Regulated Proenkephalin Expression in Human Skin and Cultured Skin Cells

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Skin responds to environmental stressors via coordinated actions of the local neuroimmunoendocrine system. Although some of these responses involve opioid receptors, little is known about cutaneous proenkephalin expression, its environmental regulation, and alterations in pathology. The objective of this study was to assess regulated expression of proenkephalin in normal and pathological skin and in isolated melanocytes, keratinocytes, fibroblasts, and melanoma cells. The proenkephalin gene and protein were expressed in skin and cultured cells, with significant expression in fibroblasts and keratinocytes. Mass spectroscopy confirmed Leu- and Met-enkephalin in skin. UVR, Toll-like receptor (TLR)4, and TLR2 agonists stimulated proenkephalin gene expression in melanocytes and keratinocytes in a time- and dose-dependent manner. *In situ* Met/Leu-enkephalin peptides were expressed in differentiating keratinocytes of the epidermis in the outer root sheath of the hair follicle, in myoepithelial cells of the eccrine gland, and in the basement membrane/basal lamina separating epithelial and mesenchymal components. Met/Leu-enkephalin expression was altered in pathological skin, increasing in psoriasis and decreasing in melanocytic tumors. Not only does human skin express proenkephalin, but this expression is upregulated by stressful stimuli and can be altered by pathological conditions.

Journal of Investigative Dermatology (2011) **131,** 613–622; doi:10.1038/jid.2010.376; published online 30 December 2010

INTRODUCTION

The skin is the largest body organ and is continuously exposed to environmental stress, including solar radiation; physical, chemical, and biological insults; and antigenic stimuli (Slominski and Wortsman, 2000; Slominski *et al.*, 2000c, 2008b; Tobin, 2006). To protect local and global homeostasis, the skin responds to these insults through complex biological, biochemical, and chemical activities that are coordinated by the local neuroendocrine and immune systems (Slominski and Wortsman, 2000; Slominski *et al.*, 2000c, 2004, 2008b; Arck *et al.*, 2006; Elias, 2007). An example of this local activity is the local production of proopiomelanocortin-derived peptides (Slominski *et al.*, 2000c), corticotropin-releasing factor and urocortins (Slominski *et al.*, 2000a, b), acetylcholine (Grando and Horton, 1997), catecholamines (Schallreuter *et al.*, 1995), serotonin (Slominski *et al.*, 2005), and melatonin (Slominski *et al.*, 2008a). These neurohormones, neurotransmitters, and neuropeptides regulate the local homeostasis through interaction with corresponding receptors expressed on skin cells or nerve endings, allowing local neural regulation or transmission to the brain (Slominski and Wortsman, 2000).

The enkephalins belong to one of three well-characterized families of endogenous opioid peptides produced by the body, with the Met-enkephalin peptide sequence coded for by both the enkephalin gene and the proopiomelanocortin gene, while the Leu-enkephalin peptide sequence is coded for by both the enkephalin gene and the dynorphin gene. The receptors for enkephalin include μ-, κ-, and δ-opioid receptors, which are expressed on skin cells and on sensory nerve endings in the skin, and their activation can regulate keratinocyte and melanocyte activities (Zagon *et al.*, 1996; Nissen and Kragballe, 1997; Bigliardi *et al.*, 1998, 2003; Bigliardi-Qi *et al.*, 1999). Proopiomelanocortin-derived β-endorphin, one of their endogenous ligands (Bigliardi-Qi *et al.*, 2000, 2004; Kauser *et al.*, 2003, 2004; Tobin, 2006), is locally produced in the skin (Slominski *et al.*, 1992, 1993; Furkert *et al.*, 1997; Slominski, 1998) and stimulates epidermal and follicular melanogenesis (Kauser *et al.*, 2003, 2004). Although there are reports demonstrating the presence of Met-enkephalin antigen in adult mammalian skin (Weihe *et al.*, 1983; Chew and Leung, 1991; Zagon *et al.*, 1996; Nissen *et al.*, 1999), there is a shortage of information on local proenkephalin gene expression and its relationship to

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Abbreviations: FP, follicular papilla; mRNA, messenger RNA; PENK, proenkephalin; TLR, Toll-like receptor

Received 9 March 2010; revised 14 October 2010; accepted 29 October 2010; published online 30 December 2010

skin pathology and the environmental regulation of proenkephalin (PENK) expression.

PENK is a large protein precursor that is highly conserved from Xenopus to humans (Comb *et al.*, 1983; Martens and Herbert, 1984; Felig and Frohman, 2001). It is processed through the action of PC1 and PC2 convertases to produce several copies of Met-enkephalin and Leu-enkephalin in a 4:1 ratio, as well as the opioids hepta- and octapeptide (Met-enkephalin–Arg–Phe and Met-enkephalin–Arg–Gly–Leu, respectively) and C- or N-terminally extended variants of these peptides (Goumon *et al.*, 2000; Kieffer *et al.*, 2003; Peinado *et al.*, 2003; Ozawa *et al.*, 2007). PENK-derived peptides exhibit opiate-like activity; act as neurotransmitters, neuromodulators, and neurohormones; and are involved in analgesia, responses to stress and pain, and regulation of appetite and sleep (Felig and Frohman, 2001; Le Merrer *et al.*, 2009). In addition to its role in the central and peripheral nervous system, the *PENK* gene is expressed in several nonneuronal tissues, including endocrine glands such as the adrenal medulla, cells of the immune system, and embryonal skin mesenchymal cells (Blalock, 1989; Kew and Kilpatrick, 1990; Polakiewicz and Rosen, 1990; Behar *et al.*, 1991, 1994; Kuis *et al.*, 1991; Polakiewicz *et al.*, 1992; Rosen *et al.*, 1995). Thus, depending on the site of production or target cells, the PENK-derived peptides can act in a para- or autocrine manner affecting immune activities, having direct antimicrobial activities, regulating cell proliferation and differentiation, and playing a role in brain development (Nissen and Kragballe, 1997; Goumon *et al.*, 1998; Kamphuis *et al.*, 1998; Metz-Boutigue *et al.*, 2000; Tasiemski *et al.*, 2000a; Kieffer *et al.*, 2003).

To address some significant gaps in our current knowledge and to further define elements of the local neuroimmunoendocrine system, we have carefully investigated the expression of *PENK* gene and protein with corresponding Met- and Leu-enkephalin peptides in human skin under physiological and pathological conditions and in cultured normal and malignant keratinocytes, melanocytes, and fibroblasts, using a range of molecular, analytical, cell biology, and *in situ* techniques. To clarify the regulatory mechanisms of PENK expression, we tested the effects of UVB, the Toll-like receptor 4 (TLR4) agonist lipopolysaccharide, and the TLR2 agonist PAM3CSK4 on PENK expression in epidermal melanocytes and keratinocytes.

RESULTS

PENK gene and protein are expressed in the human skin and skin cells

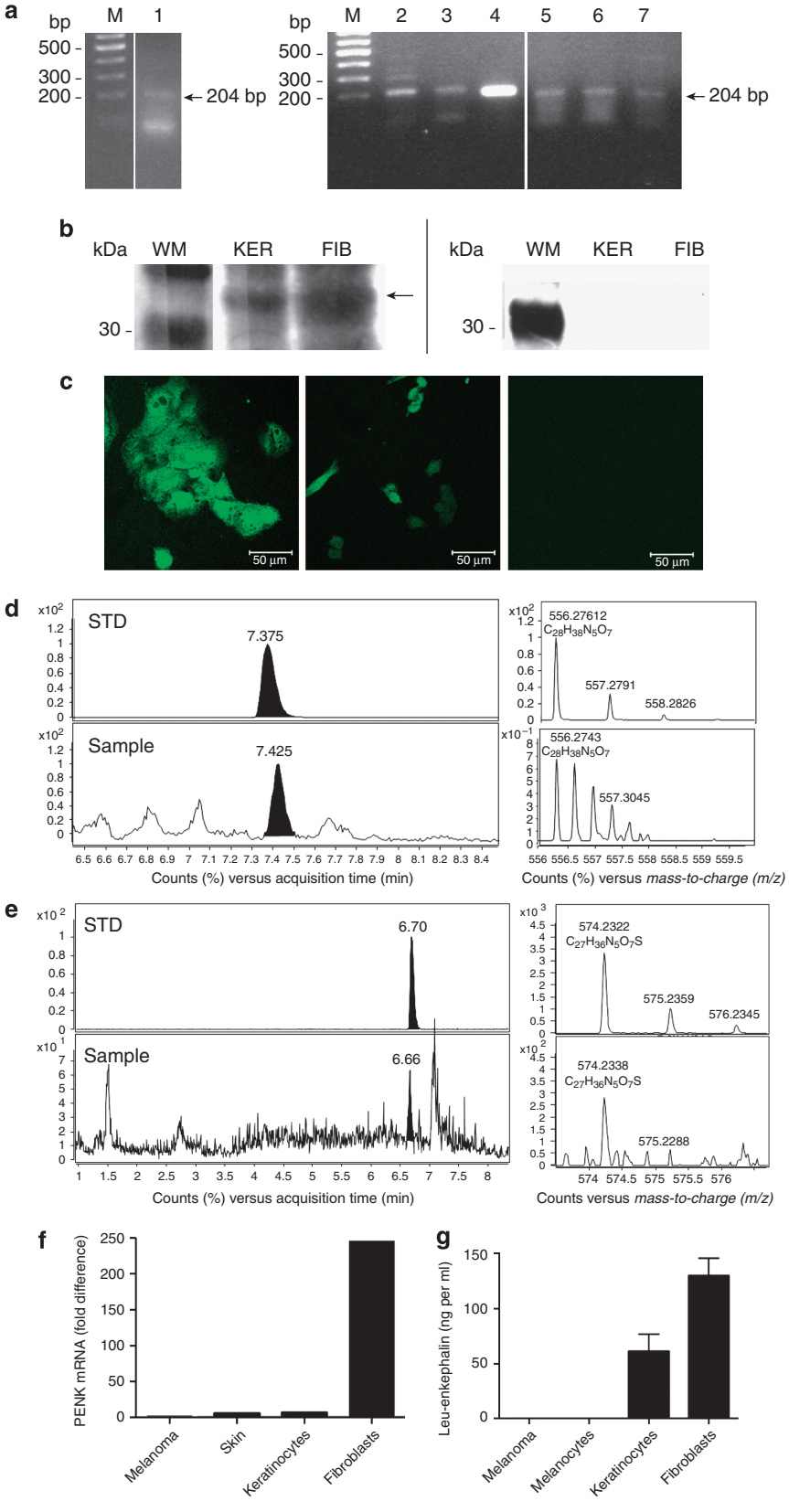
RT-PCR amplification (30 cycles) of complementary mRNA from skin biopsy specimens and cultured normal and immortalized epidermal keratinocytes, melanocytes, and dermal fibroblasts and six melanoma lines derived from melanomas at radial, vertical, and metastatic growth phases all demonstrated a 204-kb transcript with 100% homology with the corresponding *PENK* gene fragment (Figure 1a). Western blot and immunofluorescence studies demonstrated that this PENK messenger RNA (mRNA) expression is associated with production of the corresponding protein (Figure 1b and c). Figure 1b and Supplementary Figure S1 online demonstrate the presence of a PENK protein precursor with a molecular weight slightly above 30 kDa in human normal epidermal and fibroblasts (Figure 1b, left panel); it was absent in membranes incubated with nonimmune serum (Figure 1b, right panel). The band of the same size was seen in AtT-20 pituitary cells transfected with PENK plasmid (not shown). Supplementary Figure S1 online again demonstrates the presence of PENK with a molecular weight slightly above 30 kDa in human normal epidermal keratinocytes and melanocytes, HaCaT keratinocytes, dermal fibroblasts, and melanoma cells, as well as additional double bands of higher molecular weight (>40 kDa) in fibroblasts and normal and immortalized keratinocytes, which may represent post-translational processing of the protein (e.g., glycosylation).

Mass spectroscopy analysis confirmed the presence of Leu- and Met-enkephalin in skin extracts (Figure 1d and e). Specifically, a mass of 556.27 with a retention time of 7.4 minutes in skin extract was identical to the *m/z* at 556.27 and retention time of 7.4 minutes of the Leu-enkephalin standard (the calculated mass of Leu-enkephalin is 556.27711) (Figure 1d). The predicted C_{13} isotope at *m/z* 557.23 in skin extract was identical to the standard C_{13} isotope (Figure 1d, right). The software predicted a chemical formula of $C_{28}H_{38}N_5O_7$. Similarly, an *m/z* at 574.23 and a retention time of 6.7 minutes was detected in skin extract (Figure 1e) and dermal fibroblasts extract (not shown). It was identical to the mass 574.23 and retention time 6.7 minutes of the Met-enkephalin standard (the accurate mass of Met-enkephalin is 574.223) (Figure 1e). The predicted C_{13} isotope at *m/z* 575.23 was also identical to the standard C_{13} isotope (Figure 1e, lower right). On the basis of the above mass, the software predicted a chemical formula of $C_{27}H_{36}N_5O_7S$. Thus, by matching the predicted chemical formulas and the extracted chromatograms of the skin and standards, Met- and Leu-enkephalin were identified in the human skin.

We also performed real-time quantitative reverse-transcription–PCR to quantify PENK mRNA in selected skin samples and found the highest expression of PENK mRNA in fibroblasts, lower levels in keratinocytes similar to in those in

Figure 1. Expression of proenkephalin (PENK) in human skin. (**a**) RT-PCR detection of PENK mRNA. HaCaT keratinocytes (1), melanocytes (2), adult epidermal keratinocytes (3), dermal fibroblasts (4), and WM98 (5), WM164 (6), and WM1341 (7) melanomas; markers (M). (**b**) Western blot detection of PENK (left, arrow), and negative control incubated with nonimmune serum (right). The dividing line in the left panel separates the HaCaT sample from molecular markers, which, in the right panel, shows the normal skin cells from melanomas. (**c**) PENK immunoreactivity in HaCaT keratinocytes (left panel), neonatal epidermal melanocytes (middle panel), and negative control (HaCaT keratinocytes) (right panel). Bar = 50 μm. (**d**, **e**) Time-of-flight liquid chromatography mass spectrometry identification of Leu-enkephalin (**d**) and Met-enkephalin (**e**) in skin (sample). Peptide standard (STD). Left: retention times; right: *m/z* of peptides with C_{13} isotopes. (**f**, **g**) Relative expression of PENK mRNA in comparison with melanoma (=1) (**f**) and Leu-enkephalin (**g**) was measured using Taqman technology and ELISA, respectively. Data are means ± SEM (n = 4–8). FIB, dermal fibroblasts; KER, epidermal keratinocytes.

skin biopsy specimens, and very low levels, comparatively, in melanoma cells (Figure 1f). Concerning detection of Leu-enkephalin by ELISA in the culture medium above skin cells, its levels were negligible in melanoma cells and melanocytes, whereas high Leu-enkephalin levels were detected in the medium above keratinocytes and fibroblasts, with an



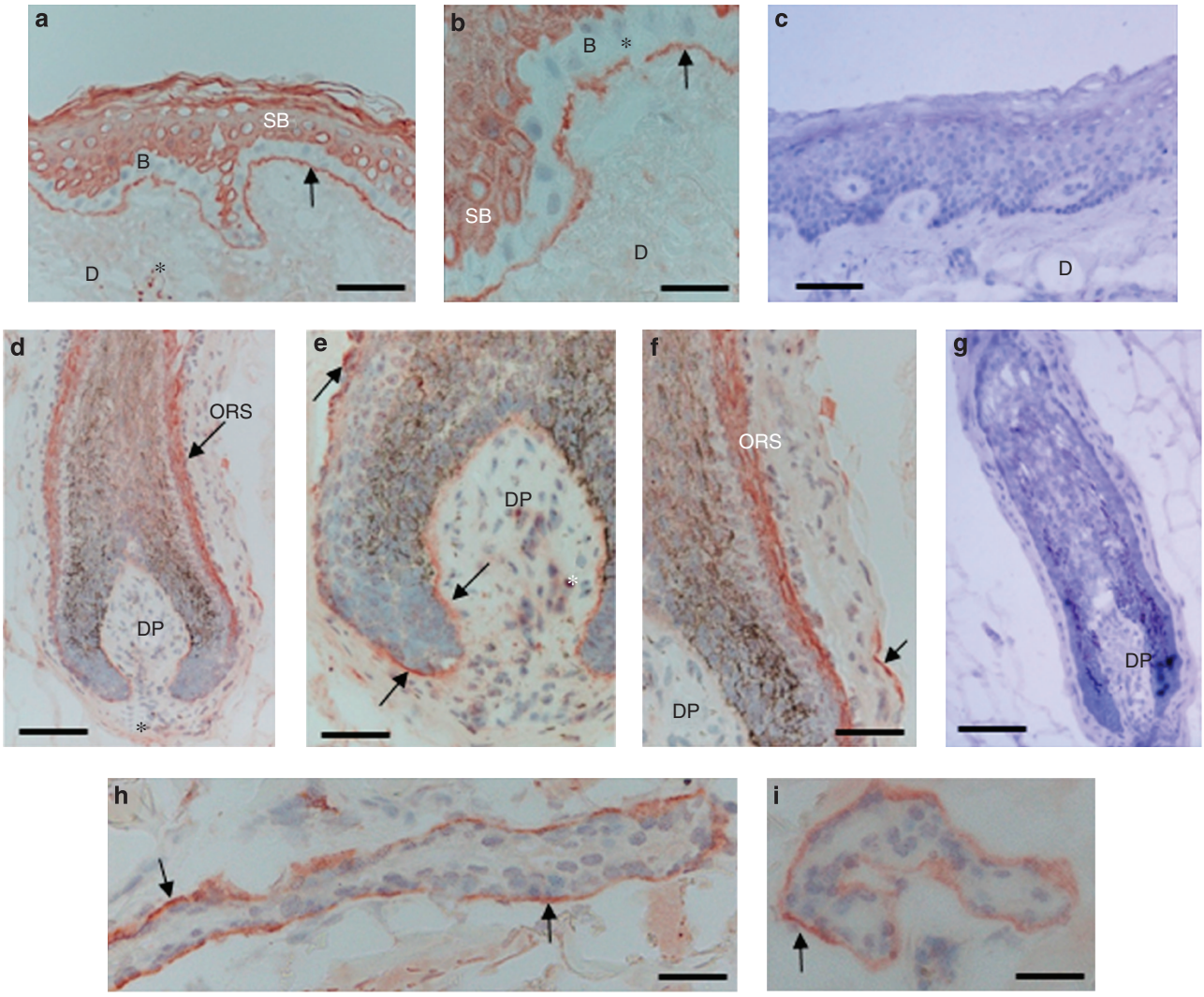


Figure 2. Localization of proenkephalin (PENK)-derived Met- and Leu-enkephalin peptides in normal human scalp skin. (**a**, **b**) PENK expression in suprabasal keratinocytes (SB), base membrane zone (arrow), and dermis (D, asterisk) and its absence in basal keratinocytes (B, asterisk). (**c**) Negative control for PENK (peptide block) in the skin, i.e., pre-absorption of primary antibody with 1:1 mix of Leu/Met-enkephalin (see Materials and Methods). (**d**–**f**) PENK peptides in the outer root sheath (ORS) (arrow, **d**, **e**) of anagen VI hair follicle (HF), basal lamina (arrow, **e**), fibroblasts of the dermal sheath (asterisk, **d**, arrow, **f**), and follicular dermal papilla (DP, **e**). (**g**) Negative control for PENK (peptide block) in HF. (**h**, **i**) PENK peptides in myoepithelial cells (arrows) of the eccrine glands. (**a**) Bar = 60 μm; (**b**) bar = 20 μm; (**c**) bar = 60 μm; (**d**) bar = 100 μm; (**e**, **f**) bar = 50 μm; (**g**) bar = 100 μm; (**h**, **i**) bar = 20 μm.

approximately twofold difference between the two cell types (Figure 1g).

PENK protein is expressed in a tissue-specific pattern in human skin

PENK-derived peptide expression (as assessed by immunohistochemistry in normal human scalp frozen sections using antibodies that recognize both Met- and Leu-enkephalins) was seen predominantly in epidermal and follicular keratinocytes (Figure 2a, b, d–f, h, i), being absent in appropriate controls incubated with antibodies preabsorbed with enkephalin peptides (Figure 2c and g). In the epidermis, PENK immunoreactivity was markedly restricted to differentiating keratinocytes of the stratum spinosum and granulosum, whereas the proliferating basal keratinocytes were negative (Figure 2a and b). Interestingly, an intense expression of Met/Leu-enkephalin was detected below the basal membrane zone and a faint stain was seen in the dermis (asterisk, D), indicating potential local release of the enkephalin peptides (Figure 2a and b). In the hair follicle, the cytoplasmic stain was seen mainly in keratinocytes of the outer root sheath and basal lamina of the anagen hair follicle (Figure 2f and g). Antigen expression was also seen in fibroblasts of both the perifollicular dermal sheath and the follicular papilla (FP) (Figure 2e and f). Again, a very faint stain was seen in the extracellular region (Figure 2e and f), indicating potential local release of the peptides. In addition, immunoreactivity was also detected in myoepithelial cells of the eccrine glands (Figure 2h and i).

The above pattern (predominant cytoplasmic stain in epidermal and follicular keratinocytes) was conserved in

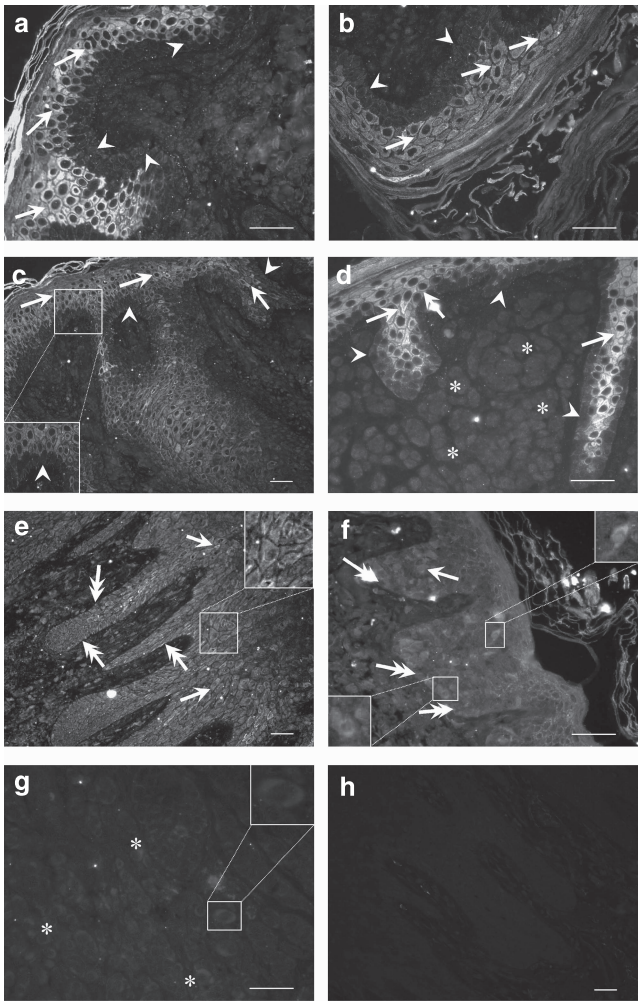


Figure 3. Localization of proenkephalin (PENK) antigens in pathological human skin. Immunolocalization of PENK in granuloma annulare (**a**), lichenoid keratosis (**b**), dermatofibroma (**c**), melanocytic nevus (**d**), psoriasis (**e**), melanoma *in situ* (**f**), and nodular malignant melanoma (**g**). Negative control represents skin with psoriasis (see **e**) in which primary antibodies were omitted (**h**). Arrows indicate PENK staining in the suprabasal layer, arrowheads indicate negative PENK staining in the basal layer, double arrowheads show basal PENK staining; asterisks indicate weak stain in the intradermal nevocytes and melanoma cells. Skin sections were stained with anti-PENK antibody, followed by secondary antibody linked to fluorescein. Insets show high magnification of cells indicated by squares. Bar = 50 μm.

formalin-fixed paraffin-embedded samples of diseased skin (Figure 3; Table 1), but with some differences depending on the diagnostic features. For example, strong to moderate and exclusively cytoplasmic stain was seen in keratinocytes of the stratum spinosum and granulosum (arrow), with very low or absent immunoreactivity in the stratum basale (arrowheads) of reviewed cases of seborrheic keratosis, lichenoid keratosis, melanocytic nevi, and granuloma annulare (Figure 3a–d, Table 1). Variable intradermal stain was present only in occasional inflammatory cells. By contrast, both basal and suprabasal keratinocytes showed increased cytoplasmic (arrows; double arrowheads for stratum basale) expression of PENK in psoriatic skin; no epidermal gradient stain was seen in the normal scalp or in the lesions listed above (Table 1, Figure 3e). Very low cytoplasmic expression was detected in nevocytes of melanocytic nevi (asterisks in Figure 3d) and in melanomas (*in situ* and nodular, asterisks) (Table 1, Figure 3f and g). Interestingly, epidermal keratinocytes in melanocytic nevus exhibited strong suprabasal expression, which was in contrast to melanomas, in which epidermal keratinocytes of all layers of the epidermis showed weaker staining. Neither intradermal spindle cells of the dermatofibroma nor histiocytic cells of the granuloma annulare expressed detectable PENK (Table 1). Interestingly, a faint stain was present in the extracellular matrix of the dermis of pathological skin in some cases.

PENK expression is regulated in melanocytes and keratinocytes

The observed increased expression of PENK in keratinocytes of psoriatic skin, together with its inflammatory basis, suggested that PENK expression may be regulated by inflammatory stimuli. Thus, we sought to determine whether PENK expression in keratinocytes can be regulated by TLR agonists such as lipopolysaccharide (TLR4) and PAM3CSK4 (TLR2). Lipopolysaccharide induced *PENK* gene expression in a time- and dose-dependent manner (Figure 4a–c), with a maximal effect (3.45 ± 0.78-fold) at 1 hour of incubation and at a concentration of 1 μg ml^{-1} (Figure 4a and b). Moreover, flow-cytometric analysis of keratinocytes stimulated with lipopolysaccharide also showed significantly increased expression of intracellular PENK protein (Figure 4c). This experiment was repeated three times with similar results. We also tested the effect of the TLR2 agonist PAM3CSK4 on normal human epidermal keratinocytes and found that, at a concentration of 1 μg ml^{-1}, it also stimulated PENK mRNA expression, with a maximal effect (2.91 ± 0.58-fold) at 1 hour of incubation (Figure 4d).

Another environmental stressor of the skin, UVB, caused a time- and dose-dependent stimulation of PENK mRNA expression in both epidermal keratinocytes and melanocytes (Figure 5a–d). In melanocytes this effect was maximal (2.64 ± 1.02-fold) at 6 hours and at a dose of 100 mJ cm^{-2} (Figure 5c and d), whereas in epidermal keratinocytes the effect was maximal (8.18 ± 0.45-fold) after 1 hour and at a UVB dose of 25 mJ cm^{-2} (Figure 5a and b). To obtain additional proof for the concept that UVB can stimulate *PENK* gene transcription, we transfected AtT-20 cells with a PENK-luc construct and demonstrated that UVB stimulated the promoter activity in a dose-dependent manner (Figure 5f). The use of AtT-20 cells was dictated by good transfection efficiency in these cells, which contrasted with the poor transfectability of normal epidermal cells.

DISCUSSION

The skin plays a significant part in the protection of both local and central homeostasis. There is increasing support for a contribution by the cutaneous neuroendocrine system, including the opiate system (Slominski and Wortsman, 2000; Slominski *et al.*, 2000c; Bigliardi-Qi *et al.*, 2004; Feldman *et al.*, 2004; Kauser *et al.*, 2004). In this study, we demonstrated a differential and cell type–dependent

Table 1. Results of staining with enkephalin antibody

Sample	Skin disease	PENK immunostaining
1	Granuloma annulare	Homogenously stained (+++) epidermal suprabasal keratinocytes; only occasional inflammatory cells are positive in the dermis
2	Cellular hemorrhagic cellular dermatofibroma	Heterogeneous (+), (++), or (+++) stain of epidermal keratinocytes; occasional (+) single spindle cells in the dermis
3	Squamous cell carcinoma	Heterogeneous (+) stain of malignant keratinocytes; (++) of hair follicle keratinocytes
4	Lichenoid keratosis	Homogenous (++) to (+++) stain in epidermal suprabasal keratinocytes, (+) in follicular keratinocytes
5	Seborrheic keratosis	Homogenous (++) stain in suprabasal epidermal and follicular keratinocytes
6	Psoriasis	Irregular (+++) stain in basal and (++) stain in spinous layers of keratinocytes; (+) stain in sparse inflammatory cells in the dermis
7	Psoriasis	(++) Stain in basal and spinous layers keratinocytes; (+) stain in sparse inflammatory cells in dermis
8	Intradermal melanocytic nevus	(−) Nevus cells
9	Intradermal melanocytic nevus, neurotized	Heterogeneous, predominantly (−) to (+) or occasionally (++) stain in nevus cells
10	Intradermal melanocytic nevus	(+) Nevus cells
11	Melanocytic nevus, compound lentiginous type	Heterogeneous (−) or (+) stain in nevus cells, (+++) stain in suprabasal keratinocytes
12	Intradermal melanocytic nevus	(+) Stain in nevus cells, (++) stain in epidermal keratinocytes
13	Invasive melanoma, VGP	Heterogeneous, predominantly (−) with occasional (−/+) stain in melanoma cells, (+) stain in keratinocytes in stratum spinosum and granulare
14	Melanoma *in situ*	(−) Stain in melanoma cells, heterogeneous (+) stain in suprabasal epidermal keratinocytes
15	Invasive melanoma, VGP	Heterogeneous, (−) to (+) stain in melanoma cells, (+) in epidermal keratinocytes
16	Nodular malignant melanoma	(−/+) Stain in melanoma cells, (++) in occasional inflammatory cells

Abbreviation: VGP, vertical growth phase.
In all cases, staining was granular and cytoplasmic.
Immunostain: (−) negative, (+) weakly positive, (++) positive, (+++) strongly positive.

expression of PENK mRNA, protein, and Leu- and Met-enkephalin peptides in human skin and in cultured human normal and malignant skin cells. Using skin biopsies, we localized *in situ* Leu- and Met-enkephalin antigens to epidermal and outer root sheath follicular keratinocytes, myoepithelial cells of eccrine glands, and some fibroblasts of the follicular dermal sheath and FP of normal human skin. This spatial expression pattern and intensity were considerably altered by skin pathology. Furthermore, physical (UVB) and biological (lipopolysaccharide and TLR2 agonist PAM3CSK4) stressors demonstrated cell type–specific time- and dose-dependent stimulation of PENK expression.

So far, the best documentation for local cutaneous production of endogenous peptide with opioid activity has been provided for β-endorphin, which results from regulated expression and processing of the proopiomelanocortin precursor (Slominski *et al.*, 1992, 2000c; Furkert *et al.*, 1997; Slominski, 1998; Mazurkiewicz *et al.*, 2000; Kauser *et al.*, 2004). The present study clearly demonstrates that human skin and cultured skin cells (normal and pathological) have the capability to transcribe and translate the *PENK* gene, with its subsequent processing to Leu- and Met-enkephalins, in a cell type–dependent manner, as demonstrated by RT-PCR, quantitative real-time PCR, western blotting, immunocytochemistry, time-of-flight/liquid chromatography mass spectrometry, and ELISA (Figures 1–3). The highest expression of PENK mRNA was found in cultured dermal fibroblasts in comparison with epidermal keratinocytes and normal skin. This would be consistent with predominant expression of *PENK* gene in cells of mesenchymal origin during embryogenesis or its expression in mesenchymal cells of embryonal skin, both of which have undifferentiated potential (Polakiewicz and Rosen, 1990; Polakiewicz *et al.*, 1992). However, the difference in Leu-enkephalin production/secretion between cultured fibroblasts and keratinocytes was much lower—e.g., a difference of only twofold (Figure 1g)—indicating similar or only moderately different potency to produce and release peptides.

In situ analysis showed much stronger expression of PENK antigen in epidermal (suprabasal) and follicular keratinocytes (outer root sheath) in comparison with dermal fibroblasts; the latter was restricted to cells of the perifollicular dermal sheath and the FP. This pattern indicates *in vivo* compartmentalization of Met- and Leu-enkephalin antigens, with strong expression in differentiating epidermal (no expression in basal) and follicular keratinocytes (outer root sheath) and selective expression in cells of mesenchymal origin, including specialized fibroblasts of the perifollicular dermal sheath and FP and myoepithelial cells of the eccrine glands. Faint, extracellular accumulation of Met- and Leu-enkephalin

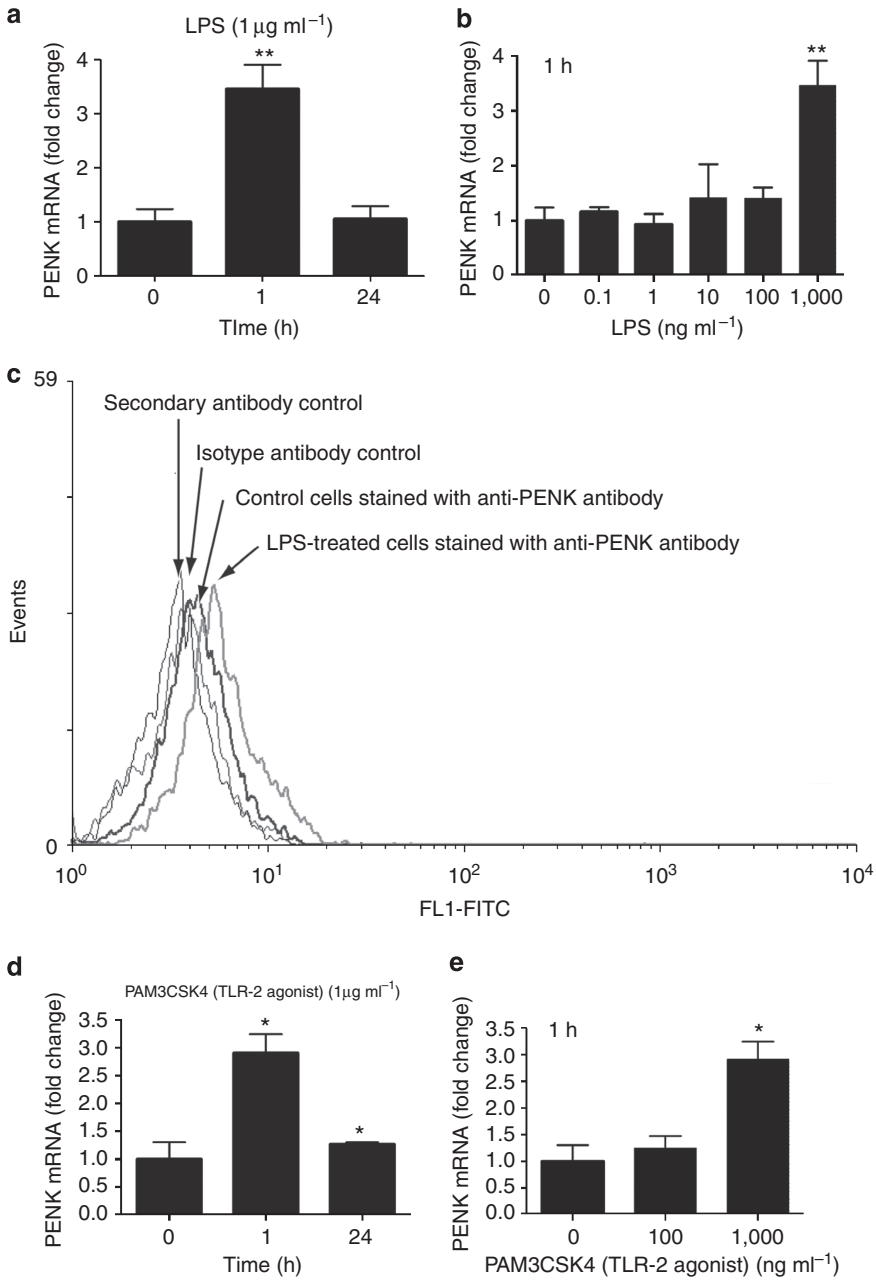


Figure 4. Lipopolysaccharide (LPS) stimulates expression of proenkephalin (PENK) in normal human epidermal keratinocytes. LPS-induced time (at 1 μg ml^{-1}) (**a**) and dose (at 1 hour) (**b**) responses of PENK mRNA expression were measured using Taqman technology. Data are means ± SEM ($n = 3$). $^{**}P < 0.005$ versus control. Flow cytometry detection of LPS- (1 μg ml^{-1}) stimulated expression of intracellular PENK protein measured (**c**). The results are representative of three independent experiments. PAM3CSK4-induced time (at 1 μg ml^{-1}) (**d**) and dose (at 1 hour) (**e**) responses of PENK mRNA. Data are means ± SEM ($n = 3$). $^{*}P < 0.05$ versus control. TLR2, Toll-like receptor 2.

antigens in the papillary dermis and perifollicular location and in FP (see the asterisks in Figure 2b and d) also suggests secretion of final peptides by fibroblasts to the extracellular environment.

Epidermal keratinocytes are responsible for building a dynamic barrier protecting the skin and the body from environmental insults, including physical and biological factors (Elias, 2005), whereas melanocytes protect against solar damage via production of melanin pigment (Slominski *et al.*, 2004). Therefore, we used both cell types to measure the effects of UVR on the expression of the *PENK* gene. The UVB stimulation of proenkephalin mRNA expression in melanocytes and keratinocytes in a time- and dose-dependent manner shows that solar radiation can act as an inducer of *PENK* gene expression (Figure 5). This has been substantiated by the demonstration that UVR also stimulates PENK promoter activity in a dose-dependent manner (Figure 5f). These findings are in agreement with an earlier study

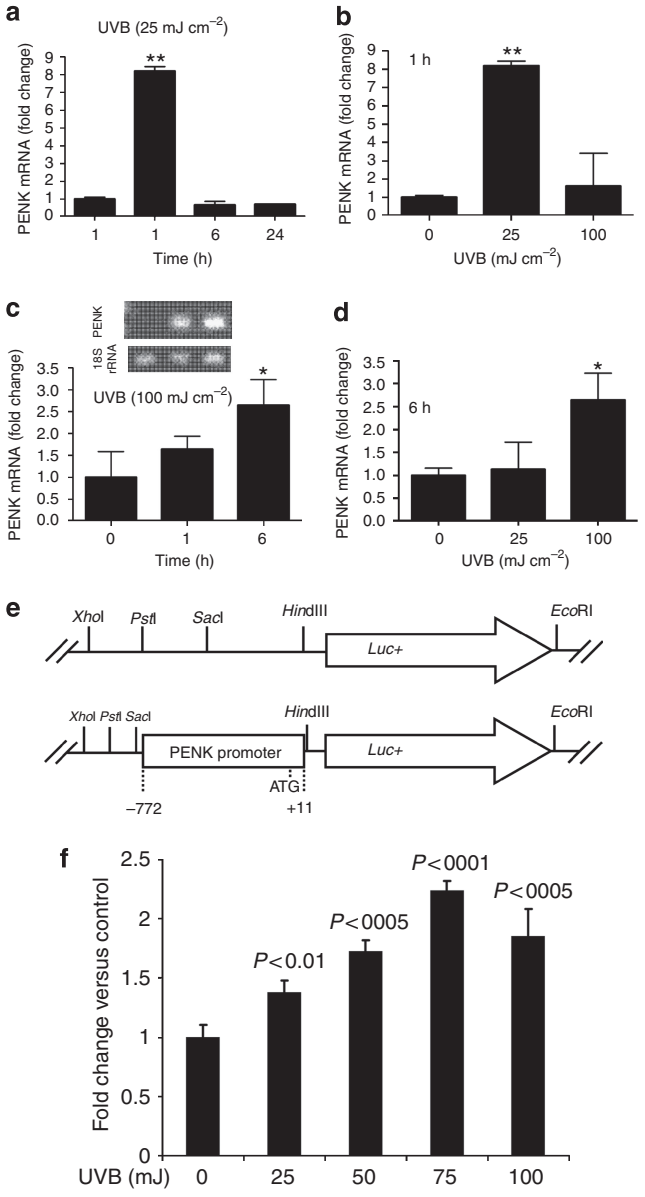


Figure 5. UVB stimulates expression of proenkephalin (PENK) mRNA in normal human epidermal keratinocytes and melanocytes. *Keratinocytes*: time (at 25 mJ cm^{-2}) (**a**) and dose (at 1 hour) (**b**) responses of PENK mRNA expression were measured using Taqman technology. *Melanocytes*: time (at 100 mJ cm^{-2}) (**c**) and dose (at 6 hours) (**d**) responses of PENK mRNA levels were measured using Sybr Green technology. Data are means ± SEM ($n = 3$). *$P < 0.05$ versus control. (**e**) PENK-luc reporter plasmid (lower panel) containing PENK promoter sequence (−772 to +11 bp) and pLINTER cloning vector coding promoterless firefly luciferase (upper panel). (**f**) UVB stimulates the PENK promoter in AtT-20 cells. After transfection with pPENK-luc, cells were treated with UVB at 25, 50, 75, and 100 mJ cm^{-2}. Results represent a fold change in luciferase activity in comparison with control.

showing that UVR wavelengths of solar radiation stimulate Met-enkephalin deposition in the human epidermis as well as increase the serum concentration of the peptide (Nissen *et al.*, 1998). In this context, UVR-induced PENK expression may represent part of the complex local neuropeptide responses to noxious physical stimuli, which already involve UVR stimulation of the proopiomelanocortin and corticotropin-releasing factor signaling systems (Slominski and Wortsman, 2000; Slominski *et al.*, 2000c, 2006). This is particularly relevant for epidermal keratinocytes, as these cells express opioid receptors and respond to enkephalins (Zagon *et al.*, 1996; Nissen and Kragballe, 1997). In the analogous, and already well-documented, cutaneous proopiomelanocortin system, the processing of a large precursor protein generates the neuropeptides α-MSH, adrenocorticotropin, and β-endorphin, with differential activity aimed at protection against solar radiation (Slominski *et al.*, 2004, 2008b). Also, activation of skin nerve endings by β-endorphin may directly transmit signals to the brain via local nociceptive or opioid effects induced by solar radiation (Slominski and Wortsman, 2000; Slominski *et al.*, 2000c). In the above context, enkephalins could represent a part of additional UVB-induced skin responses that would involve activation of opioid receptors on the cutaneous sensory nerves, a subject that deserves future study.

We also have found that lipopolysaccharide and PAM3CSK4 (TLR4 and TLR2 agonists, respectively) stimulate *PENK* gene and protein expression (Figure 4), which is consistent with stimulation of *PENK* expression by lipopolysaccharide in immune cells and adrenal glands, antimicrobial functions of PENK-derived peptides (Goumon *et al.*, 1998; Metz-Boutigue *et al.*, 2000, 2003a, b; Tasiemski *et al.*, 2000a; Kieffer *et al.*, 2003), and their immunomodulatory functions (Kamphuis *et al.*, 1997, 1998; Stefano *et al.*, 2005). Therefore, we propose that activation of PENK in keratinocytes by TLR2 and TLR4 agonists represents a part of the epidermal innate immune response (Metz-Boutigue *et al.*, 2003b) to protect against biological stressors and maintain the integrity of local homeostasis (Slominski *et al.*, 2008b). The role of enkephalins in the innate immune response is highly conserved, being found even in invertebrates, where lipopolysaccharide also stimulates enkephalins (Tasiemski *et al.*, 2000b). With respect to human skin, we observed a striking gradient of Met- and Leu-enkephalin expression in the epidermis, with the highest expression being in their outer differentiated keratinocyte layers and along the basement membrane separating the nonvascular epidermis from the highly vascularized dermis. In our opinion, this intriguing architectural pattern may reflect involvement of PENK-derived peptides in building an antimicrobial barrier to protect deeper skin layers from microbial invasion and further colonization of the dermal compartment, where access to the systemic circulation is readily available. In addition, this characteristic epidermal gradient of enkephalin expression, including accumulation in the stratum corneum, is consistent not only with a role of PENK-derived peptides in building innate immunity but also with their direct antibacterial and antifungal activity that has been conserved in various organisms for more than 500 million years of evolution (Tasiemski *et al.*, 2000b; Metz-Boutigue *et al.*, 2003b). Additionally, this barrier can function to alleviate systemic effects such as responses to pain via the nociceptive actions of enkephalin peptides (Wilson *et al.*, 1999; Yeomans *et al.*, 2004), as sensory nerve endings can penetrate into the epidermis and there is

significant innervation along the basement membrane zone (Slominski and Wortsman, 2000; Arck *et al.*, 2006).

Concerning skin pathology, it is remarkable that PENK expression is increased, with a disappearance of the epidermal gradient, in psoriasis; e.g., it is also expressed in basal proliferating keratinocytes. This is consistent with a previous report on increased expression of enkephalin antigens in this disease (Nissen *et al.*, 1999). This diffuse pattern also appears to be consistent with the opinion that a pathologically hyperactive epidermal/immune response to bacterial superantigens is an important mechanism in the etiology of psoriasis (Travers *et al.*, 2001). In contrast to a remarkable expression of PENK in inflammatory skin disorders (Table 1), it is significantly decreased in melanocytic nevi, with further decrease or loss in advanced melanomas. Interestingly, PENK expression decreases in keratinocytes of melanoma-involved skin and loses its characteristic gradient. The significance of these findings awaits further investigation on a much larger number of skin biopsies with specific focus on defined pathology. However, based on this limited number of formalin-fixed, –paraffin-embedded sections, one can safely conclude that PENK expression is deregulated in pathological skin in a pattern dependent on the nature of pathology.

In conclusion, we document that human skin is a site of proenkephalin expression, showing cell-type and tissue-compartment specificity. This expression can be regulated by solar radiation or biological factors (TLR2 and TKR4 agonists) and deregulated by pathology. These findings indicate paracrine and autocrine modes of action for PENK-derived peptides, with a potential role in the formation of the antimicrobial and biological protective barrier and the regulation of local homeostasis in addition to being a contributor to locally generated nociceptive or opioid effects.

MATERIALS AND METHODS

Human skin samples

Normal adult skin was obtained after elective surgery and neonatal skin after circumcision. Pathological skin samples for immunocytochemical and immunofluorescence analyses, obtained from paraffin blocks containing excision specimens, were retrieved from the surgical pathology files at the University of Tennessee Health Science Center (see Supplementary Table S1 online). The diagnoses were made by a board-certified dermatopathologist. The studies were approved by the local institutional review board. Other details are available in the Supplementary material online.

Leu-enkephalin ELISA

Leu-enkephalin levels in supernatants from stimulated melanocytes, keratinocytes, fibroblasts, and melanoma cells were quantitated using Leucine-Enkephalin EIA kit EK-024-21 (Phoenix Pharmaceuticals, Belmont, CA) following the manufacturer's instructions.

Statistical analyses

Data are presented as means ± SEM ($n = 3$–4) and were analyzed using the Student *t*-test (for two groups) or one-way analysis of variance with appropriate *post-hoc* tests (for more than two groups) using Prism 4.00 (GraphPad Software, San Diego, CA). Statistically significant differences between control and treated groups are denoted by asterisks: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by the National Science Foundation (grant IOS-0918934) and the National Institutes of Health (grant AR052190).
This study was conducted in Memphis, Tennessee, USA, and Bradford, UK.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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