significant, [c]$p = 0.158$, marginally significant, effects of 24 h compared to 72 h time of tyrosine exposure.

Table 3. Melanocyte bioassay: comparison between the effects of UVA *vs* UVB irradiation after previous tyrosine exposure on cell viability, melanin content and the enzymatic activity of tyrosinase, SOD, Cat

Tyrosine concentration (mM)	Radiation type	Total melanin content (μg/culture)	Enzymatic activity of tyrosinase (U)	Superoxide dismutase (U/mg protein)	Catalase (U/mg protein)	Viability (%)
		Median/range	Median/range	Median/range	Median/range	Median/range
0.397	UVA	3.75/4.4	28.44/57.52	1274.84/3618.66	27.07/17.49	88.825/14.59
	UVB	3.39/4.7[a]	13.46/31.08[a]	1378.5/3618.66[a]	30.425/43.04[a]	91.475/10.78[a]
0.5	UVA	3.165/2.3	29.35/37.48	2720.90/1699.31	79.84/218.05	
	UVB	2.875/2.5[a]	37.96/41.48[b]	1365.85/1200.0[b]	27.10/21.63[b]	
1	UVA	2.57/1.7	19.86/20.14	2127.48/1793.78	61.325/184.63	
	UVB	2.215/1.8[b]	30.74/17.44[b]	3404.78/1300[b]	68.67/176.59[a]	
2	UVA	4.75/2.2	46.44/79.92	407.145/1581.42	24.32/51.46	88.96/7.39
	UVB	4.2/3.9[a]	28.44/79.2 [a]	384.455/2504.43[a]	36.285/43.04[a]	88.15/12.94[a]

[a]$p > 0.05$, not statistically significant, [b]$p < 0.05$, statistically significant, UVA compared to the UVB effects on the previously tyrosine exposed cultures.

Table 4. Melanocyte bioassay: comparison between the effects of the UVA respective UVB irradiation energies after previous tyrosine exposure on melanin content and the enzymatic activity of tyrosinase, SOD, Cat

Radiation type	Doses of radiation (mJ/cm^2)	Total melanin content (μg/culture)	Enzymatic activity of tyrosinase (U)	Superoxide dismutase (U/mg protein)	Catalase (U/mg protein)
		Median/range	Median/range	Median/range	Median/range
Unirradiated cultures	0	2.4/1.6	12.24/91.44	1363.91/4422.4	20.66/61.95
UVA	20	2.53/2.5[a]	17.64/45.8[a]	1778.3/1157.77[a]	38.41/107.36[b]
	30	2.7/2.7[b]	19.4/53.24[a]	2735.02/3032.05[a]	33.34/203.35[a]
	40	3.76/2.7[b]	36.71/79.92[a]	2560.02/3192.75[a]	43.31/243.42[b]
UVB	20	2.42/2.5[a]	26.77/64.24[a]	1848.4/1826.41[a]	32.39/93.16[c]
	30	2.4/4.8[a]	29.52/79.2[a]	2022.13/3698.8[a]	26.98/138.9[a]
	40	3.36/4[b]	38.9/82.76[a]	2393.95/2342.12[a]	37.19/170.86[b]

[a]$p > 0.05$, not statistically significant, [b]$p < 0.05$, statistically significant, [c]$p = 0.109$, marginally significant, compared with the unirradiated cultures for UVA irradiation, respective for UVB irradiation.

SOD activity was directly correlated ($r = 0.732$, $p = 0.000$) with the enzymatic activity of tyrosinase, when the melanocytes were treated with 1 mM tyrosine (Fig. 5).

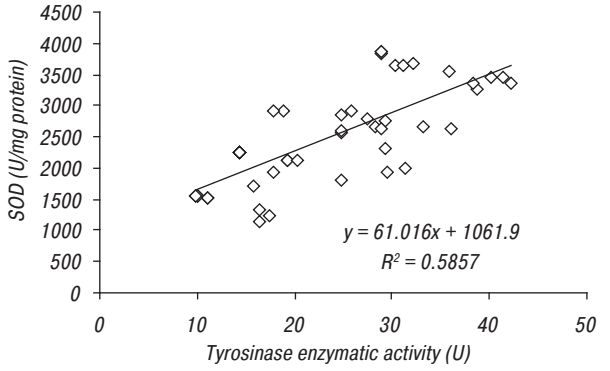

Fig. 5. Correlation between SOD and tyrosinase enzymatic activity in the melanocyte cultures exposed to 1 mM tyrosine and irradiated

However, when the melanocytes were exposed to 2 mM tyrosine, SOD and enzymatic tyrosinase activity were indirectly correlated ($p = 0.011$. $r = -0.488$) (Fig. 6).

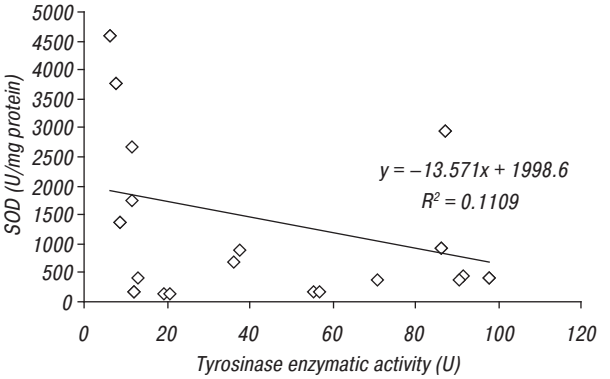

Fig. 6. Correlation between SOD and tyrosinase enzymatic activity in the melanocyte cultures exposed to 2 mM tyrosine and irradiated

SOD activity was also directly correlated ($r = 0.227$, $p = 0.099$) with the total melanin content of the cultures treated with 1 mM tyrosine (Fig. 7).

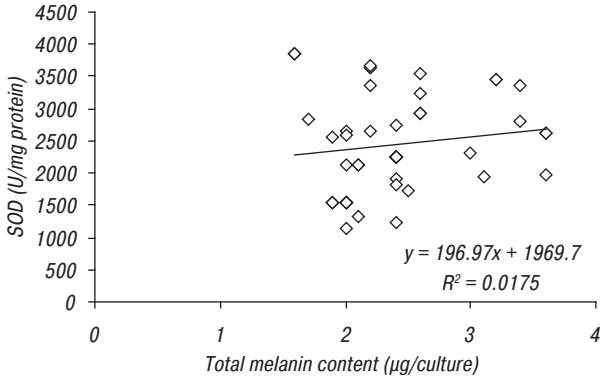

Fig. 7. Correlation between SOD enzymatic activity and total melanin content in the melanocyte cultures treated with 1 mM tyrosine and irradiated

After exposure to 2 mM tyrosine SOD activity was indirectly correlated with melanin content ($p = 0.022$, $r = -0.446$) (Fig. 8). There were no correlations between SOD and tyrosinase activity, respective melanin content in the cultures treated with 0.5 mM tyrosine.

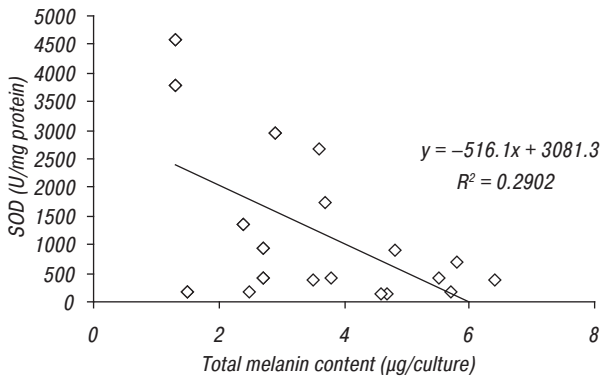

Fig. 8. Correlation between SOD enzymatic activity and total melanin content in the melanocyte cultures treated with 2 mM tyrosine and irradiated

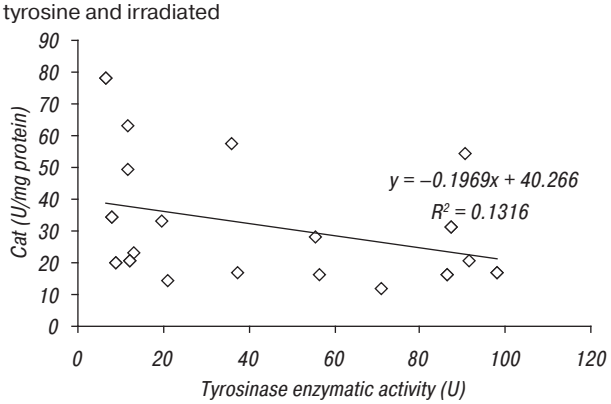

Fig. 9. Correlation between Cat and tyrosinase enzymatic activity in the melanocyte cultures treated with 2 mM tyrosine and irradiated

Cat indirectly correlated with tyrosinase activity when the melanocytes were treated to 2 mM tyrosine ($p = 0.045$, $r = -0.397$), but not with melanin production (Fig. 9). There were no correlations between the activity of Cat and tyrosinase when the cultures were exposed to lower tyrosine concentrations.

DISCUSSION

We observed the effects of oxidative stress on the rate of proliferation, melanogenesis, using normal human melanocyte cultures as cellular models. There were no significant differences in reaction between the adult and newborn epidermal melanocytes in the studied conditions. In our experiments, irradiation of the melanocytes was expected to increase oxidative stress defense enzymes directly and by triggering melanogenesis. This effect was modulated by tyrosine exposure before UV irradiation [4, 15–18, 34].

Proliferation studies showed a nonlinear decrease of proliferation with concentration, enhanced by time exposure to tyrosine, as previously described [15].

Exposure to lower concentrations of tyrosine (0.5 mM, 1 mM) discretely diminished the proliferation rate of the melanocytes, while the higher concentrations (2 mM, 3.4 mM) proved to be toxic for the cells. The cells were still viable, as shown by the trypan blue staining, but they had no ability to proliferate.

Only small doses of UVA respective UVB (20, 30 and 40 mJ/cm^2) were used in our experiments, comparable to physiologic sun exposure. There were no important differences of the irradiation time with UVA *vs* UVB, as the intensities of the light generated by the lamp were similar.

UVB decreased proliferation relative to UVA when melanocytes were exposed for 72 h to high tyrosine (2 mM, 3.4 mM), but not in cultures exposed to lower tyrosine concentration (0.5 mM, 1 mM). These differences can be due to the different UV light action mechanism depending on wavelength. Most of the biologic effects of UVA radiation in the epidermal melanocytes are mediated by reactive oxygen species (ROS). ROS seem to activate growth factors' receptors and in particular those of epidermal growth factor and initiate multiple signaling responses associated with mitogenesis and cell growth regulation [35–37].

UVB radiation has been established as the main cause of nonmelanoma skin cancer, particularly squamous cell carcinoma, due to direct DNA damage [38].

ROS generated in the cells by UVA irradiation and in a lower degree UVB, did not destroy the plasma membrane integrity, but decreased cell proliferation. This effect was enhanced by previous tyrosine exposure for both UVA and UVB irradiation. This is consistent with the research done by others, who showed an increased sensitivity of the skin type I melanocytes to UVA after increasing their melanin content, with tyrosine exposure [18]. This suggests that UVA irradiated cultured melanocytes are photosensitized by their own synthesized chromophores: melanin and pheomelanin [18].

We used melanocytes from individuals with low skin phototype (II, III). They do not present an intense tanning response after UV irradiation [25]. These melanocytes synthesize eumelanin and pheomelanin, in contrast to Negroid individuals who synthesize only eumelanin [9]. Melanin in light skin could contribute to sunlight-induced genotoxicity and maybe — to melanocyte transformation [34]. Eumelanin is capable of scav-

enging the superoxide anion and hydrogen peroxide, whereas pheomelanin acts as a photosensitizing agent [39]. It has been shown that melanocytes in culture are protected against UVB-induced direct DNA damage by increased melanin synthesis [40]. However, several experiments with normal and displastic nevi melanocytes or melanoma cells have failed to demonstrate that melanin in melanocytes protects them significantly against UV-induced direct DNA damage [41, 42].

In our experiments, tyrosine exposure stimulated melanogenesis, as previously described [4, 15–18]. This effect was enhanced with the time of exposure and tyrosine concentration. UVB stimulated pigmentation more than UVA after previous tyrosine exposure with lower concentrations (0.5 mM and 1 mM), as expected, considering that UVB is three to four times more effective per unit physical dose (J/cm^2) than UVA in inducing erythema, DNA damage, tanning and skin cancer in mice [43]. However, this situation was changed at high levels of tyrosine (2 mM) in medium.

Exposure to tyrosine, even in low concentrations, was stressful for the cells, which exhibited high levels of SOD and Cat activities. This was consistent with the work of others who found that tyrosine-induced melanogenesis in melanocytes was accompanied by increased production of ROS and decreased concentration of intracellular glutathione [42]. It also increased early induction of heme oxygenase 1 gene, a typical response to oxidative stress, after UVA irradiation [34].

Considering that UVA acts mainly through the ROS generation, physiological antioxidants play a crucial role in the skin photoprotection [44].

Lower tyrosine concentrations (0.5 mM, 1 mM) stimulated the enzymatic activity of SOD and Cat, but the high tyrosine concentration (2 mM) after an initial increase at 24 h, decreased their activity dramatically with time exposure to tyrosine. That correlated with the proliferation assay, which demonstrated that this concentration of tyrosine was toxic for the melanocytes through deleterious effect on the oxidative stress defense of the cells.

In cultures exposed to tyrosine, UVA was a more efficient stimulus for the induction of the stress enzymes than UVB. This could be explained through ROS generation. UVA irradiation enhanced SOD and Cat activity after less tyrosine stimulation than with UVB, but depleted the enzymatic reserves, especially Cat, after lower tyrosine exposure relative to UVB. Overall, UVA was more effective than UVB in inducing impairment in Cat activity, as shown also in previous studies [45, 46].

Our data showed that the levels of SOD and Cat activity in the cultures after 2 mM tyrosine exposure were low, regardless of the irradiation type. Low levels of Cat activity were previously observed in different cutaneous experimental models and they were always associated with a stress-prone status [1, 24, 47–49]. In melanocytes, the role of Cat is critical because it is the major enzyme responsible for the neutralization of H_2O_2 [23], a byproduct of the melanogenic pathway [50]. Cat oxidative damage is detrimental, because when damaged it recovers slowly [45, 51]. This results in accumulation of H_2O_2 in the cell and damages of several constituents, including Cat [22, 51] and tyrosinase [11].

Enhanced proliferation at high tyrosine concentration following UVA compared with UVB increased the number of melanocytes that exhibited imbalances of the normal antioxidant mechanisms, common in human melanoma cells [52]. Although viability was not significantly altered, cells experienced further oxidative stress and were depleted of antioxidant enzymatic defenses.

The hypothesis of melanocyte carcinogenesis states that an essential part of melanocytes' malignant transformation is a change in the redox state of melanin from a mostly antioxidant state to a prooxidant state [20]. This is supported by data that show that melanoma cells have a remarkably abnormal content of antioxidants, including vitamin E, polyunsaturated fatty acids, and catalase [4, 44, 53]. Also, on the clinical level, displastic nevi, recognized precursors of melanoma, suffer from chronic oxidative stress, even without the influence of UV radiation, due to increased pheomelanin synthesis [42].

In the cultures exposed to 1 mM tyrosine concentration the increased pigment production was directly correlated with the enzymatic activity of SOD. This is a very good indicator that melanogenesis itself directly produced oxidative stress in the cells.

The highest tyrosine concentration (2 mM) used for the melanocyte bioassay is 35–40 times higher than the physiological one and exerted a strong proliferation inhibition of the melanocytes, while modulating melanogenesis [5, 15]. In these cultures, increase of melanogenesis was correlated with the decrease of SOD and Cat activities that depleted the defense mechanisms against oxidative stress and proved to be damaging for the cells. This effect was enhanced by longer tyrosine exposure, which stimulated melanogenesis and triggered early senescent aspect of the melanocytes. Exhausting of the cell defense mechanisms rendered the melanocytes incapable of neutralizing the free radicals generated by UV exposure and melanin production. Also, synthesis of pheomelanin consumes cysteine and this may limit the capacity of the cellular antioxidative defense [42]. This hypothesis is sustained by the dynamics of the oxidative stress defense enzymes activity and the proliferation rate of the melanocytes.

The few types of melanocytes examined do not allow us to draw general conclusions. Our data indicate that in low phototype melanocytes, pigment formation, either following UV irradiation, or stimulated by tyrosine exposure is inducing oxidative stress defence mechanisms activation. In the studied conditions, UVA was more efficient in stimulating the activity of the stress enzymes but also in depleting enzymatic defenses against oxidative stress (especially Cat) compared with UVB. Pigment formation was detrimental for the cells, when exposed to high tyrosine concentrations, by reducing the activity of Cat and SOD, the natural antioxidants.

The physiologic response to UV light may be an adjunctive risk factor for people with low phototype for developing a melanoma, when exposed to UV irradiation.

How these findings relate to an enhanced skin carcinogenesis in low phototype individuals needs further investigation using long-term irradiation experiments.

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