Applying the basic knowledge about regulation of pigmentation towards development of strategies for cutaneous hypopigmentation

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Abstract

The extensive variation in human cutaneous pigmentation is mainly due to differences in the rate of melanin synthesis by epidermal melanocytes, the relative amounts of eumelanin and pheomelanin synthesized, and the manner and rate of transfer of melanosomes from melanocytes to keratinocytes. Pigmentation is a complex trait that is regulated genetically and environmentally. One gene that has been receiving a lot of attention is the gene for the melanocortin 1 receptor. The extensive polymorphism of this gene in human populations suggests its significance in the diversity of pigmentation. Exposure to solar ultraviolet radiation (UV) results in increased synthesis of a variety of growth factors, cytokines and hormones, and in modulation of their receptors in the epidermis. Knowledge about the regulation of pigmentation has led to strategies for clinical treatment of hyperpigmented skin lesions. Three main strategies are: 1) the use of chemicals that interfere with the melanin synthetic pathway, 2) the design of peptides or peptide-mimetics based on the structure of hormones that regulate eumelanin synthesis, and 3) the use of agents that reduce melanosome transfer from melanocytes to keratinocytes. All three strategies are expected to induce hypopigmentation, by inhibiting total melanin synthesis, eumelanin production, or the epidermal melanin unit, respectively.

EDUCATION

1974 B.S. Biology American University of Beirut, Beirut, Lebanon
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PROFESSIONAL AND RESEARCH EXPERIENCE

1974 Laboratory Assistant, Hematology Section of the Department of Clinical Pathology American University Medical Center, Beirut, Lebanon
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1993 Research Associate Professor of Dermatology,Department of Dermatology, University of Cincinnati College of Medicine, Cincinnati, Ohio

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PUBLICATIONS

Author or co-author of more than 50 publications in international peer reviewed journals.
Author and editor of several scientific books.
Author of more than 180 oral presentations.
Author of more than 80 poster presentations.
More than 90 times invited speaker.

SCIENTIFIC AWARDS

1990 The Vitiligo Research Award sponsored by the Skin Disease Society and awarded by the PanAmerican Society for Pigment Cell Research

MEMBERSHIP

2002 Vice-President of the Federation of Pigment Cell Societies

2002 President of Pan American Society for Pigment Cell Research

1999 President-elect of Pan American Society for Pigment Cell Research

1996 American Society for Photobiology, Member

1992 Council Member of Pan American Society for Pigment cell Research

1989 American Association for the Advancement of Science, Member
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국문요약

인체피부의 색소침착에 있어서 다양한 차이는 주로 표피의 멜라닌생성 세포에 의한 멜라닌 합성 비율, 합성된 eumelanin과 pheomelanin의 상대적인 양과 melanocyte에서 keratinocyte로 melanosomes의 이동 속도와 그 방법에서 기인된다.

색소침착은 유전적, 환경적으로 조절되어지는 복합적 특성이다. 많은 관심이 집중되었던 하나의 유전자인 melanocortin 1 receptor 유전자가 있다. 인간집단에서 이 유전자의 다양한 polymorphism은 색소침착의 다양성에 있어서 중요하다. 자외선(UV)에 대한 노출은 다양한 성장 요인, cytokines과 호르몬의 합성이 증가되고, 표피에서 그들 수용체들의 환경적응등이 나타난다. 색소침착 조절에 관한 정보는 과다색소 침착된 피부손상의 임상치료를 위한 전략들에서 이끌어 냈다. 주된 3개의 전략은 다음과 같다. : 1) melanin 합성경로를 방해하는 화합물의 사용 2) eumelanin 합성을 조절하는 호르몬의 구조에서 기인된 peptides 또는peptide-mimetics개발 3) melanocytes에서 keratinocytes로의 melanosome 이동을 감소시키는 물질의 개발.

이 모든 3가지의 전략은 각각 전체 멜라닌 합성, eumelanin 생성 또는 피부의 멜라닌 단위를 억제시킴으로 미백작용을 유도시킬 것으로 기대되어 진다.

Applying the Basic Knowledge About Regulation of Pigmentation Towards Development of Strategies for Cutaneous Hypopigmentation

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Regulation of Human Cutaneous Pigmentation

Human cutaneous pigmentation is primarily determined by three factors, the rate of melanin synthesis by epidermal melanocytes, the relative amounts of eumelanin and pheomelanin synthesized by melanocytes, and the rate of transfer of melanin-containing melanosomes from melanocytes to adjacent keratinocytes (1). These three factors are controlled genetically and environmentally. Human pigmentation is regulated by at least 70 genes that code for enzymes, structural proteins, transcription factors, growth factors and receptors. The main environmental factor that regulates pigmentation is solar ultraviolet radiation (UV). A hallmark of sun exposure is tanning, caused by stimulation of melanin synthesis and increased melanin transfer to keratinocytes.

The ability to culture human melanocytes have allowed for extensive progress in understanding the regulation of human pigmentation. Primary cultures of human melanocytes are an optimal *in vitro* model for investigating human pigmentation, since melanocytes continue to express in culture the pigmentary phenotype of their skin of origin for their entire life span (2). It is established that total melanin content in cultured

human melanocytes correlates directly with the activity of tyrosinase, the rate-limiting enzyme in the melanin synthetic pathway (2,3). Also, total melanin content correlates with the protein levels of tyrosinase, tyrosinase-related protein (TRP)-1 and-2 (4). Melanocytes cultured from dark skin consistently express higher tyrosinase activity, and higher protein levels of tyrosinase, TRP-1 and TRP-2 than melanocytes cultured from lightly pigmented skin. The concentrations of L-tyrosine, the precursor for melanin, and the first substrate for tyrosinase, and L-cysteine, which is required for pheomelanin synthesis, determine the amounts of eumelanin, the brown-black pigment, and pheomelanin, the red-yellow pigment, synthesized by melanocytes (5). Higher eumelanin to pheomelanin ratios are found in dark skin than in light skin (6). This is also true for melanocytes derived from dark skin in comparison to melanocytes cultured from lightly pigmented skin (6) (Abdel-Malek & Ito, unpublished results). It has become evident that the synthesis of eumelanin requires the availability of high concentrations of tyrosine in melanosomes, and the expression of high enzymatic activities and protein levels of tyrosinase, TRP-1 and TRP-2 (5,7). The requirements of pheomelanin synthesis are less stringent than those of eumelanin, and low levels of tyrosinase, TRP-1 and TRP-2 in the presence of low concentrations of tyrosine, are permissive for pheomelanogenesis (8,9). Eumelanin is synthesized and deposited in melanosomes that are elliptical in shape and have a well-organized matrix (10). Pheomelanin, on the other hand, is synthesized by smaller size spherical melanosomes, lacking an organized matrix. The manner by which melanosome transfer occurs in dark versus in lightly-pigmented skin is different. In dark skin, melanosomes that are mostly large and contain eumelanin, are transferred from melanocytes to keratinocytes as single entities. In light skin, melanosomes, most of

which are small and spherical, and presumably contain pheomelanin, are found in keratinocytes as packages (11). Similar findings have been reported for co-cultures of melanocytes and keratinocytes from dark versus light skin (12).

Stimulation of melanogenesis in human melanocytes in response to UV exposure is accompanied by increased melanosome number and rate of transfer of melanosomes from melanocytes to keratinocytes (1,11). In dark skin, melanosomes remain intact throughout the epidermal layers and form supranuclear caps in keratinocytes, shielding their nucleus from excessive DNA damage by UV (11,13). On the other hand, in light skin, intact melanosomes are rarely present in the suprabasal area of the epidermis. This is postulated to be one reason for the difference in photoprotection afforded by melanin in dark versus light skin. Exposure to UV affects the skin via two major mechanisms: direct effect on DNA, leading to DNA photoproducts and the generation of reactive oxygen radicals, and indirect effects, resulting in modulating the synthesis of a wide variety of cytokines and growth factors, and modulation of the expression of various receptors. It is now well-established that a paracrine/autocrine network exists in the human epidermis that regulates the response to UV exposure. At least some of those factors affect the response of melanocytes to UV, and include the melanocortins, endothelin-1, basic fibroblast growth factor, interleukin-1 and tumor necrosis factor-α (14-16). The melanocortins α-melanocortin (α-melanocyte stimulating hormone; α-MSH) and ACTH, and endothelin-1 are known to be mitogenic and melanogenic for, and to mediate the melanogenic response of human melanocytes to UV (15,17,18). Interleukin 1 and tumor necrosis-α inhibit the proliferation and melanogenesis of cultured human melanocytes, and are presumed to have a negative feedback effect on the melanogenic and mitogenic

responses to various stimulatory factors (19). As has been shown decades ago in mouse follicular melanocytes, treatment of human melanocytes with α-MSH stimulates eumelanin synthesis (20) (Abdel-Malek and Ito, unpublished results). Recent preliminary data from our laboratory indicate that combined treatment with α-MSH and endothelin-1 further increases this effect (Abdel-Malek and Ito, unpublished results). Stimulation of melanogenesis, as with treatment with α-MSH, increases melanosome number and dendricity of melanocytes, which are expected to enhance melanosome transfer to keratinocytes (17). From these observations, it can be concluded that the melanogenic response of human melanocytes to UV is the outcome of increased melanin, particularly eumelanin, synthesis and melanosome transfer.

Common Strategies for Cutaneous Hypopigmentation

Knowledge about the regulation of pigmentation, particularly the significance of melanin synthesis and transfer, and about the physiologic factors that regulate melanogenesis have led to various strategies for manipulating cutaneous pigmentation. To induce hypopigmentation, the main strategies that have been employed include: 1) interfering with the melanin synthetic pathway, e.g. by using chemicals that act as pseudo-substrates for tyrosinase, 2) reducing the transfer of melanosomes to keratinocytes by inhibiting the activation of PAR-2, receptors important for this process, 3) designing peptides or peptidomimetics that antagonize physiological melanogenic factors such as α-MSH.

Phenols and catechols were first discovered as depigmenting agents based on the observations that industrial exposure to monobenzyl ether of hydroquinone, paratertiary

butylphenol or paratertiary amylphenol formaldehyde resin resulted in leukoderma. A compound that has been widely used clinically for skin hypopigmentation is hydroquinone. The hypopigmentary effect of hydroquinone is thought to be due to its action as an alternative substrate for tyrosinase, since no melanin is formed *in vitro* when tyrosinase was incubated with tyrosine and hydroquinone (21). Also, by HPLC analysis, hydroquinone was shown to be preferentially oxidized instead of tyrosine. Another possible mechanism of action of hydroquinone is the formation of reactive oxygen species that might damage lipid membranes (22). Additionally, hydroquinone might deplete glutathione (23), thus disrupting the antioxidant defenses of melanocytes. Combined treatment with hydroquinone and S-*n*-butyl homocysteine sulfoximine (BSO), an inhibitor of γ-glutamyl cysteine synthase, results in potentiation of the hypopigmentary effect of hydroquinone *in vitro* as well as *in vivo* (24). In our experience treatment of human melanocytes with BSO, which depletes glutathione, results in increased pheomelanin content, which in turn is expected to reduce pigmentation (Abdel-Malek and Ito, unpublished results). We postulate that this effect of BSO is due to increased availability of cysteine for utilization in the synthesis of pheomelanin, rather than glutathione.

Other hydroquinone-related products that have been investigated and used as hypopigmentary agents include arbutin, kojic acid and alkyl esters of gentisic acid. Arbutin, a naturally occurring β-D-glucopyranoside of hydroquinone, was found to act as a competitive inhibitor of tyrosinase in human melanocytes, at non-cytotoxic doses (25). Arbutin is thought to compete with L-tyrosine for binding to the active site of tyrosinase and to reduce the amounts of melanin intermediates, such as DHICA. These results

suggest that the hypopigmentary effect of arbutin is mediated by inhibition of tyrosinase as well as post-tyrosinase steps in the melanogenic pathway, and is not due to cytotoxicity of melanocytes. Alkyl esters of the naturally-occurring product of gentisic acid, which is similar in structure to hydroquinone, are selective inhibitors of tyrosinase activity, are less cytotoxic than hydroquinone, and lack mutagenic potential (26). Kojic acid (5-hydroxy-2-hydroxymethyl-4-H-pyran-4-one) is a copper chelator which functions as a slow-binding competitive inhibitor of tyrosinase and as an inhibitor of DHICA formation (27,28). Comparison of gentisic acid, hydroquinone, kojic acid and arbutin revealed that hydroquinone and arbutin are relatively poor tyrosinase inhibitors (26).

Design of peptide analogs or peptidomimetic compounds based on the structure of physiologic regulators of pigmentation has been extensively used as a strategy to develop hyper- or hypopigmentary agents. The hormone that has been mostly utilized as a basis for developing agonists or antagonists is α-MSH. The physiological α-MSH consists of 13 amino acids, which makes it feasible to synthesize numerous analogs by manipulating the structure of the native peptide, and designing either full-length or fragment analogs. This approach was initially undertaken for the purpose of understanding structure-activity relationships of the hormone, and later, synthesized analogs were investigated for possible application for modulating human pigmentation (29).

It is now established that α-MSH elicits its pigmentary effects on human melanocytes by binding to the melanocortin 1 receptor (MC1R) (30,31). Thus, it is expected that inhibition of the MC1R will reduce eumelanin synthesis and result in hypopigmentation. A multi-use-library consisting of over 31,000 structurally different peptides was generated based on the α-MSH-[5-13] peptide sequence (32). This led to

the identification of α-MSH antagonists, the most potent of which was Met-Pro-D-Phe-Arg-D-Trp-Phe-Lys-Pro-Val-NH2 (153 N-6). Based on this, it was discovered that the residues in positions 5, 6, 7, 9, and 10 of the α-MSH sequence are crucial determinants for potent antagonist activity. Later, 153 N-6 was tested for its potential antagonism of the human MC1R. Comparison of the binding affinity of 153 N-6 to the different melanocortin receptors, MC1R, MC3R, MC4R, and MC5R, revealed that it binds MC1R and MC4R with equal affinity, has a lower affinity to MC3R, and least affinity to MC5R (33). The high Ki of 153 N-6 (439 times greater than that of α-MSH) made it impractical for use *in vivo* as an antagonist. Further investigation of this peptide showed that its antagonistic activity is mainly due to Pro^6 and phe^{10} substitutions (34). The use of a combinatorial diffusion assay led to the identification of an antagonist of α-MSH on frog melanophores and cloned human MC1R (35).

Another hormone that received interest as a lead compound for α-MSH antagonists is agouti signaling protein (Asp), the physiological antagonist of α-MSH. Agouti signaling protein abrogates the melanogenic and mitogenic effects of α-MSH on human melanocytes by acting as a competitive inhibitor of α-MSH binding to the MC1R. The human agouti signaling protein consists of 182 amino acids, a secretion signal sequence, an internal basic region, and a carboxyl terminal cysteine-rich region (36). The carboxyl region is required for agouti activity, the basic region is thought to influence the secreted protein, or modulate protein-protein interactions and thus the antagonism of the MC1R (37). Attempts have been made to design fragment analogs of ASP that possess antagonistic activity for α-MSH, and thus cause hypopigmentation. Within the mouse agouti peptide, a peptide consisting of residues 77-91 was found effective in significantly

inhibiting eumelanogenesis (38). Within this sequence, the pentapeptide KVARP had potent antagonistic effect at submicromolar concentrations. This sequence corresponds to KVVRP in the human ASP. Our previous findings that recombinant mouse and human ASP have similar potencies on human melanocytes suggest that KVARP and KVVRP will have similar anti-eumelanogenic effects on those cells.

Agouti-related protein (AGRP) is the physiological antagonist of the MC4R, which is involved in the regulation of food intake and energy homeostasis in the hypothalamus (39,40). AGRP also antagonizes the MC3R, but unlike ASP, does not function as MC1R antagonist or affect pigmentation. Despite these properties of AGRP, there have been attempts to develop analogs based on its structure for the purpose of antagonizing MC1R. The decapeptide hAGRP (109-118) proved to be an affective antagonist of the murine MC1R as well as MC4R (41).

The pleiotropic effects of α-MSH and the existence of 5 different MCR has made it difficult to develop α-MSH- or ASP-based compounds that can solely inhibit the MC1R. The efficacy of such compounds for transdermal delivery depends on the small size of the compound and its lipophilicity. Their effectiveness and safety depend on their antagonistic activity at a low concentration, and specificity to the MC1R, respectively. Testing for these compounds has to be done on human melanocytes, human skin or human skin equivalents, since the human MC1R differs in its pharmacological properties from other mammalian or vertebrate MC1R.

The third approach to induce hypopigmentation is based on inhibiting melanosome transfer from melanocytes to keratinocytes. The exact mechanism and the molecular events involved in melanosome transfer are not yet fully understood, and this

is now an area of intense investigation. In melanocytes, melanosomes are translocated to the tip of the dendrites by microtubular motors, myosin Va and rab 27 a (42). SNARE, receptors involved in membrane fusion, and rab 3 a, a GTP-binding protein involved in exocytosis in neuronal cells, are thought to play a role in melanosome transport to the plasma membrane of melanocytes and possibly in melanosome transfer to keratinocytes (43). Protease-activated receptor 2 (PAR-2) is expressed on keratinocytes and is important for melanosome transfer. Activation of PAR-2 increases melanosome uptake by keratinocytes, while inhibition of its activation by protease inhibitors blocks melanosome transfer and reduces keratinocyte phagocytosis (44). Accumulation of melanosomes within melanocytes is thought to reduce melanosome formation and decrease melanin synthesis via a negative feedback mechanism. Further evidence for the significance of PAR-2 in melanosome transfer came from the observation that PAR-2 expression in human epidermis is upregulated 24 and 96 hours after UV exposure, and the demonstration that PAR-2 expression is upregulated in human keratinocytes following UV exposure (45). This knowledge about PAR-2 has been exploited by using protease inhibitors for inducing depigmentation. Naturally occurring soybean-derived serine protease inhibitors have proven effective in inducing hypopigmentation and in inhibiting UV-induced pigmentation (46). Soybean-derived trypsin inhibitor and Bowman-Birk inhibitor elicit their depigmenting effects by inhibiting PAR-2 activation in the skin. These effects require keratinocyte-melanocyte contact, and have no direct effect on melanocytes.

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Applying the Basic Knowledge about Regulation of Pigmentation towards Development of Strategies for Cutaneous Hypopigmentation

by:

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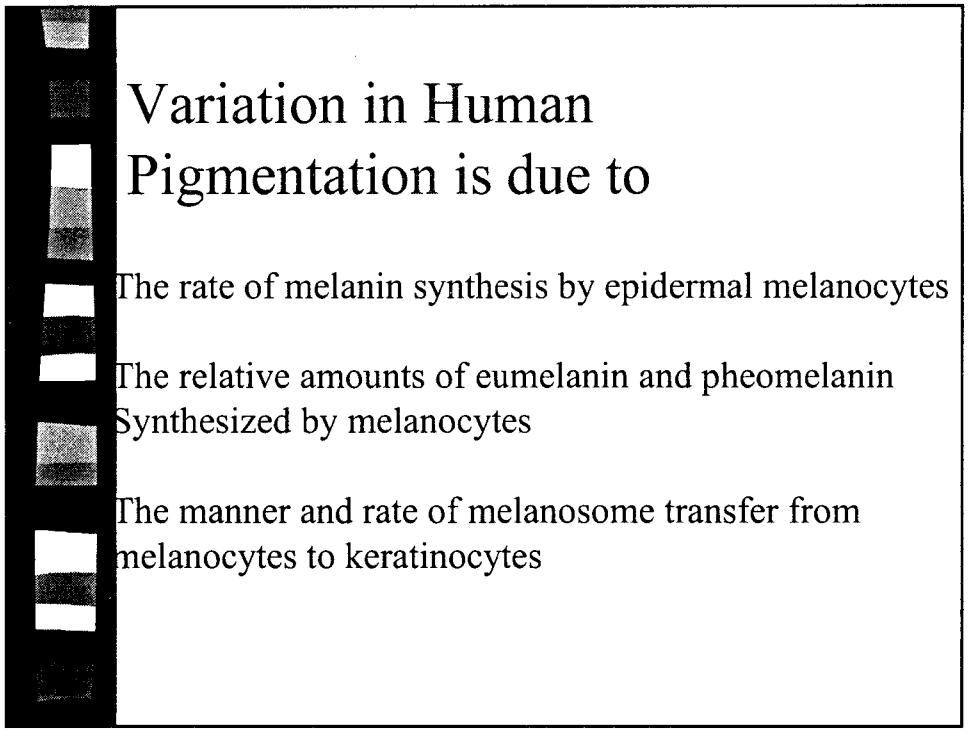
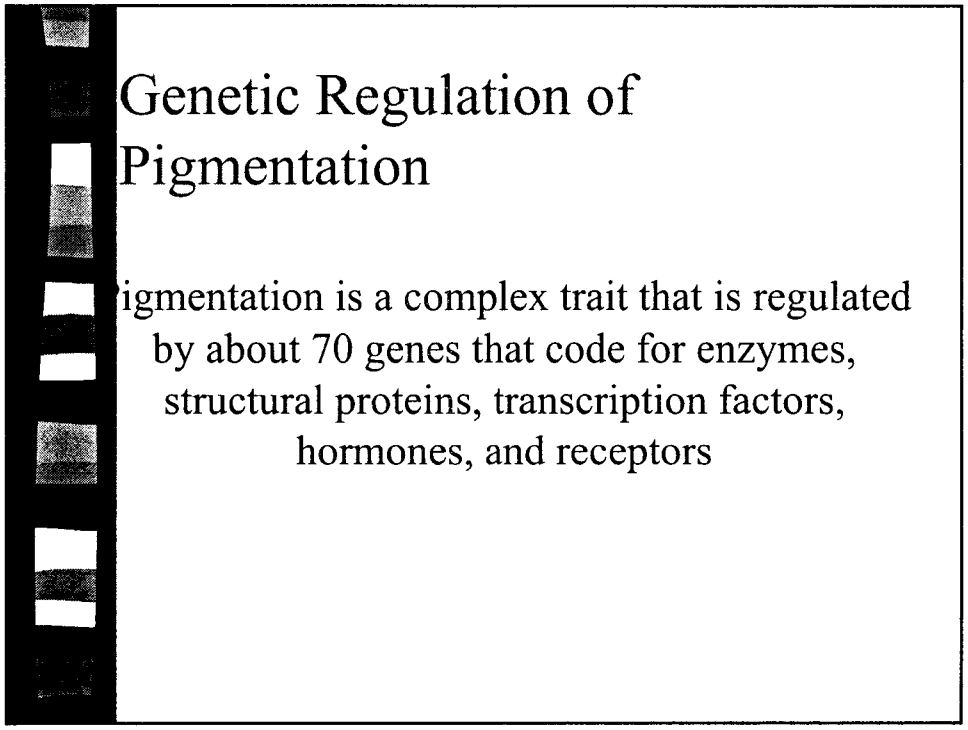
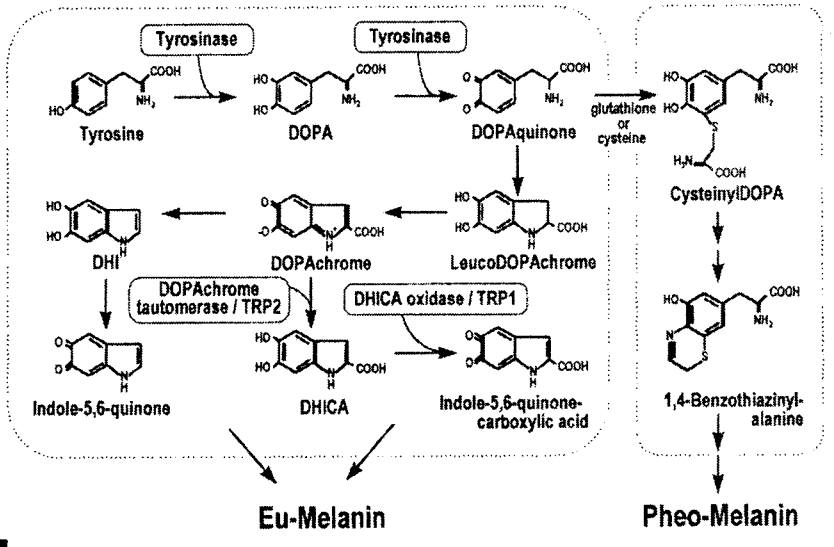

Variation in Human Pigmentation is due to
The rate of melanin synthesis by epidermal melanocytes
The relative amounts of eumelanin and pheomelanin Synthesized by melanocytes
The manner and rate of melanosome transfer from melanocytes to keratinocytes


Genetic Regulation of Pigmentation
igmentation is a complex trait that is regulated by about 70 genes that code for enzymes, structural proteins, transcription factors, hormones, and receptors

The Melanocortin 1 Receptor Gene

An important determination of constitutive pigmentation in humans

The Melanin Synthetic Pathway



Eumelanin Vs Pheomelanin Synthesis

- Eumelanin synthesis requires a high concentration of tyrosinase, and high protein levels of tyrosinase, TRP-1 and TRP-2
- Pheomelanin synthesis requires the availability of cysteine and occurs in the presence of low levels of tyrosinase, TRP-1 and TRP-2

Dark Skin Differs from Light Skin in having

- Higher melanin content
- Higher eumelanin to pheomelanin ratio
- Higher tyrosinase and protein levels of tyrosinase, TRP-1 and TRP-2

Melanosome transfer in Dark Versus Light Skin

- In dark skin, melanosomes are transferred individually to keratinocytes
- In light skin, melanosomes are smaller in size and are trasferred as membrane-bound packages to keratinocytes

Environmental Agents that Affect Pigmentation

- Solar ultraviolet radiation
- Naturally occurring metals, such as arsenic
- Industrial chemicals, such as phenolic compounds

Effects of UV on the Skin

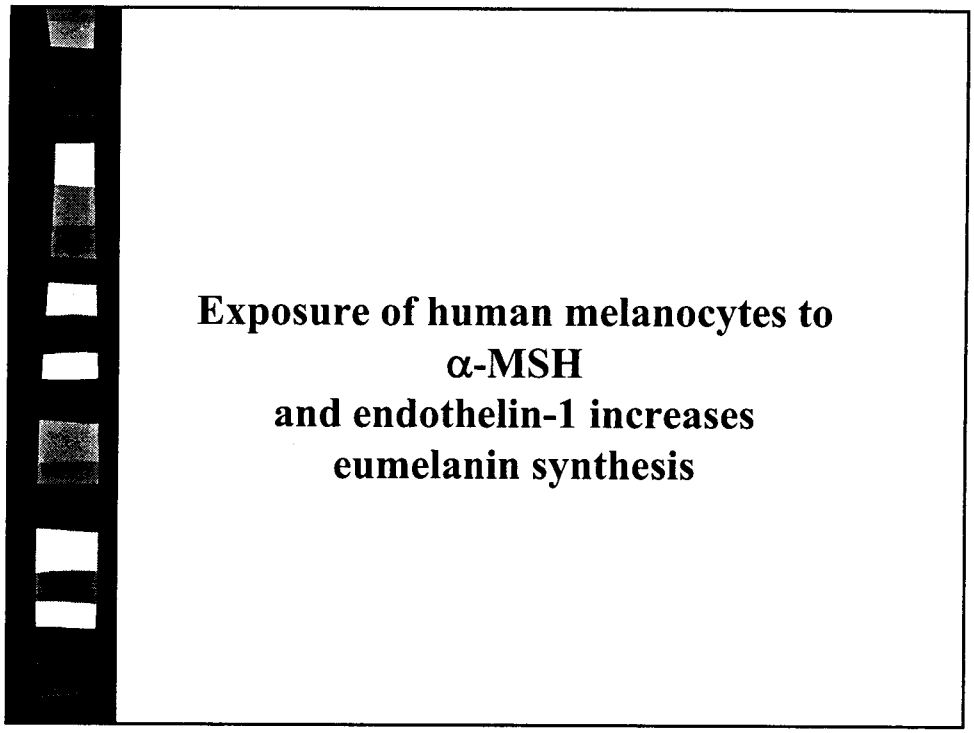
- Direct effects, e.g. generation of reactive oxygen radicals and DNA photoproducts
- Indirect effects, via stimulation of the synthesis of epidermal cytokines and growth factors

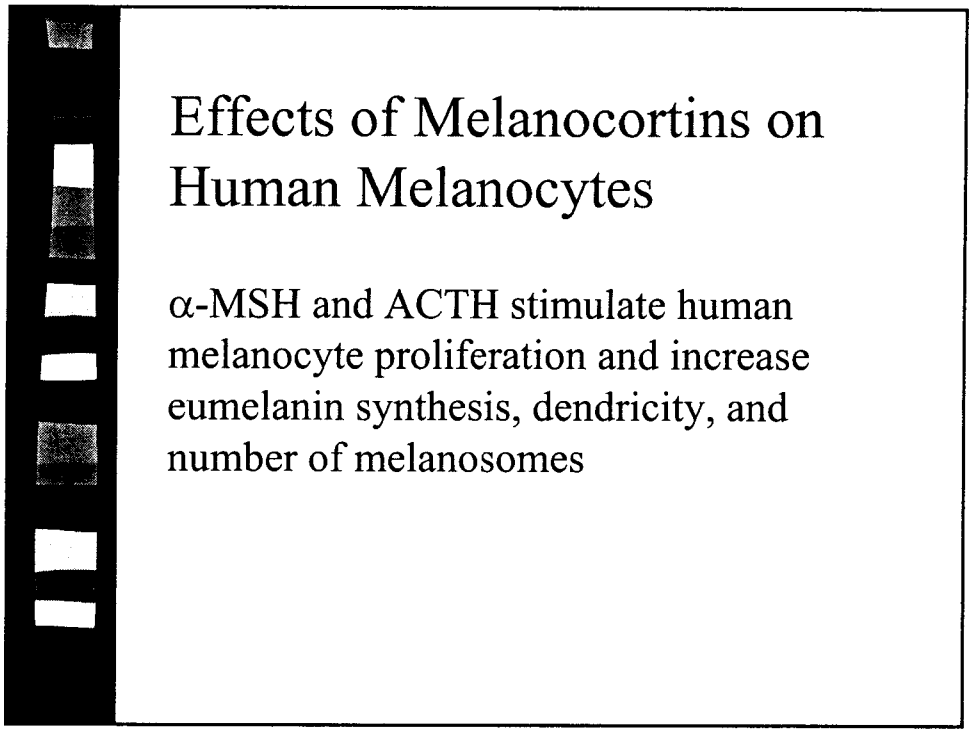
The tanning response to UV is due to

Stimulation of melanogenesis by melanogenic factors, such as α-MSH, ACTH, and endothelin-1

Increased melanosome formation and transfer

DNA damage ??


Exposure of human melanocytes to
α-MSH
and endothelin-1 increases
eumelanin synthesis


Effects of Melanocortins on
Human Melanocytes
α-MSH and ACTH stimulate human
melanocyte proliferation and increase
eumelanin synthesis, dendricity, and
number of melanosomes

Hypopigmentation of the skin can be achieved by:

- Inhibiting melanogenesis
- Reducing eumelanin to pheomelanin ratio
- Reducing melanosome transfer from melanocytes to keratinocytes

Phenols and Catechols Induce Skin Hypopigmentation

Industrial exposure to monobenzyl ether of hydroquinone, paratertiary butylphenol, or paratertiary amylphenol formaldehyde resin result in leukoderma

Strategies for Inducing Hypopigmentation

Interfering with the melanin synthetic pathway, e.g. by using chemicals that act as pseudosubstrates for tyrosinase, such as hydroquinone

Mechanism of Action of Hydroquinone

- Competition with tyrosine as an alternative substrate for tyrosinase
- Increased formation of reactive oxygen species
- Depletion of glutathione

Hydroquinone-Related Agents

- Arbutin
- Kojic acid
- Alkyl esters of gentisic acid

Design of Compounds that Inhibit Eumelanin Synthesis

- α-MSH antagonists
- Agouti protein analogs
- Agouti-related protein analogs

Amino Acid Sequence of α-MSH

Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2

Design of α-MSH Antagonists

Residues in positions 5, 6, 7, 9 and 10 of α-MSH sequence (Glu-His-Phe and Trp-Gly) are crucial for antagonist activity

Pro^6 and Phe^{10} substitutions (in place of His and Gly, respectively) confer potent antagonist activity

Human Agouti Signaling Protein

Consists of 132 amino acids, and has a secretion signal sequence an internal basic region, and a carboxyl terminal cysteine-rich region

Is a competitive inhibitor of α-MSH binding to the melanocortin 1 receptor

Melanocortin 1 Receptor Antagonists Based on the Structure of Agouti Signaling Protein

ASP 77-91 (KVARP in mouse ASP; KVVRP in human ASP)inhibits eumelanogenesis at submicromolar concentrations

Agouti-Related Protein-Based Antagonists

The human AGRP (109-118) effectively antagonizes the melanocortin 1 receptor

Induction of Hypopigmentation by Inhibition of Melanosome Transfer to Keratinocytes

Inhibition of PAR-2 expressed on keratinocytes by soybean trypsin inhibitors reduces keratinocyte phagocytosis and melanosome uptake

Regulation of PAR-2 Expression by UV

Increased PAR-2 expression in the epidermis and in cultured human keratinocytes after UV exposure