

Human *melanocortin 1 receptor* variants, receptor function and melanocyte response to UV radiation

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Summary

Cutaneous pigmentation is determined by the amounts of eumelanin and pheomelanin synthesized by epidermal melanocytes and is known to protect against sun-induced DNA damage. The synthesis of eumelanin is stimulated by the binding of α -melanotropin (α -melanocyte-stimulating hormone) to the functional melanocortin 1 receptor (MC1R) expressed on melanocytes. The human *MC1R* gene is highly polymorphic and certain allelic variants of the gene are associated with red hair phenotype, melanoma and non-melanoma skin cancer. The importance of the *MC1R* gene in determining skin cancer risk led us to examine the impact of specific polymorphisms in this gene on the responses of human melanocytes to α -melanotropin and UV radiation. We compared the ability of human melanocyte cultures, each derived from a single donor, to respond to α -melanotropin with dose-dependent stimulation of cAMP formation, tyrosinase activity and proliferation. In each of those cultures the *MC1R* gene was

sequenced, and the eumelanin and pheomelanin contents were determined. Human melanocytes homozygous for Arg160Trp, heterozygous for Arg160Trp and Asp294His, or for Arg151Cys and Asp294His substitutions, but not melanocytes homozygous for Val92Met substitution, in the MC1R demonstrated a significantly reduced response to α -melanotropin. Additionally, melanocytes with a non-functional MC1R demonstrated a pronounced increase in their sensitivity to the cytotoxic effect of UV radiation compared with melanocytes expressing functional MC1R. We conclude that loss-of-function mutations in the *MC1R* gene sensitize human melanocytes to the DNA damaging effects of UV radiation, which may increase skin cancer risk.

Key words: Melanocortin 1 receptor, Human melanocytes, MC1R variants

Introduction

The significance of human cutaneous pigmentation lies in its protective role against sun-induced DNA damage and photocarcinogenesis (Kaidbey et al., 1979; Pathak et al., 1980; Pathak, 1995). Total melanin content and the relative amounts of eumelanin, the black-brown pigment, and pheomelanin, the red-yellow pigment, synthesized by human epidermal and follicular melanocytes are important determinants of skin and hair color, respectively. Fair skin and red hair, characterized by a low melanin content and a low eumelanin to pheomelanin ratio, is associated with poor tanning ability following sun exposure and with increased risk of skin cancer (Fitzpatrick et al., 1976; Pathak et al., 1980). Melanin-containing granules, or melanosomes, form supranuclear caps in keratinocytes, thus shielding the DNA from excessive exposure to UV radiation (UVR) (Pathak et al., 1971; Kobayashi et al., 1998). In dark skin with a high eumelanin relative to pheomelanin content, melanosomes are found throughout the epidermal layers (Pathak et al., 1971). However, in fair skin, melanosomes are absent from the suprabasal layer, allowing for increased UVR

penetration and DNA damage, and for increased susceptibility to sun-induced genotoxicity and carcinogenesis. Resistance of eumelanin to photodegradation and its ability to scavenge radicals (Menon et al., 1983) suggest that stimulation of eumelanin synthesis by epidermal melanocytes enhance photoprotection.

Eumelanin synthesis in melanocytes is stimulated by activation of the α -melanocyte stimulating hormone (α -MSH) receptor, termed the melanocortin 1 receptor (MC1R) (Geschwind et al., 1972; Tamate and Takeuchi, 1984; Hunt et al., 1995). The human *MC1R* is more polymorphic than several other pigment genes, including *tyrosinase*, suggesting its importance in determining constitutive pigmentation in humans (Sturm et al., 1998; Rees, 2000). Furthermore, mutations in the gene for proopiomelanocortin, the precursor for melanocortins and other bioactive peptides, result in red hair color in addition to metabolic abnormalities, including adrenal insufficiency and obesity (Krude et al., 1998). This underscores the significance of melanocortins in regulating eumelanin synthesis in humans.

The MC1R is a G-protein-coupled receptor with seven transmembrane domains (Chhajlani and Wikberg, 1992; Mountjoy et al., 1992). Binding of α -MSH to this receptor activates adenylate cyclase and increases cAMP formation (Suzuki et al., 1996). More than 30 allelic variants of the human *MC1R* have been identified mainly in northern European populations and in Australia (Valverde et al., 1995; Box et al., 1997; Smith et al., 1998; Rees, 2000). However, the consequences of these variants on the physiological function of the MC1R remain poorly understood. Among the variants so far reported, Arg142His, Arg151Cys, Arg 160Trp and Asp294His are the mutations mostly associated with the red hair phenotype and reduced tanning ability in several populations (Box et al., 1997; Smith et al., 1998; Healy et al., 2000). This supports the notion that α -MSH and its receptor significantly affect the response of melanocytes to UVR (Pawelek et al., 1992; Im et al., 1998). The above four *MC1R* variants are common in melanoma patients, and increase the risk of melanoma by more than twofold (Palmer et al., 2000). Expression of those variants in heterologous cell cultures reduced the functional coupling of the MC1R to adenylate cyclase (Frändberg et al., 1998; Schiöth et al., 1999). As yet, no studies have shown how allelic variants of *MC1R* would affect the function of the receptor in human melanocytes, a main physiological target for melanotropins in the skin. Therefore, we sought to analyze the biological consequences of *MC1R* mutations by investigating the responses of genetically different epidermal melanocyte cultures to α -MSH and UVR.

Materials and Methods

Melanocyte culture

Primary human melanocyte cultures were established from neonatal foreskins with different pigmentation, and maintained as described previously (Abdel-Malek et al., 1993; Abdel-Malek et al., 1995). Obtaining neonatal foreskins for this purpose was approved by the University of Cincinnati Medical Center Institutional Review Board. Bovine pituitary extract contained in the melanocyte growth medium contains high concentrations of melanotropins (Abdel-Malek et al., 1995), thus it was removed from the culture medium 2 to 3 days prior to, and for the duration of, each experiment in which the effects of α -MSH were tested.

Determination of tyrosinase activity and proliferation

To determine the effects of α -MSH on melanocyte proliferation and tyrosinase activity, melanocytes were plated onto 60 mm dishes at a density of 2.5×10^5 cells. 72 hours later, and every other day thereafter for a total of 6 days, the growth medium was changed and melanocytes were treated with 0 (control), 0.1, 1 or 10 nM α -MSH, or with 1 μ M forskolin ($n=3$ dishes per group) (both were obtained from Sigma Chemical Company, St Louis, Missouri). The tyrosine hydroxylase activity of tyrosinase was determined as described previously (Pomerantz, 1969; Abdel-Malek et al., 1992). The cell number was determined using a Coulter Counter. Each experiment was repeated at least twice for each melanocyte culture. Statistical analysis was carried out using the Student's *t*-test to compare the effects of different concentrations of α -MSH on each individual melanocyte culture. Two-way ANOVA was also used to compare the responses to different concentrations of α -MSH of melanocyte cultures that expressed wild-type MC1R and their MC1R mutant counterparts, which had reduced response to α -MSH.

Sequencing of the *MC1R* gene

PCR amplification, sequencing, and restriction fragment length polymorphism (RFLP) analysis of the *MC1R* gene were carried out as follows. Total RNA was purified from cultured human melanocytes using an RNA Easy Kit (Qiagen, Valencia, CA). The entire coding region of the MC1R was amplified using reverse transcriptase and nested PCR amplification. 3 μ g of total RNA was amplified in a PCR reaction containing standard concentrations of reverse transcriptase, Taq DNA polymerase, MgCl₂, RNase inhibitor, dNTPs, buffer and primers, as described previously (Koppula et al., 1997) in a total volume of 50 μ l. The complementary strand was synthesized at 43°C for 1 hour, and the *MC1R* was amplified for 35 cycles (1 minute at 94°C, 1 minute at 60°C, and 2 minutes at 72°C) in an automated thermal cycler (Gene Amp PCR System 9600, Perkin Elmer, Boston, MA). 1-5 μ l of the first reaction was amplified in two separate reactions using two sets of M13-adjointed nested primers, first half N-terminal primer+M13up (5'-TGTA AACGACGGCCAGTCC-TGGCAGCACCATGA ACTAAGC-3'); first half C-terminal primer+M13rp (5'-CAGGAAACAGCTATGACCTGGTCGTAGTAGGCGATGAAGAGC-3'); second half N-terminal primer+M13up (5'-TGTA AACGACGGCCAGTCCGCTACATCTCCATCTTCTACGCAC3'); and second half C-terminal primer+M13rp (5'-CAGGAAACAGCTATGACCTCTGCCACACTTAAAGC 3'). The standard concentrations of PCR reagents were added to the first reaction and amplified for 25 cycles (30 seconds at 94°C, 1 minute at 62°C and 1 minute at 72°C). The final reaction yielded a 640 (first half) and a 560 (second half) nucleotide product flanked by the M13 primers. 5 μ l of the PCR reaction was electrophoresed on a 2% agarose gel. The remainder was purified in a Centri-Spin Column (Princeton Separations, Adelphia, NJ). The PCR products were sequenced by dye primer chemistry. Briefly, M13 forward and reverse primers were labeled with four fluorescent dyes in four separate base-specific tubes. The products were electrophoresed and read by an automated sequencing machine (Perkin Elmer/Applied Biosystems models 373A or 377). We confirmed some mutations by restriction fragment length polymorphism analysis for variants at codons 151 (*Hha*I), 160 (*Ssr*II) and 294 (*Taq*I). The PCR products were digested using standard conditions and run on 1-3% agarose gels.

Determination of cAMP levels

The dose-dependent effect of α -MSH on cAMP formation in human melanocytes was determined using a cyclic AMP radioimmunoassay kit (Dupont-New England Nuclear, Boston, MA), as recommended by the manufacturer and as described previously (Suzuki et al., 1996). Duplicate samples were assayed from each culture well (triplicate wells/group) after the appropriate dilution (1:10 for groups treated with 10 nM α -MSH, and 1:5 for the remaining groups). Each culture was tested twice in two separate experiments. The data were analyzed by two-way ANOVA and Student's *t*-test, as described above.

Analysis of eumelanin and pheomelanin content and total melanin content

Melanocytes were lyophilized, and eumelanin and pheomelanin contents were analyzed using a microassay developed previously (Ito and Fujita, 1985). Duplicate samples of melanocytes deprived of bovine pituitary extract (approximately 1×10^6 melanocytes/sample) were oxidized by permanganate to pyrrole 2,3,5-tricarboxylic acid (PTCA) and analyzed by HPLC with UV detection to determine eumelanin content. Identical duplicate samples were hydrolyzed with hydriodic acid to aminohydroxyphenylalanine (AHP), and analyzed by HPLC with electrochemical detection to determine pheomelanin content. Variations of PTCA and AHP values were approximately 10% or less when determined on separate occasions in this study. The amount of eumelanin can be obtained by multiplying the amount of PTCA by a conversion factor of 160, while the amount of

Table 1. Determination of *MC1R* genotype and relative eumelanin and pheomelanin contents in human melanocyte cultures

Melanocyte	<i>MC1R</i> genotype	Eumelanin ($\mu\text{g}/10^6$ cells)	Pheomelanin ($\mu\text{g}/10^6$ cells)	Eumelanin/ pheomelanin	Total melanin ($\mu\text{g}/10^6$ cells)
729-b	Consensus sequence	26.7	4.29	6.23	49.3
747-c	Consensus sequence	1.23	1.43	0.86	14.7
751-b	Consensus sequence	ND	ND	ND	23.8
731-c	Heterozygous for Arg151Cys	ND	ND	ND	5.73
754-b	Heterozygous for Val92Met	34.4	2.49	13.8	41.8
755-c	Homozygous for Val92Met	2.58	2.15	1.20	11.7
765-c	Heterozygous for Val92Met	0.83	0.55	1.51	4.20
777-c	Heterozygous for Val60Leu	1.46	3.01	0.49	14.6
790-c	Heterozygous for Arg163Gln	1.25	0.87	1.44	8.81
780-b	Heterozygous for Phe196Leu	25.9	3.05	8.49	41.7
753-c	Homozygous for Arg160Trp	0.66	0.86	0.77	3.3
830-c	Compound heterozygous for Arg160Trp and Asp294His	2.30	1.48	1.55	4.60
849-b	Compound heterozygous for Arg151Cys and Asp294His	14.9	3.64	4.09	33.8

The *MC1R* genotype of 13 melanocyte cultures was determined by sequence analysis of the entire coding region of the *MC1R* gene, as described in Materials and Methods. The genotypes for NHM 830-c and 849-c were further confirmed by RFLP analysis. The melanocyte cultures are grouped according to whether they are homozygous for the consensus *MC1R*, heterozygous for a *MC1R* variant, or homozygous or compound heterozygous for *MC1R* variants. Cultures were analyzed for eumelanin, pheomelanin and total melanin contents. ND, not determined.

pheomelanin can be obtained by multiplying the amount of AHP by a conversion factor of 10 (Ozeki et al., 1996). Statistical significance of differences was assessed with the Mann-Whitney test. Differences were considered to be significant when *P* values were less than 0.05.

Total melanin content was determined in $0.5\text{-}1\times 10^6$ melanocytes. Cells were harvested, centrifuged, washed twice with PBS, counted and centrifuged. The cell pellets were solubilized in 0.2 M NaOH (1×10^6 cells/ml) for 1 hour, and melanin content was determined spectrophotometrically by reading the absorbance at 475 nm. Melanin content was calculated using a standard curve generated from the absorbance of known concentrations of synthetic melanin, as described previously (Barker et al., 1995).

Response of melanocytes to UVBR

The response of melanocytes to UVBR was determined by plating the cells in complete growth medium at a density of 1×10^5 cells/60 mm dish. 72 hours later, melanocytes were irradiated once with $21\text{ mJ}/\text{cm}^2$ UVBR as described previously (Barker et al., 1995). The UV source is a bank of six FS20 sun lamps (Westinghouse) with 75% emission in the UVB and 25% emission in the UVA range. The peak emission of the lamps is at 313 nm. Percent cell death was determined on days 2 and 4 after UV irradiation by calculating the number of dead melanocytes that detached and incorporated Trypan blue dye and the number of viable cells that remained attached to the culture dish and excluded Trypan blue, as described before (Barker et al., 1995). The responses to UVR of the cultures with functional *MC1R* were compared with those of the cultures with reduced response to α -MSH using one-way ANOVA.

Results

Characterization of *MC1R* genotype and eumelanin to pheomelanin ratios

We established 35 human melanocyte (NHM) cultures from light-colored foreskins (NHM-c) and eight cultures from dark foreskins (NHM-b), and compared their ability to respond to α -MSH with dose-dependent increases in proliferation and in the activity of tyrosinase, the rate-limiting enzyme in the melanin synthetic pathway. Of those, 33 NHM-c and seven NHM-b demonstrated dose-dependent increases in tyrosinase activity and proliferation in response to α -MSH over the range

of doses tested. The remaining three cultures had no, or minimal response to α -MSH. We sequenced the entire coding region of the *MC1R* in the three cultures with impaired responsiveness to α -MSH and in ten α -MSH-responsive cultures (four NHM-b and six NHM-c). We analyzed 11 of those 13 cultures for their eumelanin and pheomelanin contents to assess the differences in their constitutive pigmentation. A higher number of NHM-c than NHM-b cultures was included since variation in the *MC1R* is expected to be higher in individuals with fair skin and red hair phenotype than in individuals with dark skin and hair color (Valverde et al., 1995; Smith et al., 1998; Harding et al., 2000).

The sequencing data presented in Table 1 revealed that NHM 753-c was homozygous for Arg160Trp, NHM 830-c was heterozygous for Arg160Trp and Asp294His, and NHM 849-b was heterozygous for Arg151Cys and Asp294His substitutions in *MC1R*. All three cultures failed to respond to α -MSH with a significant dose-dependent increase in tyrosinase activity (Fig. 1B). NHM 755-c was homozygous for Val92Met substitution, and homozygous for a silent mutation, Thr314Thr, in the *MC1R*. Four other cultures were heterozygous for Arg151Cys (NHM 731-c), Val60Leu (NHM 777-c), Arg163Gln (NHM 790-c) and Phe196Leu (NHM 780-b) substitutions. Two cultures were heterozygous for Val92Met substitution (NHM 754-b and 765-c). NHM 754-b and 780-b were heterozygous for a silent mutation, Thr314Thr and Thr177Thr, respectively. Only three cultures, NHM 729-b, 747-c and 751-b carried the wild-type *MC1R* genotype.

Analysis of eumelanin to pheomelanin content showed that NHM-b cultures consistently expressed higher eumelanin to pheomelanin ratios than NHM-c cultures. The mean (\pm s.d.) content of eumelanin of NHM-b cultures (25.5 ± 8.02 ; $n=4$) was more than tenfold higher ($P\leq 0.01$) than that of NHM-c cultures (1.47 ± 0.72 ; $n=7$). Also, the mean (\pm s.d.) content of pheomelanin of NHM-b cultures (3.37 ± 0.77) was significantly higher ($P\leq 0.01$) than that of NHM-c (1.48 ± 0.86). As a result, the mean (\pm s.d.) eumelanin to pheomelanin ratio of NHM-b (8.15 ± 4.17) was sevenfold higher ($P\leq 0.01$) than that of NHM-c (1.12 ± 0.41).

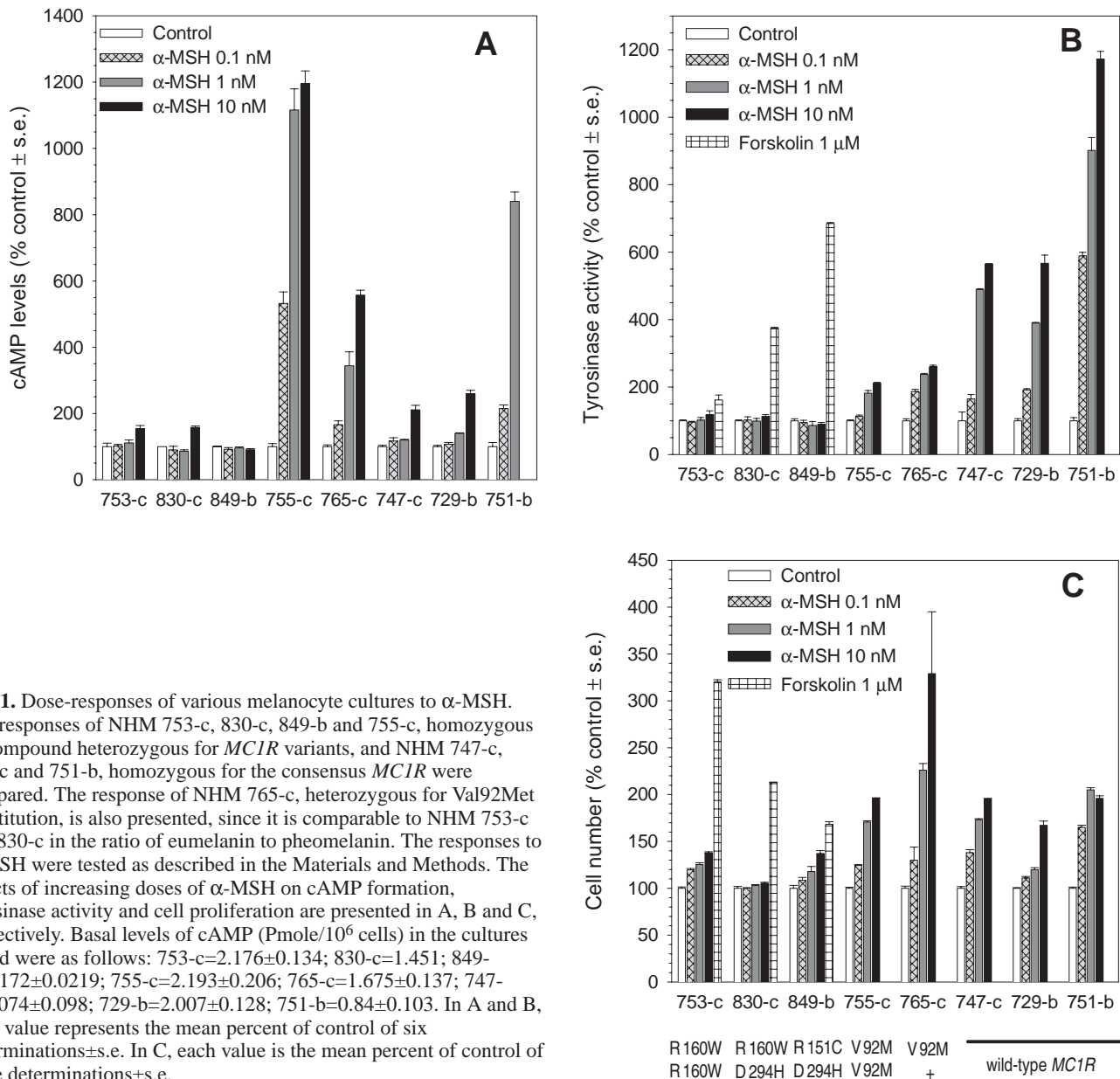


Fig. 1. Dose-responses of various melanocyte cultures to α -MSH. The responses of NHM 753-c, 830-c, 849-b and 755-c, homozygous or compound heterozygous for *MC1R* variants, and NHM 747-c, 729-c and 751-b, homozygous for the consensus *MC1R* were compared. The response of NHM 765-c, heterozygous for Val92Met substitution, is also presented, since it is comparable to NHM 753-c and 830-c in the ratio of eumelanin to pheomelanin. The responses to α -MSH were tested as described in the Materials and Methods. The effects of increasing doses of α -MSH on cAMP formation, tyrosinase activity and cell proliferation are presented in A, B and C, respectively. Basal levels of cAMP (Pmole/ 10^6 cells) in the cultures tested were as follows: 753-c=2.176 \pm 0.134; 830-c=1.451; 849-b=1.172 \pm 0.0219; 755-c=2.193 \pm 0.206; 765-c=1.675 \pm 0.137; 747-c=1.074 \pm 0.098; 729-b=2.007 \pm 0.128; 751-b=0.84 \pm 0.103. In A and B, each value represents the mean percent of control of six determinations \pm s.e. In C, each value is the mean percent of control of three determinations \pm s.e.

Response of melanocytes with known *MC1R* genotype to α -MSH

Melanocyte cultures homozygous for the consensus *MC1R* or heterozygous for one variant of the *MC1R* (represented by the data for NHM 765-c) responded to α -MSH with dose-dependent increases in cAMP levels, tyrosinase activity and proliferation (Fig. 1). Variations in the magnitude of the response to α -MSH among cultures may be attributed to differential expression of other genes involved in the regulation of pigmentation. NHM 753-c, homozygous for Arg160Trp, 830-c, heterozygous for Arg160Trp and Asp294His, and 849-b, heterozygous for Arg151Cys and Asp294His substitutions in *MC1R* were unresponsive, or 100-times less responsive to α -MSH than melanocytes wild-type or heterozygous for *MC1R* variants (Fig. 1). Comparing the responses to α -MSH of NHM 753-c, 830-c and 849-b to that of NHM 765-c demonstrated significant differences. NHM 753-c, 830-c and 849-b showed

no significant change in cAMP levels after treatment with α -MSH, as determined using Student's *t*-test. (Fig. 1A). In contrast, NHM 765-c, heterozygous for Val92Met substitution and with a low eumelanin to pheomelanin ratio, responded to 0.1 or 10 nM α -MSH with significant increases in cAMP levels above control (66% and 4.5-fold, respectively; $P \leq 0.001$, as determined by Student's *t*-test). NHM 755-c homozygous for the Val92Met substitution responded to 0.1 nM and 10 nM α -MSH with a 4-fold and 11-fold increase in cAMP formation (Fig. 1A).

Additionally, NHM 753-c, 830-c and 849-b showed no change, while NHM 765-c showed significant increases, in tyrosinase activity in response to 0.1 or 1 nM α -MSH (about 90% or 130% increase, respectively; $P < 0.0001$ using Student's *t*-test) (Fig. 1B). NHM 753-c, 830-c and 849-b showed no significant stimulation, while NHM 765-c showed a 160% increase in tyrosinase activity after treatment with 10 nM α -

MSH. NHM 753-c, 830-c and 849-b responded to 1 μ M forskolin, an activator of adenylate cyclase, with remarkable increases in proliferation and tyrosinase activity. This indicated that their adenylate cyclase is inducible and that their inability to respond to α -MSH is due to a defect that lies upstream of adenylate cyclase (Fig. 1B,C). NHM 755-c responded dose-dependently to 1 or 10 nM α -MSH, with an 80 or 110% increase in tyrosinase activity, respectively ($P < 0.001$, as determined by Student's *t*-test).

As expected, the cultures wild-type for *MC1R* responded with a dose-dependent increase in proliferation beginning at a dose of 0.1 nM (Abdel-Malek et al., 1995; Suzuki et al., 1996). NHM 753-c and 849-b demonstrated a significant increase (38% above control) in cell number only following treatment with 10 nM α -MSH ($P < 0.01$); NHM 830-c showed no stimulation of proliferation at any of the concentrations of α -MSH that were used (Fig. 1C). In comparison, NHM 765-c demonstrated a 30 ($P < 0.1$), 126 or 229% ($P < 0.0001$) increase, and NHM 755-c, homozygous for Val92Met substitution, demonstrated a 25, 70 or 96% increase in cell number above control ($P < 0.0001$) in response to 0.1, 1 or 10 nM α -MSH, respectively. Further statistical analysis using two-way ANOVA showed that the effects of 1 and 10 nM α -MSH on cAMP levels, tyrosinase activity and proliferation of NHM 753-c, 830-c and 849-b were significantly different than the effects on NHM 747-c, 729-b and 751-b with wild-type *MC1R* ($P \leq 0.01$).

MC1R genotype and the responses of melanocytes to UVR

We assessed the survival of melanocyte cultures following a single exposure to a dose of 21 mJ/cm² UVBR. This treatment resulted in a 28, 31 and 34% cell death of NHM 753-c, 830-c and 849-b, respectively, compared with only 6% cell death of NHM 765-c and 755-c, at 2 days after irradiation (Fig. 2). NHM 753-c, 830-c and 849-b encountered a 17, 22 and 27% cell death, respectively, on day 4 after UVB exposure, compared with 8 and 5% cell death in NHM 765-c and 755-c, respectively (Fig. 2). The differences in the extent of cell death between the cultures that were unresponsive to α -MSH and those with functional *MC1R* were statistically significant, as determined by one-way ANOVA, which took into account the responsiveness to α -MSH ($P < 0.0001$). The responses of the latter two cultures is comparable with that of many other cultures with functional *MC1R* that we have tested. We did not detect significant differences in the amounts of UVB-induced cyclobutane pyrimidine dimers or pyrimidine 6,4-pyrimidone photoproducts in NHM 753-c with loss-of-function *MC1R* and NHM 765-c with functional *MC1R* that have comparable melanin contents (data not shown). Melanocyte cultures respond to UVB-irradiation with a linear dose-dependent increase in the generation of extracellular hydrogen peroxide. We did not detect significant differences in the amounts of extracellular hydrogen peroxide generation in NHM 830-c with a loss-of-function *MC1R* and a NHM-c with a functional *MC1R* and comparable melanin content (data not shown).

Discussion

An important determinant of skin cancer risk is cutaneous

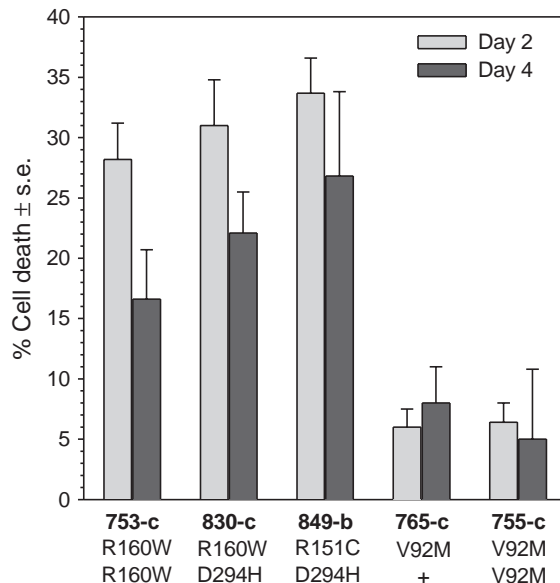


Fig. 2. Cytotoxic effects of UVBR on melanocytes with different *MC1R* genotypes. We compared the response to UVBR of NHM 753-c, 830-c and 849-b homozygous or heterozygous for loss-of-function mutations in *MC1R* to that of NHM 765-c and NHM 755-c, heterozygous or homozygous for Val92Met substitution, respectively. All melanocyte cultures were irradiated a single time with UVBR and cell number and viability were determined on days 2 and 4 after irradiation, as described in Materials and Methods. Each value represents the mean percent cell death of three determinations \pm s.e.

pigmentation and the ability to tan upon sun exposure. Several studies have suggested a crucial role for *MC1R* in regulating eumelanin synthesis and the pigmentary response of human skin to UVR. Allelic variants of the *MC1R*, particularly Arg142His, Arg151Cys, Arg160Trp and Asp294His, are strongly associated with red hair phenotype, reduced tanning ability, melanoma and possibly other skin cancers (Box et al., 1997; Box et al., 2001; Smith et al., 1998; Healy et al., 2000; Palmer et al., 2000; Bastiaens et al., 2001; Kennedy et al., 2001).

The current study is the first to elucidate the impact of various allelic variants of the human *MC1R* on the response of human melanocytes to α -MSH and UVR. Genetic sequencing of the *MC1R* in 13 different melanocyte cultures revealed extensive polymorphism, with 31% of the cultures homozygous for a *MC1R* allelic variant or compound heterozygous for two different allelic variants, and 54% of the cultures heterozygous for one *MC1R* variant (Table 1). Unexpectedly, NHM 849-b, with a relatively high eumelanin to pheomelanin ratio (4.09), was heterozygous for Arg151Cys and Asp294His substitutions in *MC1R* (Table 1), confirming that *MC1R* variants are necessary but not sufficient for the red hair phenotype (Sturm et al., 1998).

All 13 melanocyte cultures were compared for their ability to respond to α -MSH with dose-dependent stimulation of cAMP formation, tyrosinase activity and proliferation (Fig. 1). Binding of α -MSH to the *MC1R* stimulates cAMP formation, a major pathway for stimulation of melanogenesis, particularly eumelanin synthesis, and proliferation in human melanocytes (Abdel-Malek et al., 1992; Suzuki et al., 1996; Sakai et al.,

1997). Our finding that increases in tyrosinase activity did not correlate perfectly with increases in cAMP following treatment of responsive melanocyte cultures to α -MSH suggest that the MC1R activates other pathways, such as protein kinase C β (Park et al., 1996). As expected, melanocyte cultures homozygous for wild-type *MC1R* demonstrated a typical dose-dependent response to α -MSH beginning at a dose of 0.1 nM (Fig. 1) (Abdel-Malek et al., 1995; Suzuki et al., 1996). All cultures heterozygous for a *MC1R* variant had dose-dependent responses to α -MSH similar to those of cultures expressing the wild-type gene, showing that a single wild-type functional copy of MC1R is sufficient for receptor function. In contrast, NHM 753-c, homozygous for Arg160Trp, 830-c and 849-b, heterozygous for Arg160Trp and Asp294His and for Arg151Cys and Asp294His, respectively, had a drastically reduced response to α -MSH (Fig. 1). The inability of NHM 753-c, 830-c and 849-b to respond to α -MSH with a dose-dependent stimulation of cAMP formation is not due to lack of expression of the *MC1R* gene, as all three cultures expressed *MC1R* mRNA as determined by northern blot analysis (data not shown), nor to a defect in adenylate cyclase since they were responsive to forskolin (Fig. 1B,C). The high eumelanin to pheomelanin ratio of 849-b is not the result of a constitutively active MC1R that could not be stimulated by α -MSH, as in the sombre mouse (Robbins et al., 1993; Abdel-Malek et al., 2001). Basal cAMP level in 849-b was comparable with the levels in 747-c and 751-c, which expressed the consensus MC1R, as described in the legend for Fig. 1A. Our findings are corroborated by previous reports and demonstrate that Arg151Cys, Arg160Trp, and Asp294His substitutions in the MC1R significantly diminish the functional coupling of the receptor as shown by poor stimulation of intracellular cAMP production in response to α -MSH (Fig. 1A) (Frändberg et al., 1998; Schiöth et al., 1999). In transfected cells, those allelic variants did not significantly reduce the binding affinity of α -MSH for MC1R since Arg151Cys and Arg160Trp lie within the second intracellular loop of the MC1R, a region unlikely to be involved in receptor binding (Prusis et al., 1997).

One report (Xu et al., 1996) suggested that the Val92Met substitution reduces the binding affinity of MC1R for α -MSH, while another found no alteration in the functional coupling of the receptor to adenylate cyclase (Koppula et al., 1997). This variant was identified in Chinese individuals, and therefore is not associated with a red hair phenotype (Box et al., 1997). Here, we showed that NHM 755-c, homozygous for Val92Met substitution, responds dose-dependently to α -MSH with stimulation of cAMP formation, tyrosinase activity and proliferation, suggesting that this polymorphism does not represent a loss-of-function in the *MC1R* (Fig. 1A-C).

A role for the melanocortins and MC1R in the response of melanocytes to UVR and in determining the tanning ability of individuals has been proposed (Pawelek et al., 1992; Im et al., 1998; Healy et al., 2000). The observed exaggerated sensitivity of melanocyte cultures with loss-of-function MC1R to UVR emphasizes the significance of the *MC1R* in the cutaneous response to UVR (Fig. 2). Comparison of the UV responses of NHM 765-c and 755-c with functional MC1R to the UV responses of NHM 753-c and 830-c with loss-of-function MC1R revealed a striking difference in the extent of cell death. Despite similar melanin contents in all four melanocyte cultures, NHM 753-c and 830-c demonstrated a greater extent

of cell death than NHM 765-c and 755-c. Additionally, the observation that NHM 849-b, with a high eumelanin to pheomelanin ratio and loss-of-function MC1R, is more sensitive to UVR-induced cytotoxicity than NHM 765-c or 755-c suggests that regardless of constitutive pigmentation, inability of melanocytes to respond to α -MSH reduces their defense mechanisms against UVR genotoxicity. The mechanism for the increased susceptibility of these melanocytes to UVR is now being investigated. Our preliminary results showed that the amounts of cyclobutane pyrimidine dimers and pyrimidine(6-4)pyrimidone photoproducts, the major types of DNA damage induced by UVR (Brash, 1988), were not increased in NHM 753-c. However, the rate of removal of these photoproducts is yet to be determined. Our results suggest that the extensive cell death observed in NHM 753-c and, by extension, in NHM 830-c and 849-b, possibly result from impaired DNA repair and/or oxidative DNA damage. Our preliminary data show a linear dose-dependent increase in the generation of hydrogen peroxide by UVB-irradiated melanocytes, suggesting an induction of a prooxidant state. Recently, it was reported that α -MSH protects from oxidative stress that may result from exposure of cells to UVR (Haycock et al., 2000).

This report is significant since it is the first to document the effects of four known *MC1R* variants, namely Arg151Cys, Arg160Trp, Asp294His and Val92Met, on the responses of human melanocytes to α -MSH and UVR. Our results suggest that the *MC1R* genotype is a useful marker that is more reliable than melanin content for predicting the sensitivity of individuals to sun exposure and their susceptibility to skin cancer.

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