Published in final edited form as:

FASEB J. 2007 June ; 21(8): 1844–1856.

Proopiomelanocortin (POMC), the ACTH/melanocortin precursor, is secreted by human epidermal keratinocytes and melanocytes and stimulates melanogenesis

Karine Rousseau^{*,1}, Sobia Kauser^{†,1}, Lynn E. Pritchard^{*}, Anne Warhurst^{*}, Robert L. Oliver^{*}, Andrzej Slominski[‡], Edward T. Wei[§], Anthony J. Thody^{||}, Desmond J. Tobin[†], and Anne White^{*2}

**Faculties of Life Sciences and Medical and Human Sciences, Stopford Building, University of Manchester, Manchester, UK*

†Medical Biosciences Research, University of Bradford, West Yorkshire, UK

‡Department of Pathology and Laboratory Medicine, University of Tennessee Health Science Center, Memphis, Tennessee, USA

§School of Public Health, University of California, Berkeley, California, USA

||Dermatological Sciences, University of Newcastle upon Tyne, UK

Abstract

Proopiomelanocortin (POMC) can be processed to ACTH and melanocortin peptides. However, processing is incomplete in some tissues, leading to POMC precursor release from cells. This study examined POMC processing in human skin and the effect of POMC on the melanocortin-1 receptor (MC-1R) and melanocyte regulation. POMC was secreted by both human epidermal keratinocytes (from 5 healthy donors) and matched epidermal melanocytes in culture. Much lower levels of α -MSH were secreted and only by the keratinocytes. Neither cell type released ACTH. Cell extracts contained significantly more ACTH than POMC, and α -MSH was detected only in keratinocytes. Nevertheless, the POMC processing components, prohormone convertases 1, 2 and regulatory protein 7B2, were detected in melanocytes and keratinocytes. In contrast, hair follicle melanocytes secreted both POMC and α -MSH, and this was enhanced in response to corticotrophin-releasing hormone (CRH) acting primarily through the CRH receptor 1. In cells stably transfected with the MC-1R, POMC stimulated cAMP, albeit with a lower potency than ACTH, α -MSH, and β -MSH. POMC also increased melanogenesis and dendricity in human pigment cells. This release of POMC from skin cells and its functional activity at the MC-1R highlight the importance of POMC processing as a key regulatory event in the skin.—Rousseau, K., Kauser, S., Pritchard, L. E., Warhurst, A., Oliver, R. L., Slominski, A., Wei, E. T., Thody, A. J., Tobin, D. J., White, A. Proopiomelanocortin (POMC), the ACTH/melanocortin precursor, is secreted by human epidermal keratinocytes and melanocytes and stimulates melanogenesis.

Keywords

α -MSH; hair follicle melanocytes; CRH receptors

2Correspondence: Endocrine Sciences, Faculties of Life Sciences and Medicine and Human Sciences, Stopford Bldg., University of Manchester, Manchester M13 9PT, UK. E-mail: anne.white@manchester.ac.uk.

¹These authors contributed equally to this work.

Differential Enzymatic Cleavage of proopiomelanocortin (POMC) by prohormone convertases (PCs) results in the production of ACTH, α -melanocyte-stimulating hormone (α -MSH), β -MSH, γ -MSH, and other hormones that include β -endorphin (β -END) and β -lipotropic hormone (β -LPH) (1,2). Depending on the tissue, different POMC-derived peptides are produced that act on specific melanocortin receptors (MC-Rs) (1). In the human pituitary, POMC is processed to ACTH but not to α -MSH, whereas in the rat hypothalamus we identified both ACTH and α -MSH (3).

POMC processing is incomplete in pituitary cells and hypothalamic neurons. The POMC precursor peptide is secreted from the pituitary and is found in the human circulation (4,5). In addition, POMC is released by the hypothalamus and is present in both human (6) and rat cerebrospinal fluid (CSF) (3).

It is well recognized that POMC is expressed locally in the skin (reviewed in ref. 1). POMC processing in human skin appears to resemble that of the hypothalamus in that ACTH, α -MSH, and β -END have been found in melanocytes and keratinocytes, and in mammalian skin biopsies (reviewed in ref. 1, ref. 7–ref. 10). In addition, ACTH, α -MSH, and β -END have been identified in cultured human epidermal melanocytes (EM), normal and malignant, and in keratinocytes (10–15).

Whether there is incomplete processing of POMC in the skin, as seems to occur in other tissues, is not yet clear. Indeed, little is known of the regulation of the intracellular events involved in POMC processing and how these processes compare at different sites and different cell types. Corticotrophin-releasing hormone (CRH) has been found in human skin (13) and has been shown to stimulate production and secretion ACTH (16). Therefore, it is important to determine whether it can regulate the processing of POMC-derived peptides in this organ and how this might differ in keratinocytes and melanocytes.

The prohormone convertase, PC1, cleaves POMC ACTH, β -LPH, and an N-terminal peptide, N-POMC. PC2 then cleaves ACTH, which is further processed by amidation and acetylation, to α -MSH. Both PC1 and PC2 are expressed in human and rodent skin (17,18). Expression of PC1, PC2, and 7B2 (the PC2-specific modulator protein) was also detected in cultured epidermal melanocytes (18). Human dermal fibroblasts and follicular dermal papilla cells also express the POMC processing machinery (19–21). In human follicular melanocytes, PC1, PC2, and 7B2 are differentially expressed according to cell differentiation status (20). Therefore, the skin and the hair follicle have the full potential to process POMC to ACTH and β -LPH, and subsequently to α -MSH and β -END (1).

The function of α -MSH is mediated by the MC-Rs, and most of the actions of α -MSH in the skin are *via* the MC-1R. It is assumed that α -MSH is the natural ligand for MC-1R, but if processing of POMC to ACTH and subsequently α -MSH is not complete, then POMC and ACTH will be present in the skin. Given that they all have the core His-Phe-Arg-Trp residues important for melanocortin agonists, their relative activity at the MC-1R is key to understanding their physiological roles. At human MC-4R, ACTH has a potency similar to that of α -MSH (22), and even POMC stimulates cAMP production, albeit with low potency (unpublished data). At the MC-1R, α -MSH is one of the most potent agonists, although ACTH also has marked activity terms of adenylate cyclase activation (23). Moreover, some peptides (*e.g.*, des- α -MSH) act as partial agonists at the MC-1R and block the actions of α -MSH (24). Thus, processing of POMC and generation of the POMC-derived peptides is likely to be an important regulatory step for actions at the melanocortin receptors.

The importance of POMC peptides in skin and hair pigmentation is evidenced clinically in patients with POMC gene mutations, who exhibit pale skin and red hair in addition to early-onset obesity and adrenal insufficiency (25). ACTH and α -MSH can produce significant skin

darkening when administered to humans (26), and prolonged administration of ACTH to humans induces hyperpigmentation (27). In addition, some patients with Addison's disease and Nelson's syndrome, where excessive concentrations of POMC are detectable in the circulation, have marked hyperpigmentation (28). There are also numerous reports of the stimulation of human melanocyte tyrosinase and melanogenesis by POMC peptides (10,11, 20,27,29–31)

It has been difficult, if not impossible, using immunochemistry alone to distinguish the relative amounts of POMC in skin cells in relation to its cleavage products ACTH and α -MSH. Antibodies to ACTH and α -MSH are not specific and will detect POMC, but to an unknown degree. Most antibodies have not been tested for their ability to cross-react with POMC because it is difficult to obtain purified or synthetic preparations of POMC. The corollary is that for immunocytochemistry there are no antibodies that specifically recognize the precursor without also detecting the constituent peptides. The studies by Slominski's group (1) have used liquid chromatography-mass spectroscopy to identify ACTH and α -MSH in extracts from skin, but this does not yield information on the skin epidermal cell types; human dermal fibroblasts also secrete these peptides. Western blotting can distinguish precursors from derived peptides, but it is relatively insensitive and may miss subtle changes in processing. We have developed a two-antibody "sandwich" assay where one antibody binds the ACTH region in POMC and the other antibody binds the N-POMC region. One antibody labels POMC and the other antibody captures the complex. A signal is only generated when both antibodies are bound, and therefore it is specific for POMC and does not recognize ACTH and α -MSH (32). This is particularly valuable in addressing questions regarding the degree of processing and how it is regulated.

The present study was designed to compare levels of POMC and the POMC-derived peptides, ACTH and α -MSH, in fully matched cultures of human epidermal melanocytes and keratinocytes and in hair follicle melanocytes in order to determine how POMC is cleaved and which of the peptides are released. There are now substantial data suggesting that the skin and its principal appendage, the hair follicle, have a system analogous to the hypothalamic-pituitary-adrenal (HPA) axis such that there is evidence for expression of CRH and detection of cortisol in addition to POMC (16,33,34). It is thought that this enables the skin to regulate local responses to stress (16). Therefore, it is important to understand whether CRH stimulates POMC and POMC-derived peptides. Having found that POMC was the predominant peptide released from the melanocytes and keratinocytes, we investigated the biological activity of POMC in recombinant cells stably expressing both MC-1R and cAMP-responsive reporter constructs and in human pigment cells that express human MC-1R.

MATERIALS AND METHODS

Cell culture conditions

Epidermal keratinocytes and melanocytes and hair follicle melanocytes—All cell culture reagents were obtained from Invitrogen Ltd. (Paisley, UK) unless otherwise stated. Epidermal keratinocytes (EK) were established from normal human haired scalp tissue of five normal healthy donors (4 female, 1 male; mean age 53 years) as described previously (11) and grown in keratinocyte serum-free medium (K-SFM; Invitrogen) supplemented with 25 μ g/ml bovine pituitary extract (BPE), 0.2 ng/ml rEGF, penicillin (100 U/ml)/streptomycin (100 μ g/ml), and 2 mM L-glutamine. Culture medium was replenished every second day. Matched EM cultures were established from the above tissue (11) and grown in a mixture of K-SFM and Eagle's minimal essential medium (EMEM) supplemented with 2% FBS, 1 $\times$ concentrated nonessential amino acid mixture, penicillin (100 U/ml)/streptomycin (100 μ g/ml), 2 mM L-glutamine, 5 ng/ml basic fibroblast growth factor (bFGF), and 5 ng/ml endothelin-1 (Sigma, Poole, Dorset, UK). Cells were incubated at 37°C in a 5% CO₂ atmosphere and medium was replenished every third day. Hair follicle melanocytes were established from normal human

haired scalp tissue (3 females 1 male; 55–66 years, mean 59 years) as described previously (20,35,36) and grown as for epidermal melanocytes above.

AtT20 cells—The mouse pituitary adenoma cell line (AtT20) was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and grown in Dulbecco's modified Eagle medium (DMEM) (Sigma) supplemented with 10% fetal calf serum, 4 mM glutamine, and 1 mM pyruvate.

DMS-79 cells—The DMS-79 cell line is derived from a human small cell lung carcinoma that expresses the POMC gene and secretes almost exclusively unprocessed POMC (37). The cell line was kindly donated by Prof. O. Pettengill, Dartmouth Medical School (Hanover, NH, USA). Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mmol/L glutamine, and penicillin (100 U/ml)/streptomycin (100 µg/ml) (Sigma). DMS-79 cells grow as aggregates in suspension (28).

FM55 human melanoma cells—Human melanoma FM55 cells were established from metastatic melanoma nodules and were a gift from Dr. A. F. Kirkin (Danish Cancer Center). Cells were grown under the same conditions as DMS79 cells

S91 mouse melanoma cells—Cloudman S91 melanoma cells (clone M-3) were purchased from ATCC. Cells were grown in Kaighn's modification of Ham's F12 medium (F12K) with 2 mM L-glutamine modified by ATCC to contain 1.5 g/L sodium bicarbonate, and supplemented with 15% horse serum and 2.5% FBS (Sigma). POMC is present in S91 mouse melanoma cells, but is not secreted into culture medium (POMC<10pmol/L *n*=3 separate experiments).

Detection of peptides

Detection of POMC, ACTH, and α -MSH in matched human epidermal keratinocytes and melanocytes—To detect POMC, ACTH, and α -MSH, epidermal melanocytes and keratinocytes were switched from fully supplemented medium to medium lacking FCS and BPE for 24 h before the experiment. This was to deplete all cells and medium of exogenous POMC and POMC-derived peptides. Both EK and EM were incubated for a further 24 h in this basal medium containing 0.25% BSA (Sigma) to reduce nonspecific binding of POMC peptides to the culture flask. The medium was collected and immediately stored at -80°C until assayed; the cells were extracted on ice in radio-immunoprecipitation assay buffer (Sigma) and snap-frozen in liquid nitrogen until assayed. Protein content was assayed using DC protein assay kit (Bio-Rad, Hemel Hempstead, Herts, UK).

POMC, ACTH, and α -MSH assays—Cell culture media, cell extracts, and fractions from the POMC purification were analyzed for the presence of POMC with an ELISA based on the immunoradiometric assay as described previously (32) and with the same format as the OCTEIA POMC kit (IDS Ltd., Boldon, UK). The ELISA utilizes purified human POMC (prepared as described below) as a standard. Monoclonal antibody N1C11, which recognizes the γ -MSH region of POMC, is labeled and a monoclonal antibody (A1A12), which recognizes the ACTH 10–18 region of POMC, is coated on the ELISA plate. Binding of both antibodies is required to generate a signal in the assay. Therefore, the POMC assay does not cross-react with ACTH (<3.6%) or α -MSH (<2.2%), but does cross-react 100% with POMC and pro-ACTH. Assay sensitivity during the study was 10 pmol/L.

The ACTH assay we developed is an ELISA format of the immunoradiometric assay described previously (4,38). It is based on a monoclonal antibody (A2A3) to the C-terminal of ACTH, which is labeled, and the monoclonal antibody A1A12, which recognizes the ACTH 10–18

region of POMC and is coated on the ELISA plate. As with the POMC assay, both antibodies have to bind to generate a signal so the assay does not cross-react with α -MSH, ACTH (18–39), or ACTH (1–24). It has < 0.1% cross-reactivity with POMC. Assay sensitivity was 3 pmol/L.

α -MSH was measured using a RIA (Euro-diagnostica AB, Malmö, Sweden). The α -MSH assay does not cross-react with ACTH, but does cross-react 100% with desacetyl- α -MSH.

Immunocytochemical detection of PC1, PC2, and 7B2 in matched epidermal keratinocytes and melanocytes and the detection of MC-1R in epidermal melanocytes

Matched EM and EK cultures (passages 2–5) were seeded onto 8-well Lab-Tek® chamber slides (ICN Biomedicals, Inc. Aurora, OH, USA) at 5000 cells/well and cultured for 2–3 days. FCS and BPE were omitted from the culture media 48 h prior to immunostaining to remove all exogenous sources of POMC and POMC-derived peptides. Cells were rinsed in PBS for 5 min and fixed in ice-cold methanol for 10 min at -20°C . Cells were blocked in 10% normal goat serum for 1 h, then rinsed briefly in PBS and incubated with PC1, PC2 and 7B2-specific antibodies (1:200) (a gift from Prof. NG Seidah, Clinical Research Institute of Montreal, Canada).

To detect MC-1R, cells were fixed in 5% buffered paraformaldehyde for 30 min at room temperature, rinsed with PBS, incubated with an antibody to MC-1R 1:50 (gift from Dr. M Böhm, Department of Dermatology, University of Münster, Germany). Cells were also incubated with positive control antibodies to gp100 (NKI/beteb; Monosan, Netherlands), tyrosinase (Novacastra, Newcastle on Tyne, UK), and a keratin-specific antibody AE3 (a gift from T-T. Sun, NYU Medical Center, New York, NY, USA) at 4°C for 18 h. Subsequent steps in immunostaining were performed using the DAKO LSAB®2 HRP kit and DAKO AEC substrate system (DAKO, Carpinteria, CA, USA) according to the manufacturer's instructions. Negative controls included the omission of primary antibody and replacement with preimmune serum from secondary antibody host and inclusion of secondary antibodies.

CRH stimulation of human hair follicle melanocytes

Follicular melanocyte cultures ($n=7$) were maintained without FCS and BPE for 48 h prior to stimulation for 24, 48, and 72 with native CRH (American Peptide Company, Sunnyvale, CA, USA) or its modified peptides [D-Glu²⁰]-CRH, [D-Pro⁵]-CRH, and [D-Pro⁴]-r-urocortin at 10^{-7} M to 10^{-10} M concentrations, as described previously (39). Briefly, D-Glu²⁰-CRH is 25-fold more selective for CRH-R1 than CRH; [D-Pro⁵]-CRH is 5-fold more selective for CRH-R2; [D-Pro⁴]-r-urocortin is almost wholly selective for CRH-R2. Medium was collected from the cells at each of the above time points and immediately stored at -80°C until assayed for POMC, ACTH, α -MSH.

Purification and concentration of POMC

We previously produced a monoclonal antibody (N1C11) to the γ -MSH region of POMC (32). This antibody was coupled to a solid-phase support (Sephacryl S-300, GE Healthcare, Buckinghamshire, UK) as described previously (32) for affinity purification of POMC. The solid-phase antibody was stored at 4°C . Supernatant media from confluent DMS-79 cells were collected by centrifugation and used immediately or frozen at -20°C . Aliquots of media (400 ml) were incubated overnight at 4°C with 6.0 ml 50% N1C11 solid phase on a shaking platform. N1C11 solid phase was collected by centrifugation and washed three times with PBS. POMC peptide was eluted with 3.5 M MgCl₂; the fractions collected were concentrated using Amicon Ultra filters (Millipore UK Ltd., Watford, UK) and stored at -80°C . Fifty microliters of purified POMC was subjected to analytical size exclusion chromatography on a 2.4 ml Superdex 75 column (Amersham Biosciences, Chalfont St. Giles, UK) equilibrated in 50 mM Tris-HCl and

0.15 M NaCl (pH7.4). The column was eluted with the same buffer at 50 μ l/min. Fractions were analyzed for POMC, ACTH, and α -MSH as described above.

In addition, 10 μ l purified POMC and 10 μ l of 100 μ M ACTH (Sigma) and β LPH (kindly supplied by Dr. A. Parlow, Pituitary Hormone and Antisera Center, UCLA Medical Center, Los Angeles, CA, USA) were electrophoresed on a 10% Bis-Tris NuPage® acrylamide gel (Invitrogen, Paisley, UK) and transferred onto PVDF membrane using an Xcell Surelock® transfer module (Invitrogen). POMC was detected by Western blot following standard protocol, using A1A12, a monoclonal antibody directed to ACTH 10–18, which recognizes POMC (32).

Activation of MC-1R

CHOK1 cells were stably transfected with full-length human MC-1R (the MC-1R sequence was identical to the ncbi sequence accession number: NM_002386, except for a previously described polymorphism: Thr155Ile) and a cAMP reporter construct consisting of a cAMP response element (CRE) and three vasoactive intestinal peptide (VIP) enhancer elements upstream of a lac Z reporter gene (kindly provided by Drs. M. Needham and D. Scanlan, AstraZeneca, Cheshire, UK). Cells were grown to complete confluency in DMEM (Sigma), 10% fetal calf serum, 1% HT supplement (Life Technologies, Inc.), 1% nonessential amino acids (Life Technologies, Inc.), 200 μ g/ml G418 (Life Technologies, Inc.), and 500 μ g/ml hygromycin B (Roche Applied Science, Lewes, East Sussex, UK). RT-PCR was undertaken on clones of CHOK-1 cells transfected with MC-1R to demonstrate that they expressed the receptor and that untransfected cells did not endogenously express MC-1R.

POMC-derived peptides, apart from POMC itself, were purchased from Sigma (Poole, Dorset, UK). Ligand stocks (2 $\times$) were prepared in indicator-free DMEM and 50 μ l aliquots were added, in quadruplicate, onto polylysine-coated 96-well plates. CHOK1 cells expressing the human MC-1R were added to each well at a density of 50,000 cells/well and the plates were incubated for 5 h at 37°C/5% CO₂. Cyclic AMP was detected by addition of 1 mM chlorophenol red- β -D-galactopyranoside (CPRG) (Roche) in buffer containing a final concentration of 40 mM Na₂HPO₄, 40 mM NaH₂PO₄, 7 mM KCl, and 0.7 mM MgSO₄. β -Galactosidase converts CPRG to give a red color. Results were quantified by reading absorbance at 590 nm on a Spectrafulor (Tecan, Theale, Reading, UK) plate reader. Each experiment was performed a minimum of three times with quadruplicate wells. Dose-response data were fitted to a sigmoid curve using nonlinear squares regression (Origin 6.0, Mircocal Software, Inc., Northampton, MA, USA).

Positive clones were selected based on their response to α -MSH in the cyclic AMP reporter assay. Untransfected cells did not respond to α -MSH, whereas cells transfected with MC-1R did respond.

Assessment of melanin content, dendricity, and proliferation

The S91 Cloudman mouse melanoma cells were seeded at a density of 0.5 to 1 $\times$ 10⁶ cells per 100 mm cell culture dish (Corning Costar Corporation, Cambridge, MA, USA) and allowed to attach overnight. Cells were treated with POMC (10⁻⁵-10⁻⁸ M) peptides in serum-free medium containing 0.25% BSA for 72 h. The peptides were then renewed for another 72 h. After 6 days incubation, the cells were trypsinized, pelleted by centrifugation, and solubilized in 1 M NaOH, for 1 h at 60°C.

The FM55 human melanoma cells were seeded at a density of 1 $\times$ 10⁵ cells in triplicate in culture flasks (Corning Costar Corporation, Cambridge, MA, USA) and allowed to attach overnight. Cells were treated with POMC (purified as described above) at 10⁻⁵-10⁻⁸ M in

serum-free medium containing 0.25% BSA (to reduce peptide adhesion to the culture flasks) and incubated for 72 h. The cells were trypsinized, pelleted by centrifugation, photographed, and solubilized in 1 M NaOH for 10 min at 100°C. Protein assessment used the Bio-Rad RC DC kit. Normal human EM were seeded at a density of 5×10^5 in triplicate in culture flasks and allowed to attach overnight. EM were incubated with 10^{-5} and 10^{-6} M POMC for 72 h, as described above. 10^{-5} and 10^{-6} M POMC was chosen for stimulation, as the greatest effects on melanogenesis and dendricity were seen at these concentrations in experiments with FM55 melanoma cells. Melanocytes from the same donor were maintained in parallel in the absence of the peptide and served as a negative control. After stimulation, cells were trypsinized and pelleted by centrifugation, photographed, and solubilized in 1 M NaOH as before.

Melanin content was measured spectrophotometrically at 475 nm. A standard curve of synthetic melanin (Sigma, Dorset, UK) 0.05–100 µg was used as a basis to determine melanin content. Protein content was determined by Bio-Rad RC DC assay and measured spectrophotometrically at 750 nm. Results were determined as microgram melanin per milligram protein or pg/cell and expressed as percentage of unstimulated controls. Representative photographs of FM55 human melanoma cells incubated in the presence or absence of POMC at 10^{-5} – 10^{-8} M were taken from up to eight random and different fields (of 20 cells for each cell line); the number of bipolar cells and the number of cells with more than three dendrites were counted and compared with controls.

After trypsinization, cells were counted using a Neubauer counting chamber. The increase in cell number was expressed as percentage increase in cell number above control unstimulated levels.

Statistical analysis

Statistical significance was assessed using one-way ANOVA with Dunnet's *post hoc* test using Prism v.3.00 or 4.00 (Graph-Pad Software Chicago, IL, USA). Statistically significant differences are denoted with asterisks: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

RESULTS

POMC is secreted by primary cultures of human keratinocytes and melanocytes

Human epidermal keratinocytes—Human epidermal keratinocytes, isolated from the scalp of five different subjects and established in culture, all secreted high concentrations of POMC into the culture medium (mean=1690, range=1120–2440 fmol/flask after 24 h) (Fig. 1A). In comparison, ACTH was below the limit of detection for this system (<30 fmol/flask) and only very low concentrations of α -MSH were found (mean=70 fmol/flask), although it was detectable in all five cultures. However, in cell extracts from cultures, levels of ACTH (mean=809, range=745–882 fmol/flask) were greater than POMC (mean=452, range=231–681 fmol/flask; $P < 0.001$) and α -MSH (mean=10, range=7–13 fmol/flask; $P < 0.001$).

Human epidermal melanocytes—Matched cultures of human epidermal melanocytes from the same five subjects showed a similar pattern for the relative concentrations of POMC and its derived peptides (Fig. 1B). POMC was the most abundant peptide secreted (mean=450, range=290–620 fmol/flask), with no ACTH or α -MSH detected. In cell extracts, POMC was less than ACTH (mean 247 and 681 fmol/flask, respectively; $P < 0.005$), but α -MSH was below the level of detection.

Comparison of keratinocytes and melanocytes—This analysis allows a direct comparison of the concentrations of POMC, ACTH and α -MSH both in the cells and secreted into the culture medium. POMC was the most abundant peptide released from both cell types.

ACTH was present intracellularly in both keratinocytes and melanocytes and found at higher concentrations than POMC. α -MSH was detected in cell extracts and media from keratinocytes but not from melanocytes. Both melanocytes and keratinocytes contain much lower levels of POMC and ACTH than the control pituitary cell line (AtT20), which had 788 and 326 pmol/flask, respectively, under equivalent conditions.

Hair follicle melanocytes—Hair follicle melanocytes isolated from four subjects also released POMC into culture medium (384 fmol/flask) over 24 h at concentrations similar to those for epidermal melanocytes. ACTH and α -MSH were not detected. After 72 h in culture, α -MSH was detected (42 fmol/flask) but was released only by melanocytes from one subject. In contrast, POMC was present at much higher concentrations (range 264–705 fmol/flask) (Fig. 1C).

Normal epidermal keratinocytes and melanocytes express the POMC processing machinery

Matched epidermal keratinocytes and melanocytes from five subjects all expressed PC1, PC2, and 7B2, the PC2 regulatory protein, predominantly in the cell cytoplasm. Levels of these peptides were variable within the cell populations and appeared to correlate with cell differentiation status. The expression of the processing machinery was strongest in more differentiated (*i.e.*, dendritic, melanocytes) (Fig. 2A, arrows) *vs.* the less differentiated at cells (Fig. 2A, arrowheads). Considerable heterogeneity in their expression was also apparent in epidermal keratinocytes, and this heterogeneous staining pattern appeared to correlate positively with morphological features of keratinocyte differentiation (Fig. 2B).

CRH increases both POMC and α -MSH in human hair follicle melanocytes

In keeping with the current hypotheses that the hair follicle has an equivalent axis to the HPA (34,40), we have now found that hair follicle melanocytes respond to 10^{-7} M CRH with a dramatic increase in the release of both POMC (50-fold) and α -MSH (22-fold) after 72 h incubation (Fig. 3A). ACTH levels, in contrast, were below the limit of detection of the assay (3.0 pmol/L).

CRH also stimulated POMC release after 24 and 48 h incubation, resulting in 97-fold and 160-fold increases, respectively (Fig. 3B). However, neither ACTH nor α -MSH could be detected at these earlier time points.

The modified CRH peptide D -Pro⁵CRH, which is 5-fold more selective for CRH-R1, also stimulated large increases in POMC secretion (Fig. 3C), maximal at 10^{-10} M, but concentrations above 10^{-9} M were less effective. A bell-shaped curve was observed for D -Glu²⁰CRH that is 25-fold more selective for CRH-R2, with peak responses at 10^{-8} M; higher concentrations of agonist inhibited POMC release. The CRH agonist D -Pro⁴-r-urocortin (which is almost wholly selective for CRH-R2) had relatively little effect on POMC release, but a 6-fold increase was observed at 10^{-8} M (Fig. 3C). This suggests that CRH is acting primarily through CRH-R1 in the hair follicle melanocytes.

POMC binds the MC-1R and stimulates cAMP production

POMC purification and characterization—POMC synthesis and secretion by the human small cell lung carcinoma cell (SCLC) line, DMS 79, has been described (32). This predominance of precursor and absence of processing has been observed in all 10 human SCLC cell lines found to produce ACTH-related peptides (41,42). For this study, we purified POMC from culture medium using our monoclonal antibody, N1C11, coupled to Sephacryl for affinity purification and monitored purification with our two-antibody assay for POMC. We achieved 80-fold purification (from 87 ± 12 nM to 7000 ± 2000 nM POMC, $n=5$). Size separation gel

chromatography and Western blot analysis of the purified preparation confirmed there was no ACTH contamination in the POMC used for subsequent experiments (Fig. 4A)

POMC binding at the MC-1R—The potencies of POMC-derived peptides at the MC-1R were compared by utilizing a CHOK1 cell clone stably transfected with the human MC-1R, together with a cAMP-responsive β -galactosidase reporter construct (Fig. 4B). Cells transfected with the cAMP reporter construct only (*i.e.*, no MC-1R) did not respond to POMC, indicating that the cells do not express endogenous melanocortin receptors.

POMC was able to activate the MC-1R with an EC_{50} of $21.4 \pm 2.5 \times 10^{-9}$ M assessed from the mean of three independent experiments. In comparison, ACTH and β -MSH were the most potent peptides with EC_{50} values of $4.5 \pm 0.1 \times 10^{-9}$ M and $4.4 \pm 0.9 \times 10^{-9}$ M, respectively, whereas α -MSH had a potency of $17.4 \pm 12 \times 10^{-9}$ M; γ -MSH was the least potent in activating the MC-1R ($116.3 \pm 9.9 \times 10^{-9}$ M), as expected from earlier studies (43,44).

POMC binds to endogenous melanocortin receptors

POMC was capable of binding to endogenous melanocortin receptors as evidenced by the induction of melanogenesis (23% increase) in the Cloudman S91 mouse melanoma cells, but ACTH had a greater effect (62% increase). However, α -MSH was the most potent of the POMC peptides in stimulating melanogenesis (85% increase at 10 nM). (Fig. 5A). At the concentrations tested, γ -MSH was not able to induce melanogenesis, in agreement with previous studies (44). At day 3 and at the end of the experiment (day 6), the POMC concentration in the medium was assayed to see whether POMC had degraded during the incubation. We found 7400 ± 400 pmol/L of POMC in the medium after 3 days incubation compared with the 10,000 pmol/L added at the beginning of the experiment, which suggests that POMC is not rapidly degraded and concentrations in the culture remain high.

To determine whether POMC is also an agonist at the endogenous MC-1R in human pigment cells, we stimulated FM55 human melanoma cells with concentrations of POMC in the range 10^{-8} to 10^{-5} M (Fig. 5B). A significant stimulation of melanogenesis was visible in cell pellets with POMC at 10^{-6} and 10^{-5} M, but not with lower concentrations (Fig. 5C). Measurement of the melanin content in the cells showed that melanin synthesis was increased by 36% and 63% above unstimulated control levels in response to 10^{-6} M and 10^{-5} M POMC, respectively.

POMC also stimulated pigment cell dendricity (Fig. 5D) in a dose-dependent manner detectable at 10^{-7} M, but with the most potent effects observed at concentrations of 10^{-6} and 10^{-5} M. Cell proliferation was also stimulated by POMC in human melanoma cells at 10^{-6} M and 10^{-5} M, producing average increases of 20% and 22%, respectively, above control unstimulated levels (data not shown).

POMC modulates human epidermal melanocyte phenotype

Human epidermal melanocytes expressing the MC1-R (Fig. 6A–C) showed an increase in dendricity in response to both 10^{-6} and 10^{-5} M POMC (Fig. 6D–F). The epidermal melanocytes were primarily bipolar in basal culture conditions, but there was a marked increase in dendricity after stimulation with POMC.

POMC at 10^{-6} and 10^{-5} M significantly increased the melanin content of epidermal melanocytes (Fig. 6G), producing an average increase of 21% and 26%, respectively, above control unstimulated levels after 72 h stimulation. The epidermal melanocytes were derived from an individual with photo skin type III. A visible increase in melanogenesis was evident in pellets from the melanocytes stimulated with POMC (Fig. 6H).

There was also a marked increase in proliferation of the melanocytes in culture after 72 h with 10^{-6} and 10^{-5} M POMC stimulation resulting in an average increase of 30% and 38%, respectively, above control stimulated levels (Fig. 6I).

Taken together, these results indicate that POMC, albeit at high concentrations, is capable of modulating changes in multiple aspects of epidermal melanocyte phenotype *in vitro*.

DISCUSSION

Our study is the first to report the secretion of POMC from human epidermal melanocytes and keratinocytes and human hair follicle melanocytes *in vitro*. Previous studies have demonstrated the presence of ACTH and α -MSH in culture medium and cell extracts from normal human keratinocytes (12) and melanocytes (45), with concentrations similar to those found in the present study. However, understanding the processing of these peptides from their precursor, POMC, and the concentration of POMC in relation to the smaller peptides derived from it was not achieved in earlier studies. This is primarily because it was difficult to assess the concentration of POMC relative to the peptides derived from it. Assays utilizing a single antibody that binds a common epitope on both the peptide and its precursor do not fully recognize the precursor and therefore underestimate the concentration of the precursor. Indeed, we have evidence that an antibody to an epitope on ACTH shows low cross-reactivity with POMC, presumably because it does not recognize the tertiary structure of the ACTH epitope in POMC (32). In this study, POMC was measured directly using a two-antibody assay that overcomes this limitation. By using specific assays for the peptides, we were able to show that POMC is present in human melanocytes and keratinocytes and is the major POMC peptide secreted by these cells.

The predominance of POMC in culture medium both basally and after stimulation with CRH cannot be attributed to a lack of processing within the cells, as ACTH was the most abundant peptide in cell extracts. Our data are the first to show that epidermal keratinocytes have the capacity to process POMC *in vitro* in that they express PC1, PC2, and 7B2, as do their matched epidermal melanocyte counterparts. We previously observed PC1 and PC2 expression in human epidermis (10). Furthermore, epidermal melanocytes have been shown to express the POMC processing machinery (18).

In the present study, the cultures of keratinocytes were relatively undifferentiated, with a “basal” cell phenotype characterized by a rapidly expanding monolayer of closely adherent small cells with a “cobblestone” appearance; in areas where there was differentiation, the cells were increasingly large and pleiomorphic, with reduced intercellular adhesion and a tendency to stratify (46). As the melanocytes differentiated, they showed increased cell dendricity (47). In areas where differentiation was apparent, PC1, PC2, and 7B2 expression appeared to correlate with this differentiation. This is consistent with the view that in both keratinocytes and melanocytes, the expression of POMC correlates with increasing cell differentiation (11, 20,36). Consequently, it would appear that POMC processing is dependent on cell status such that as the cells differentiate, they express the processing machinery and produce the smaller, more active POMC peptides. Thus, the relatively low levels of α -MSH and ACTH in the cultured melanocytes and keratinocytes could be related to their less differentiated form. It would therefore be of interest to determine whether differentiation affects the processing of POMC to α -MSH and ACTH peptides in epidermal keratinocytes and melanocytes.

The hair follicle melanocytes also released high concentrations of POMC. No α -MSH was detected after 24 h in culture, although after 72 h low levels of α -MSH were identified in the culture media. Immunocytochemical studies indicate that ACTH and α -MSH (as well as PC1, PC2, and 7B2) are undetectable in the melanogenic zone of the anagen hair bulb, the site of

the most highly differentiated (perhaps terminally differentiated) melanocytes. Thus, the ACTH/ α -MSH/MC-1R system appears to be expressed most markedly during early stages of melanocyte differentiation, becoming down-regulated during terminal differentiation in hair bulb melanocytes. The keratinocytes used in the current study were maintained as proliferating basal phenotype cells that expressed the full complement of POMC peptides, processing enzymes, and the MC1R, and may reflect a more responsive population of keratinocytes in terms of release of POMC.

In response to CRH, we observed increases in POMC and α -MSH, but not ACTH, from hair follicle melanocytes. The fact that we saw no change in ACTH could suggest that in these cells this peptide is present in secretory granules and that CRH, by activating PC2, stimulates its cleavage to α -MSH. Whatever the explanation, our results suggest that, at least in hair follicle melanocytes, CRH is capable of increasing both the release of POMC and its processing to α -MSH. CRH provoked a marked increase in POMC release from hair follicle melanocytes at all time points, and at 72 h gave rise to a significant increase at concentrations above 10 nM. This was accompanied by a smaller but significant increase in α -MSH. This may reflect the effect of CRH on POMC gene expression, as it is similar to stimulation of the POMC gene in epidermal melanocytes *in vitro* (16) and in fibroblasts (33). There were differences in the time course for POMC mRNA expression in that the peak is reached at 1 h in fibroblasts, but was delayed in melanocytes (48). In this study, the highest levels of the POMC peptide were obtained after 24 h, and this effect was sustained 72 h after addition of CRH.

Our previous data showed that hair follicle melanocytes in culture expressed both CRH-R1 and CRH-R2 and that expression correlated with more differentiated cells (34). However, normal epidermal melanocytes and keratinocytes are reported to express CRH-R1 exclusively (27). Therefore, it was important to determine which receptor subtype is involved in POMC secretion. From the data presented, it would appear that CRH is acting primarily through the CRH-R1 receptor in that [D-Pro^4]-r-urocortin, an analog that acts almost entirely *via* the CRH-R2, has relatively little effect on POMC concentrations. This is in keeping with the requirement of CRH-R1 for CRH-stimulated POMC gene expression (48). We have previously shown that stimulation of hair follicle melanocytes with CRH and urocortin peptides modulated proliferation, dendricity, and melanogenesis (34). We postulated that some of the effects may be direct but that some may occur indirectly by CRH effects on POMC. Given that the relative biological activity of the CRH analogs was similar in potency in stimulating POMC release, it is possible that CRH is primarily acting indirectly by stimulating POMC.

It may be that as yet unidentified factors such as UV irradiation stimulate POMC processing in epidermal keratinocytes and melanocytes. Thus, the release of POMC under basal conditions may occur *via* the constitutive-like pathway (49) as an “overflow” mechanism to reduce a buildup of prohormone in unstimulated cells. Another possibility is that POMC is released and processed extracellularly, possibly at the target cell surface. Such a mechanism has been proposed for N-POMC at the adrenal cortex (50) and it is thought that furin can act extracellularly to cleave POMC. Whatever the explanation, it is clear that to appreciate the importance of POMC in the skin, we need to analyze not only its expression but also the way in which this precursor is post-translationally cleaved. It is this process that will determine which bioactive peptides are produced.

The obvious excess of POMC relative to other peptides opens speculation as to the biological activity of POMC. A synthetic source of POMC was not available, and therefore it was necessary to purify POMC from a cell line. The specific monoclonal antibodies we produced earlier (32) made the purification easier. Extensive characterization confirmed that the purified POMC did not contain any ACTH or α -MSH at concentrations that would activate the MC-1R. When comparing the peptides at human MC-1R, it is clear that POMC has a much lower

potency than α -MSH. ACTH nevertheless is capable of activating adenylate cyclase, and the similarity in potency for ACTH and α -MSH at the human MC-1R has been seen before (10, 51).

To determine the biological activity of POMC, we assessed its effect on melanogenesis, dendricity, and proliferation in human pigment cells. Our results showed that POMC was functionally active, but only at concentrations in excess of 10^{-7} M. This is considerably higher than the concentrations of POMC released from the cells ($\sim 1 \times 10^{-10}$ M from epidermal keratinocytes) in the current study using mostly undifferentiated cell cultures. It is possible that POMC is degraded extracellularly to ACTH-like peptides or β -MSH in these cultures, and that the biological activity is due to the effect of these peptides. It has been shown that nanomolar or lower concentrations of α -MSH, β -MSH, and ACTH can stimulate melanogenesis, cell proliferation, dendrite formation, and cAMP production in cultured human melanocytes (27,29–31,52). However, it is unlikely that POMC is degraded to α -MSH as the PC2 cleavage, and the amidation and acetylation required to generate α -MSH can only occur intracellularly within the acidic environment of the secretory granule.

It is interesting to speculate whether POMC might act as a partial agonist at the MC-1R, and in this way either down-regulate the receptor or prevent its dimerization. As the melanocytes and keratinocytes differentiate, they begin to express the processing machinery and therefore are capable of cleaving POMC to more active peptides. Once released, these peptides could act to override the antagonistic actions of POMC, allowing the MC-1R to become activated. In an analogous way, it has been shown that γ 3-MSH can act as a partial antagonist at MC-1R and that γ 1 and γ 2 MSH can modify β -MSH action (44).

In conclusion, the present study has identified incomplete processing of POMC in human epidermal keratinocytes and melanocytes and in human hair follicle melanocytes such that little if any biologically active ACTH and α -MSH are released from cells under resting conditions. There is evidence that expression of the processing machinery is correlated with cell differentiation status. In keeping with the concept of a regulatory peptide system analogous to the HPA axis, there is evidence that CRH, acting primarily through CRH-R1, stimulates release of POMC and α -MSH. However, it does not appear to cause a major increase in processing. It is surprising that given the size of POMC, it is capable of activating the MC-1R and stimulating cAMP production, albeit with a lower potency to α -MSH. This is in keeping with the effects of POMC at high concentrations in stimulating increases in melanin and promoting increased dendricity of human epidermal melanocytes. If POMC is able to attain such high concentrations in the skin and to have the same effects on melanocytes, then it could be significant in regulating multiple aspects of the human melanocyte phenotype. This notwithstanding, it is important that we understand the mechanisms that regulate processing of POMC in order to determine how and when the biologically active POMC peptides are released from the different skin cell types and whether they are generated extracellularly in the cutaneous environment.

Acknowledgements

We are grateful to Dr. John Brennan, Rick Davies, and Catherine Schmitz from AstraZeneca for providing reagents for the analysis of MC-1R activity. We also acknowledge the support of the Wellcome Trust (A.W., A.T.) and National Institutes of Health Grant #AR047079 (A.S., D.T.) for providing funding.

REFERENCES

1. Slominski A, Wortsman J, Luger T, Paus R, Solomon S. Corticotropin releasing hormone and proopiomelanocortin involvement in the cutaneous response to stress. *Physiol. Rev* 2000;80:979–1020. [PubMed: 10893429]

2. Pritchard LE, Turnbull AV, White A. Proopiomelanocortin processing in the hypothalamus: impact on melanocortin signalling and obesity. *J. Endocrinol* 2002;172:411–421. [PubMed: 11874690]
3. Pritchard LE, Oliver RL, McLoughlin JD, Birtles S, Lawrence CB, Turnbull AV, White A. Proopiomelanocortin-derived peptides in rat cerebrospinal fluid and hypothalamic extracts: evidence that secretion is regulated with respect to energy balance. *Endocrinology* 2003;144:760–766. [PubMed: 12586751]
4. Gibson S, Crosby SR, Stewart MF, Jennings AM, McCall E, White A. Differential release of proopiomelanocortin-derived peptides from the human pituitary: evidence from a panel of two-site immunoradiometric assays. *J. Clin. Endocrinol. Metab* 1994;78:835–841. [PubMed: 8157708]
5. White A, Gibson S. ACTH precursors: biological significance and clinical relevance. *Clin. Endocrinol. (Oxford)* 1998;48:251–255.
6. Tsigos C, Gibson S, Crosby SR, White A, Young RJ. Cerebrospinal fluid levels of beta endorphin in painful and painless diabetic polyneuropathy. *J. Diabetes Complications* 1995;9:92–96. [PubMed: 7599354]
7. Thody AJ, Ridley K, Penny RJ, Chalmers R, Fisher C, Shuster S. MSH peptides are present in mammalian skin. *Peptides* 1983;4:813–816. [PubMed: 6672790]
8. Slominski A, Paus R, Wortsman J. On the potential role of proopiomelanocortin in skin physiology and pathology. *Mol. Cell. Endocrinol* 1993;93:C1–C6. [PubMed: 8391494]
9. Bhardwaj RS, Luger TA. Proopiomelanocortin production by epidermal cells: evidence for an immune neuroendocrine network in the epidermis. *Arch. Dermatol. Res* 1994;287:85–90. [PubMed: 7726641]
10. Wakamatsu K, Graham A, Cook D, Thody AJ. Characterisation of ACTH peptides in human skin and their activation of the melanocortin-1 receptor. *Pigment Cell Res* 1997;10:288–297. [PubMed: 9359624]
11. Kauser S, Schallreuter KU, Thody AJ, Gummer C, Tobin DJ. Regulation of human epidermal melanocyte biology by beta-endorphin. *J. Invest. Dermatol* 2003;120:1073–1080. [PubMed: 12787137]
12. Schauer E, Trautinger F, Kock A, Schwarz A, Bhardwaj R, Simon M, Ansel JC, Schwarz T, Luger TA. Proopiomelanocortin-derived peptides are synthesized and released by human keratinocytes. *J. Clin. Invest* 1994;93:2258–2262. [PubMed: 8182158]
13. Slominski A, Ermak G, Mazurkiewicz JE, Baker J, Wortsman J. Characterization of corticotropin-releasing hormone (CRH) in human skin. *J. Clin. Endocrinol. Metab* 1998;83:1020–1024. [PubMed: 9506767]
14. Slominski A, Heasley D, Mazurkiewicz JE, Ermak G, Baker J, Carlson JA. Expression of proopiomelanocortin (POMC)-derived melanocyte-stimulating hormone (MSH) and adrenocorticotrophic hormone (ACTH) peptides in skin of basal cell carcinoma patients. *Hum. Pathol* 1999;30:208–215. [PubMed: 10029451]
15. Wintzen M, Yaar M, Burbach JP, Gilchrist BA. Proopiomelanocortin gene product regulation in keratinocytes. *J. Invest. Dermatol* 1996;106:673–678. [PubMed: 8618003]
16. Slominski A, Zbytek B, Szczesniwski A, Semak I, Kaminski J, Sweatman T, Wortsman J. CRH stimulation of corticosteroids production in melanocytes is mediated by ACTH. *Am. J. Physiol* 2005;288:E701–E706.
17. Mazurkiewicz JE, Corliss D, Slominski A. Spatiotemporal expression, distribution, and processing of POMC and POMC-derived peptides in murine skin. *J. Histochem. Cytochem* 2000;48:905–914. [PubMed: 10858267]
18. Peters EM, Tobin DJ, Seidah NG, Schallreuter KU. Pro-opiomelanocortin-related peptides, prohormone convertases 1 and 2 and the regulatory peptide 7B2 are present in melanosomes of human melanocytes. *J. Invest. Dermatol* 2000;114:430–437. [PubMed: 10692100]
19. Schiller M, Raghunath M, Kubitscheck U, Scholzen TE, Fisbeck T, Metze D, Luger TA, Bohm M. Human dermal fibroblasts express prohormone convertases 1 and 2 and produce proopiomelanocortin-derived peptides. *J. Invest. Dermatol* 2001;117:227–235. [PubMed: 11511298]
20. Kauser S, Thody AJ, Schallreuter KU, Gummer CL, Tobin DJ. A fully functional proopiomelanocortin/melanocortin-1 receptor system regulates the differentiation of human scalp hair follicle melanocytes. *Endocrinology* 2005;146:532–543. [PubMed: 15498881]

21. Bohm M, Eickelmann M, Li Z, Schneider SW, Oji V, Diederichs S, Barsh GS, Vogt A, Stieler K, Blume-Peytavi U, Luger TA. Detection of functionally active melanocortin receptors and evidence for an immunoregulatory activity of alpha-melanocyte-stimulating hormone in human dermal papilla cells. *Endocrinology* 2005;146:4635–4646. [PubMed: 16081629]
22. Pritchard LE, Armstrong D, Davies N, Oliver RL, Schmitz CA, Brennand JC, Wilkinson GF, White A. Agouti-related protein (83–132) is a competitive antagonist at the human melanocortin-4 receptor: no evidence for differential interactions with pro-opiomelanocortin-derived ligands. *J. Endocrinol* 2004;180:183–191. [PubMed: 14709157]
23. Mountjoy KG. The human melanocyte stimulating hormone receptor has evolved to become “super-sensitive” to melanocortin peptides. *Mol. Cell. Endocrinol* 1994;102:R7–R11. [PubMed: 7926261]
24. Ancans, J.; Tsatmali, M.; Hoogduijn, MJ.; Thody, AJ. Melanocortin peptides and their control of skin pigmentation. In: Ortonne, JP.; Ballotti, R., editors. *Mechanisms of Suntanning*. London: Martin Dunitz; 2002.
25. Krude H, Biebermann H, Luck W, Horn R, Brabant G, Gruters A. Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in humans. *Nat. Genet* 1998;19:155–157. [PubMed: 9620771]
26. Lerner AB, McGuire JS. Effect of alpha- and betamelanocyte stimulating hormones on the skin colour of man. *Nature* 1961;189:176–179. [PubMed: 13761067]
27. Slominski A, Pisarchik A, Tobin DJ, Mazurkiewicz JE, Wortsman J. Differential expression of a cutaneous corticotropin-releasing hormone system. *Endocrinology* 2004;145:941–950. [PubMed: 14605004]
28. Ray DW, Gibson S, Crosby SR, Davies D, Davis JR, White A. Elevated levels of adrenocorticotropin (ACTH) precursors in post-adrenalectomy Cushing’s disease and their regulation by glucocorticoids. *J. Clin. Endocrinol. Metab* 1995;80:2430–2436. [PubMed: 7629238]
29. Hunt G, Todd C, Cresswell JE, Thody AJ. Alpha-melanocyte stimulating hormone and its analogue Nle4DPhe7 alpha-MSH affect morphology, tyrosinase activity and melanogenesis in cultured human melanocytes. *J. Cell Sci* 1994;107:205–211. [PubMed: 8175909]
30. Hunt G, Todd C, Kyne S, Thody AJ. ACTH stimulates melanogenesis in cultured human melanocytes. *J. Endocrinol* 1994;140:R1–R3. [PubMed: 8138743]
31. Abdel-Malek Z, Swope VB, Suzuki I, Akcali C, Harriger MD, Boyce ST, Urabe K, Hearing VJ. Mitogenic and melanogenic stimulation of normal human melanocytes by melanotropic peptides. *Proc. Natl. Acad. Sci* 1995;92:1789–1793. [PubMed: 7878059]
32. Crosby SR, Stewart MF, Ratcliffe JG, White A. Direct measurement of the precursors of adrenocorticotropin in human plasma by two-site immunoradiometric assay. *J. Clin. Endocrinol. Metab* 1988;67:1272–1277. [PubMed: 2848055]
33. Slominski A, Zbytek B, Semak I, Sweatman T, Wortsman J. CRH stimulates POMC activity and corticosterone production in dermal fibroblasts. *J. Neuroimmunol* 2005;162:97–102. [PubMed: 15833364]
34. Kauser S, Slominski A, Wei ET, Tobin DJ. Modulation of the human hair follicle pigmentary unit by corticotropin-releasing hormone and urocortin peptides. *FASEB J* 2006;20:882–895. [PubMed: 16675846]
35. Tobin DJ, Colen SR, Bystry JC. Isolation and long-term culture of human hair-follicle melanocytes. *J. Invest. Dermatol* 1995;104:86–89. [PubMed: 7528247]
36. Kauser S, Thody AJ, Schallreuter KU, Gummer CL, Tobin DJ. beta-Endorphin as a regulator of human hair follicle melanocyte biology. *J. Invest. Dermatol* 2004;123:184–195. [PubMed: 15191559]
37. Bertagna XY, Nicholson WE, Sorenson GD, Pettengill OS, Mount CD, Orth DN. Corticotropin, lipotropin, and beta-endorphin production by a human nonpituitary tumor in culture: evidence for a common precursor. *Proc. Natl. Acad. Sci. U. S. A* 1978;75:5160–5164. [PubMed: 217015]
38. White A, Smith H, Hoadley M, Dobson SH, Ratcliffe JG. Clinical evaluation of a two-site immunoradiometric assay for adrenocorticotrophin in unextracted human plasma using monoclonal antibodies. *Clin. Endocrinol. (Oxford)* 1987;26:41–51.

39. Wei ET, Thomas HA, Christian HC, Buckingham JC, Kishimoto T. D-amino acid-substituted analogs of corticotropin-releasing hormone (CRH) and urocortin with selective agonist activity at CRH1 and CRH2beta receptors. *Peptides* 1998;19:1183–1190. [PubMed: 9786168]
40. Ito N, Ito T, Kromminga A, Bettermann A, Takigawa M, Kees F, Straub RH, Paus R. Human hair follicles display a functional equivalent of the hypothalamic-pituitary-adrenal axis and synthesize cortisol. *FASEB J* 2005;19:1332–1334. [PubMed: 15946990]
41. Stewart MF, Crosby SR, Gibson S, Twentyman PR, White A. Small cell lung cancer cell lines secrete predominantly ACTH precursor peptides not ACTH. *Br. J. Cancer* 1989;60:20–24. [PubMed: 2553086]
42. White A, Stewart MF, Farrell WE, Crosby SR, Lavender PM, Twentyman PR, Rees LH, Clark AJ. Pro-opiomelanocortin gene expression and peptide secretion in human small-cell lung cancer cell lines. *J. Mol. Endocrinol* 1989;3:65–70. [PubMed: 2472813]
43. Kadarko AL, Kanto H, Kavanagh R, Abdel-Malek ZA. Significance of the melanocortin 1 receptor in regulating human melanocyte pigmentation, proliferation, and survival. *Ann. N. Y. Acad. Sci* 2003;994:359–365. [PubMed: 12851336]
44. Slominski A, Costantino R, Wortsman J, Paus R, Ling N. Melanotropic activity of gamma MSH peptides in melanoma cells. *Life Sci* 1992;50:1103–1108. [PubMed: 1556905]
45. Chakraborty AK, Funasaka Y, Slominski A, Ermak G, Hwang J, Pawelek JM, Ichihashi M. Production and release of proopiomelanocortin (POMC) derived peptides by human melanocytes and keratinocytes in culture: regulation by ultraviolet B. *Biochim. Biophys. Acta* 1996;1313:130–138. [PubMed: 8781560]
46. Kautsky MB, Fleckman P, Dale BA. Retinoic acid regulates oral epithelial differentiation by two mechanisms. *J. Invest. Dermatol* 1995;104:546–553. [PubMed: 7706775]
47. Buffey JA, Messenger AG, Taylor M, Ashcroft AT, Westgate GE, MacNeil S. Extracellular matrix derived from hair and skin fibroblasts stimulates human skin melanocyte tyrosinase activity. *Br. J. Dermatol* 1994;131:836–842. [PubMed: 7857836]
48. Slominski A, Zbytek B, Zmijewski M, Slominski RM, Kauser S, Wortsman J, Tobin DJ. Corticotropin releasing hormone and the skin. *Front. Biosci* 2006;11:2230–2248. [PubMed: 16720310]
49. Dumermuth E, Moore H-P. Analysis of constitutive and constitutive-like secretion in semi-intact pituitary cells. *Methods* 1998;16:188–197. [PubMed: 9790865]
50. Bicknell AB, Lomthaisong K, Woods RJ, Hutchinson EG, Bennett HP, Gladwell RT, Lowry PJ. Characterization of a serine protease that cleaves pro-gammamelanotropin at the adrenal to stimulate growth. *Cell* 2001;105:903–912. [PubMed: 11439186]
51. Thody AJ, Graham A. Does alpha-MSH have a role in regulating skin pigmentation in humans? *Pigment Cell Res* 1998;11:265–274. [PubMed: 9877097]
52. Hunt G, Kyne S, Ito S, Wakamatsu K, Todd C, Thody A. Eumelanin and pheomelanin contents of human epidermis and cultured melanocytes. *Pigment Cell Res* 1995;8:202–208. [PubMed: 8610071]

A Epidermal keratinocytes **B Epidermal melanocytes** **C Hair follicle melanocytes** **Figure 1.**

More POMC than α -MSH is secreted by epidermal kartinocytes (A), epidermal melanocytes (B), and hair follicle melanocytes (C). Results are presented as fmol/flask to allow direct comparison of peptides in extracts and media. POMC (shaded bars), ACTH (open bars), and α -MSH (hatched bars) were measured in epidermal melanocytes and matched keratinocytes from five normal volunteers after culturing for 24 h in serum-free medium. Levels of peptides in extracts from confluent cultures are compared with those secreted into culture medium (10 ml/flask). Hair follicle melanocytes from four separate subjects were cultured and the secreted levels of POMC and α -MSH were measured at 72 h. Limit of detection for ACTH and α -MSH

in culture medium are <24 fmol/flask and in cell extracts are <7 fmol/flask. Bars represent the mean $\pm$ SD ($n=3$).

Figure 2.

A) PC1, PC2, and 7B2 are expressed in epidermal melanocytes *in vitro*. The POMC processing machinery was variably expressed in cultured epidermal melanocytes (passage 3). Expression correlated positively with differentiation status. Stronger expression was observed in differentiated dendritic melanocytes (arrow); it was weaker in less differentiated (flatter) melanocytes (arrowhead) and exhibited a cytoplasmic distribution pattern. Positive staining was observed with the antibody against gp100 (melanocyte lineage-specific marker). In the negative control, the primary antibody was replaced by preimmune serum from secondary antibody host. Scale bar: 17 μ m. B) POMC processing machinery is expressed in epidermal keratinocytes *in vitro*. The expression of PC1, PC2, and 7B2 was variable in cultured epidermal keratinocytes (passage 2). Expression was higher in keratinocytes with a more differentiated phenotype (arrow) compared with those with a basal cell phenotype (arrowhead). Strong expression was confined to the perinuclear region, where it showed a granular distribution pattern. Positive staining was observed with the keratin-specific antibody, AE3. In the negative control, the primary antibody was replaced by preimmune serum from secondary antibody host. Scale bar: 17 μ m

Figure 3.

Effects of CRH on POMC release. *A*) CRH stimulation of POMC and α -MSH secreted into culture medium by human hair follicle melanocytes cultured for 72 h. Mean $\pm$ SE ($n=4$ patients); $*P < 0.05$, $**P < 0.01$ relative to unstimulated control. *B*) Time course of the responsiveness of POMC to CRH in cultured human hair follicle melanocytes. Mean $\pm$ SE ($n=4$ patients). *C*) Dose response of CRH agonists D -Pro⁵CRH, D -Pro⁴-r-urocortin, and D -Glu²⁰CRH on the release of POMC by cultured hair follicle melanocytes.

Figure 4.

Activation of MC-1R by POMC. A) Gel chromatography profile of the human POMC after purification of POMC secreted into culture medium from the small cell lung carcinoma cell line DMS 79. Fractions were assayed in specific immunoassays for POMC and ACTH. All ACTH results were below the limit of detection. A) Western blot based on an antibody to ACTH 10–18 identifies the purified POMC (lane 3) with no band at the molecular weight of ACTH compared with synthetic ACTH (lane 1) and no recognition of synthetic β -LPH (lane 2). B) ChOK1 cells stably expressing the human MC-1R and a β -galactosidase reporter construct were incubated with increasing concentrations of β -MSH ($\blacktriangle$), ACTH ($\triangle$), α MSH ($\blacksquare$), POMC ($\square$), and γ MSH ($\bullet$). Ligand binding to the MC-1R stimulates cAMP, which activates the reporter gene to synthesize β -galactosidase. When substrate is added, this generates a color

reaction that is measured as optical density (OD) units. Data points represent means of quadruplicate samples $\pm$ SD. The curves are representative of 3 independent experiments.

Figure 5.

Effects of POMC on melanoma cells. **A)** POMC, ACTH, and α -MSH bind to the endogenous MC-1R in mouse melanoma cells. Melanin content was increased in response to POMC (∇), ACTH ($\circ$), and α -MSH ($\square$) in mouse melanoma cells (S91) cultured for 6 days. Melanin content was determined spectrophotometrically at 475 nm against a synthetic melanin standard. Results were calculated as μg melanin per mg protein and expressed as % of the controls ($n=3$). **B)** POMC stimulated melanogenesis in human melanoma cells in culture. Melanin content was determined spectrophotometrically at 475 nm in cells stimulated with POMC at 10^{-8} - 10^{-5} M. Results are expressed as % increase in melanin content over control levels ($n=3$). **C)** The corresponding cell pellets revealed the melanogenic effects of POMC at the most potent concentrations. **D)** POMC stimulated dendricity in cultured human melanoma cells. Cell dendricity was assessed by counting cells with 3 or more dendrites before and after stimulation with POMC at 10^{-8} - 10^{-5} M. Melanoma cells were established in RPMI 1640 medium, FCS was omitted from the culture medium 24 h prior to stimulation and replaced with 0.25% BSA. POMC at 10^{-5} and 10^{-6} M were potent inducers of melanoma cell dendricity and subtle effects were observed at 10^{-7} M. Scale bar: 50 μm . (top right of control)

Figure 6.

Effects of POMC on human epidermal melanocytes. *A–C*) The MC-1R is present in cultured epidermal melanocytes and is found at the cell surface (arrowheads). Cells were cultured in FCS/BPE-free medium for 48 h prior to immunostaining, then fixed in 5% paraformaldehyde and the MC-1R was visualized by binding of an anti-rabbit antibody. Melanocyte identity was confirmed by gp100 expression (*B*). *D–F*) Epidermal melanocyte cultures (passage 4) were established in bFGF/ET-1 supplemented medium. FCS and BPE were omitted from the culture medium 48 h prior to stimulation. A marked increase in cell dendricity was seen 72 h after POMC stimulation at 10^{-5} and 10^{-6} M concentrations compared with melanocytes maintained under basal medium conditions. Scale bar: 50 μ m. *G*) Effect of POMC on melanin content in human epidermal melanocytes. Melanin content was determined spectrophotometrically (475 nm) after sodium hydroxide solubilization. *H*) Visible increases in melanogenesis after 10^{-5} and 10^{-6} M POMC stimulation were observed. Results are expressed as a percentage increase in melanin content over control unstimulated levels. *I*) Effect of POMC on proliferation in human epidermal melanocytes. Cell proliferation was assessed by determining cell counts before and after 10^{-5} and 10^{-6} M POMC stimulation. A marked increase in melanocyte proliferation was observed after 72 h stimulation with POMC. Results are expressed as a

percentage increase in cell number over control unstimulated levels and experiments were performed in triplicate.