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Expression of μ-Opiate Receptor in Human Epidermis and Keratinocytes

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There is increasing evidence that neurotransmitters play a crucial role in skin physiology and pathology. The expression and production of proopiomelanocortin molecules such as β-endorphin in human epidermis suggest that an opiate receptor is present in keratinocytes. In this paper we show that human epidermal keratinocytes express a μ-opiate receptor on both the mRNA level and the protein level. Performing polymerase chain reaction with cDNA libraries from human epidermal keratinocytes gave the polymerase chain reaction products of the expected length, which were confirmed as μ-opiate receptors by Southern blot analysis. Using *in situ* hybridization techniques with a specific probe for μ-opiate receptors we detected the receptor in human epidermis. There was a cytoplasmic expression in all layers of the epidermis, which was more distinct in the suprabasal layers. Immunohistochemistry using the μ-opiate receptor-specific antibody indicates that epidermis expresses protein as well, and that the protein level is more elevated in the basal layer. The correlation between the locations of both mRNA and protein expression in skin indicates that the μ-opiate receptor has not only been transcribed but also has a specific function. To prove a function of the receptor we performed a functional assay using skin organ cultures from human skin transplants. After 48 h incubation with Naloxone or β-endorphin the expression of the μ-opiate receptor in epidermis was significantly downregulated compared with the control. These results show that a functional receptor indeed exists in human epidermis. ***Key words: human keratinocytes/μ-opiate receptor.*** ***J Invest Dermatol 111:297–301, 1998***

Recent studies suggest that the nervous system and the skin interact directly through neuropeptides (Ansel *et al*, 1996). Neuropeptides are able to interact with multiple types of cells in the skin to mediate actions important in skin inflammation. Thus there is possibly not only a neuroimmunologic axis but also a neuroimmune–dermatologic axis. This system would operate multidirectionally between the skin, the immune system, and the nervous system. Such interaction may be expressed as stress-dependent neurogenic inflammation, a frequently mentioned factor in common skin diseases like psoriasis (Glinski *et al*, 1994) and atopic dermatitis (Glinski *et al*, 1995). The clinical observation of aggravation of these skin diseases by psychic stress and anxiety has been associated with anomalous neuropeptide regulation. This emphasizes the role of the skin itself as a source and target of classical stress factors like adrenocorticotropin and endorphins. Of special interest is the role of epidermal keratinocytes in the neuroimmune–dermatologic system. Human epidermal keratinocytes express different neuropeptides, including proopiomelanocortin (POMC) (Schauer *et al*, 1994). One POMC derivative is β-endorphin, which binds with high affinity to μ-opiate receptors. There are reports of elevation of β-endorphin concentration in skin diseases like psoriasis (Glinski *et al*, 1994) or atopic dermatitis (Glinski *et al*, 1995). We have therefore investigated if the major cell type in human skin, keratinocytes, express an opioid receptor for this ligand. We found that human epidermal keratinocytes indeed express a μ-opiate receptor at both the mRNA level and the protein level.

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Abbreviation: POMC, proopiomelanocortin.

MATERIALS AND METHODS

Polymerase chain reaction (PCR) and Southern blot analysis Two pairs of μ-opiate receptor-specific primers were designed. The first pair is TM1a: CATCATGGCCCTCTACTCTATC (position 418–440 of HSMOR1X) and E2: GGACCCCTGCCTGTATTTTGT (position 851–830 of HSMOR1X); the second pair is MSt1f: GTCAGTACCATGGACAGCAG (position 203–223 of HSMOR1X) and Mst1r: CTGTTAGGGCAACGGAGCAG (position 1418–1398 of HSMOR1X). The PCR reaction was conducted on a human keratinocyte cDNA library (Clontech, Palo Alto, CA). The reaction was carried out under the following conditions: 94°C 4′, 1 cycle; 94°C 1′, 37°C 1′, 72°C 1′, 10 cycles; 94°C 1′, 55°C 1′, 72°C 1′, 35 cycles. The PCR products were analyzed on a 1.0% agarose gel and were blotted onto membranes. The blots were hybridized with digoxigenin-labeled μ-opiate receptor-specific probe and detected using NBT/BCIP substrate color reaction. The southern hybridization probe was made by generating a 228 nt long PCR fragment (position 457–685 of RNMORA) from the rat MOR-1 clone, which is 93% identical with human probe. A similar PCR reaction and Southern blot were conducted on cDNA made from mRNA isolated from cultured human keratinocytes using the guanidinium thiocyanate method (Chirgwin *et al*, 1979) (Pharmacia Biotech, Uppsala, Sweden). Human epidermal keratinocytes were obtained from normal human foreskins. The keratinocytes were cultured according to Grando *et al* (1993). After one passage the keratinocytes were used to extract the mRNA. Additionally mRNA was isolated from rat brain using an extraction kit based on the guanidinium thiocyanate method (Pharmacia Biotech, Uppsala, Sweden).

***In situ* hybridization** The digoxigenin-labeled riboprobes were generated from the rat MOR-1 clone (a gift from Prof. H. Loh, Department of Pharmacology, University of Minnesota, Minneapolis, MN). The rat MOR-1 was subcloned in pBluescript II SK (+/–) phagemid and was linearized and transcribed with T3 and T7 RNA polymerase using digoxigenin-labeled UTP (Boehringer, Mannheim, Germany). The rat MOR-1 was subcloned in

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pBluescript II SK (+/–) phagemid and was linearized and transcribed with T3 and T7 RNA polymerase using digoxigenin-labeled UTP (Boehringer).

Normal human skin was taken from the edge of an excisional skin biopsy during surgical operations for nevi. The biopsies were all taken from healthy individuals without any skin diseases. Formalin-fixed, paraffin-embedded human skin biopsies were sectioned at 5 μm. All sections were treated with proteinase K (10 mg per ml) and were washed in 0.1 M triethanolamine buffer containing 0.25% acetic acid. Sections were covered with 25 ml of hybridization buffer containing anti-sense or sense RNA probe. They were incubated at 42°C for 18 h in a humidified chamber. After hybridization, the slides were washed under stringent conditions (2×10 min, 1×sodium citrate/chloride buffer, 0.1% sodium dodecyl sulfate, 2×10 min, 55°C, 0.2 × sodium citrate/chloride buffer, 0.1% sodium dodecyl sulfate). They were then incubated with the anti-DIG-alkaline phosphatase (1:3000, Boehringer) for 1 h at room temperature, followed by NBT/BCIP substrate for up to 1 h.

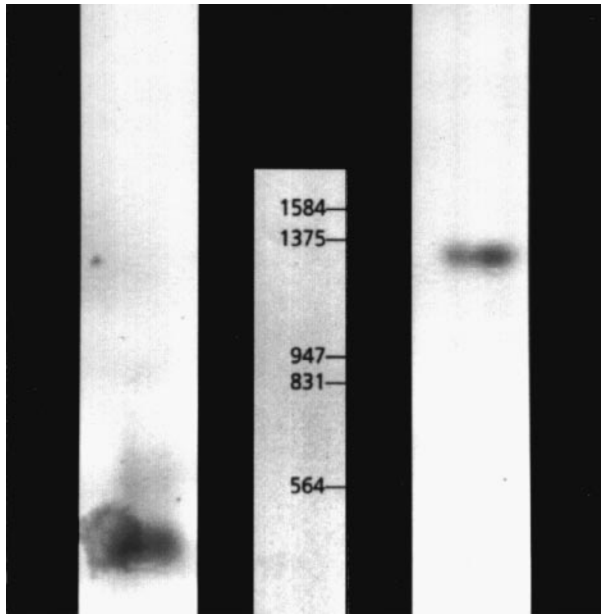

Figure 1. Human μ-opiate receptor is present in human keratinocyte cDNA library. PCR was conducted by using two different μ-opiate receptor specific primer pairs. The PCR product of the left-hand lane derived from the first primer pair, i.e., TM1a (position 418–440 of HSMOR1X) and E2 (position 851–830 of HSMOR1X); the PCR product of the right-hand lane derived from the second primer pair, i.e., MSt1f (position 203–223 of HSMOR1X) and Mst1r (position 1418–1398 of HSMOR1X). Southern blot analysis using the μ-opiate receptor specific probe confirmed the identity of the PCR fragments. The cDNA from both the human keratinocyte cDNA library and the cultured epidermal keratinocytes gave PCR fragments with the expected sizes at 433 nt and 1215 nt.

Immunohistochemistry The skin biopsies taken from the edge of excisional skin were embedded in Tissue-Tek (Miles, Elkhart, IN) and immediately snap frozen in liquid nitrogen. The frozen biopsies were cut into 6 μm sections. The sections were first fixed in acetone and blocked with 5% normal goat serum, 2% fish gelatin in Tris buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 1 h at room temperature. The antibody used to stain the μ-opiate receptor was a commercially available, affinity purified, polyclonal rabbit anti-μ-opioid receptor antibody (Pharmingen, Hamburg, Germany). This antibody was generated from an immunogen of a 17 amino acid peptide with a sequence that is 100% identical in the human, mouse, and rat (information from Pharmingen). The primary antibody was incubated with sections for 1 h at 37°C whereas the controls were left in normal goat serum. After three washes with Tris buffer, the sections were all incubated with Cy2-conjugated goat anti-rabbit IgG (H + L) (Jackson Immuno Research Laboratories, West Grove, PA) at room temperature for 30 min. The secondary antibody was tested for minimal cross-reaction to human, mouse, and rat serum proteins. The sections were visualized using immunofluorescence microscopy (Nikon, Diaphot 300, Tokyo, Japan).

Opiate receptor expression in human skin organ culture Skin grafts of about 0.5 mm thickness were obtained by a dermatome from the upper leg of an individual. To standardize the tissue volume and thereby the cell mass of cultured skin fragments, only punches of 4 mm diameter were used. Several randomized 4 mm skin punches per experimental group were placed on Anocell 10 mm tissue culture inserts (Nunc, Life Technologies, Rockville, MD). These inserts were put in a Nunclon 24 well plate containing 2 ml Dulbecco's modified Eagle's medium (with Glutamax-I, Life Technologies) supplemented with 10% fetal bovine serum and 50 μg gentamicin (Life Technologies) per ml. After addition of 10 μM β-endorphin or 100 μM naltrexone separately, the organ cultures were incubated for 48 h at 37°C in 5% CO_2 and 100% humidity. As control we incubated skin organ cultures in the culture medium only. At the end of the incubation period the organ culture pieces were embedded in Tissue-Tek and immediately snap frozen in liquid nitrogen. This method of functional assays with skin organ cultures is adapted from the methods of Paus *et al* (1994). Finally we performed immunohistochemistry with the affinity purified, polyclonal rabbit anti-μ-opioid receptor antibody (Pharmingen) and used Cy2-conjugated goat anti-rabbit IgG (H + L) (Jackson Immuno Research Laboratories) as secondary antibody as described above.

RESULTS

mRNA of human μ-opiate receptor is expressed in keratinocytes We have conducted PCR using primers specific for human μ-opiate receptor on both a human keratinocyte cDNA library and cDNA made by reverse transcriptase-PCR from human foreskin keratinocytes in culture. The cDNA from the human keratinocyte

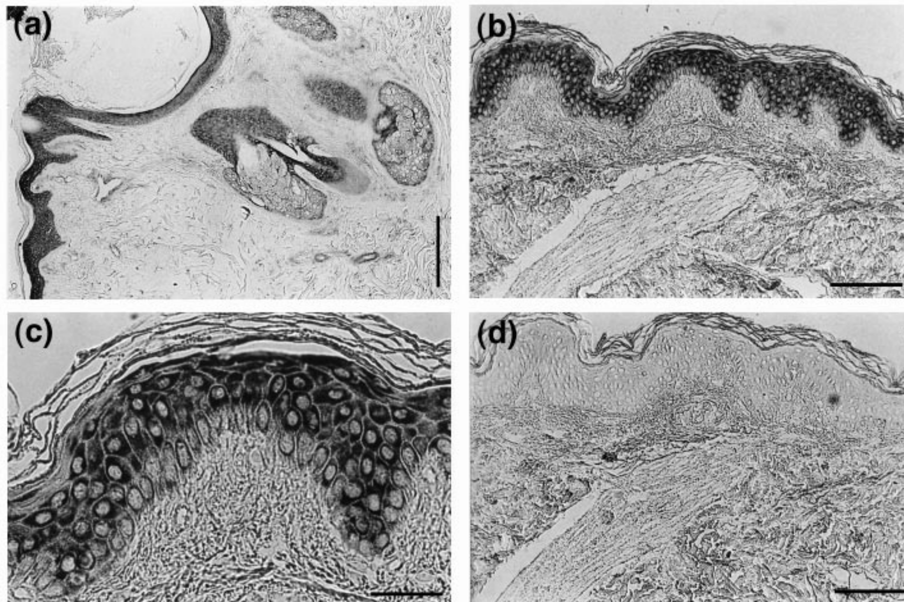

Figure 2. mRNA of μ-opiate receptor is expressed in normal human corporal skin by *in situ* hybridization with specific RNA probe. The paraffin-embedded skin biopsy was hybridized with digoxigenin-labeled anti-sense riboprobe made from rat μ-opiate receptor clone. The hybridization was conducted at 42°C overnight and detected with NBT/BCIP color substrate for alkaline phosphatase labeled anti-digoxigenin antibody. (*a*) Positive hybridization signal in the epidermis and epithelial cells of dermal adnex structures; (*b*, *c*) positive hybridization signal in cytoplasm of epithelial cells, mostly in the suprabasal layer; (*d*) negative control hybridized with sense RNA-probe had no signal on either dermis or epidermis. *Scale bars*, (*a*) 220 μm, (*b*, *d*) 110 μm, (*c*) 37 μm.

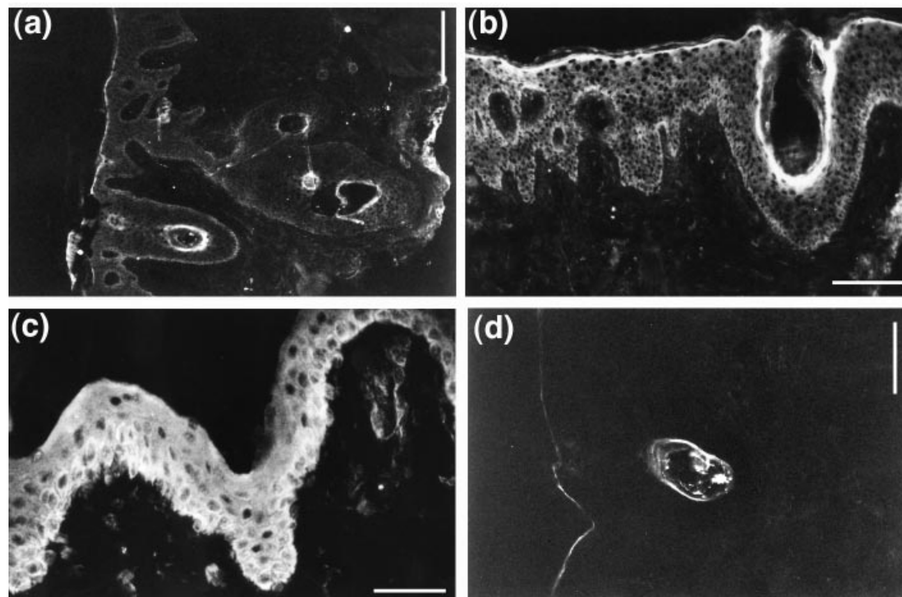

Figure 3. μ-opiate receptor protein is present in human epidermis. Cryosections of normal human skin were stained with μ-opiate receptor specific antibody. The normal human skin cryosection (6 μm) was first blocked with 5% normal goat serum and then incubated with μ-opiate receptor antibody at 37°C for 1 h. The secondary antibody labeled with Cy2 was used to detect the binding sites. (*a*) Positive staining of normal human scalp skin in interfollicular epidermis and dermal adnex structures, no specific staining of the cornified material of the stratum corneum and in the hair follicle. (*b*) Positive immunohistochemical signal in cytoplasm of epithelial cells from normal human scalp skin and (*c*) epithelial cells from normal human corporal skin. More intense immunostaining of the μ-opiate receptor on the basal layer. (*d*) Negative control biopsy was only exposed to the secondary fluorescence tagged antibody. There was no specific staining in the epidermis. *Scale bars*, (*a*) 220 μm, (*b*, *d*) 110 μm, (*c*) 37 μm.

cDNA library and cultured epidermal keratinocytes gave PCR fragments with the expected sizes at 1215 nt and 433 nt. The specificity of the PCR products was confirmed using Southern blot analysis with a μ-opiate receptor-specific probe (**Fig 1**). The 433 nt PCR fragment from cultured human foreskin keratinocyte cDNA was subcloned in TA-cloning vector and sequenced. The sequence was identical with the MOR-1 human μ-type opioid receptor. Amplification with primers specific for human δ-opiate receptors and κ-opiate receptors gave no PCR products. All these results suggest that there is only one type of opiate receptor in human keratinocytes, which is identical with the μ-opiate receptor (MOR-1) in human brain.

mRNA of μ-opiate receptor is expressed in human epidermis The expression of the μ-opiate receptor at the mRNA level *in vivo* was shown by *in situ* hybridization experiments using paraffin-embedded normal human corporal skin sections. To test the specificity of the RNA probe we first performed *in situ* hybridization with rat brain. Specific hybridization signal of nerve cells especially in the subcortical zone of the brain was observed (data not shown). The positive hybridization signal is found in the epidermis and also in epithelial cells of dermal adnex structures such as sweat gland ducts and hair follicles (**Fig 2*a***). The epithelial cells in the hair follicle are hybridized as strongly as in the epidermis. The central parts of the sebaceous glands with the disintegrating glandular cells are negative, but the epithelial cells of the lobes, especially at the periphery of the glands, are positive. The epithelial cells of the secretory sweat gland ducts are also hybridized positively. There was an expression of mRNA in all epidermal layers, except in the corneal layer. All hybridized signals are observed in the cytoplasmic region and there is no nuclear staining. Although the hybridized signal tends to be dispersed throughout the whole epidermis, it is more intense in the cells of the suprabasal region. The endothelial cells of blood vessels were negative (**Fig 2*b***, ***c***).

μ-opiate receptor protein is expressed in human epidermis Immunohistochemistry experiments were carried out to give evidence that the μ-opiate receptor is expressed on the protein level as well. To test the specificity of the μ-opiate receptor antibody, we first performed the immunostaining on human frontal cortex cryosections. We observed specific staining on nerve cells and axons (data not shown). Epidermis and dermal adnex structures expressed the μ-opiate receptor in immunohistochemistry. The cells of the external root sheath in hair follicle and the basal layer in the interfollicular epidermis both show intense staining (**Fig 3*a***); however, the staining of the hair cortex is nonspecific, as is also observed in stratum corneum. The nonspecific staining of horny material is often observed in immunohistochemistry of the skin. The central glandular cells of the sebaceous glands are negatively stained, but the peripheral epithelial cells of the glands stain positively. The secretory portions of sweat glands also have specific staining. These observations resemble the patterns found in *in situ* hybridization described above. The staining is generally dispersed in the epidermis and confined to the cytoplasmic region. In contrast to the mRNA hybridization signal, there is more immunostaining of the μ-opiate receptor on the basal layer (**Fig 3*b***, ***c***).

The expression of the μ-opiate receptor protein decreases by incubation with μ-specific ligands We studied the regulation of the μ-opiate receptor on protein level by exposing skin organ cultures taken from skin transplants to an agonist and antagonist of the μ-opiate receptor system. As control we incubated skin organ cultures in the culture medium only. We observed a strong expression of the receptor in the basal and suprabasal layers of the epidermis as expected (**Fig 4*a***). When 100 μM naltrexone, the antagonist, was added to the culture medium, the expression of the μ-opiate receptor in epidermis was almost totally blocked (**Fig 4*b***) after 48 h. After 48 h incubation of the skin organ culture with 10 μM β-endorphin the expression of the μ-opiate receptor was significantly downregulated (**Fig 4*c***). These results show that the receptor can be downregulated by ligands of the μ-opiate system and suggest that a functional active receptor indeed exists in human epidermis.

DISCUSSION

Several observations, especially about POMC peptides, suggest the presence of an opiate system in skin, although little data show that opioid receptors exist in skin (Zagon *et al*, 1996). Our results indicate that there is a μ-opiate receptor in skin both at the RNA and at the protein level. We also confirmed the existence of a μ-opiate receptor in human keratinocytes on the mRNA level. Reverse transcriptase-PCR, amplification of cDNA, and sequencing revealed a 433 nt DNA fragment identical to a μ-opiate receptor from human brain (MOR-1). Southern blot analysis confirmed both PCR products. *In situ* hybridization showed expression of the receptor in human epidermis, especially in the suprabasal layers. Some expression was also observed in the epithelial cells of adnex structures in dermis, especially sweat gland ducts and hair follicles. Interestingly, although immunohistochemistry using antibody against μ-opiate receptor resulted in strong staining in the epidermal layer, the staining was even more pronounced in the basal layer. This subtle difference between the expression of mRNA and the expression of protein could be attributed to the fact that the production of μ-opiate receptor mRNA is regulated and that mRNA is degraded faster than the protein. Within the basal layer, different cell types were hybridized to different extents. It will be

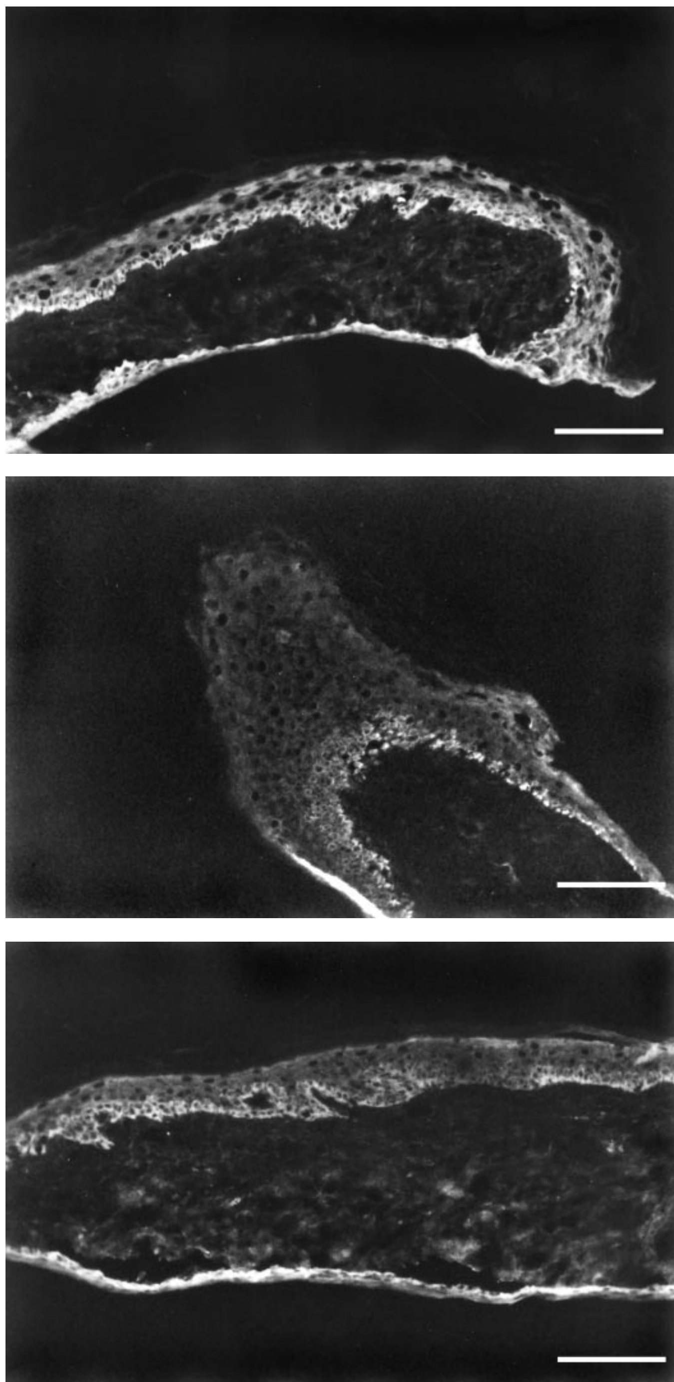

Figure 4. The expression of the μ-opiate receptor protein decreases after incubation with μ-specific ligands. The skin organ cultures were incubated with different agonist and antagonist of the μ-opiate receptor system in Dulbecco's modified Eagle's medium with supplements for 48 h. The cryosections from these organ cultures were stained by μ-opiate receptor specific antibody. (*a*) Control slide with skin organ culture incubated in modified Eagle's medium only. The receptor is expressed in basal and suprabasal layers of the epidermis. (*b*) Skin organ culture incubated with 100 μM naltrexone. The receptor in the epidermis is almost entirely downregulated. (*c*) Skin organ culture incubated with 10 μM β-endorphin. The receptor in the epidermis is significantly downregulated. The negative control biopsy for each incubation was only exposed to the secondary antibody with fluorescence label. There was no specific staining in the epidermis (data not shown). *Scale bars*, 110 μm.

interesting to investigate further whether the negative hybridizing cells in the basal layer region are melanocytes, Langerhans cells, or keratinocytes in a different mitotic stage.

We investigated additionally if the μ-opiate receptor is functionally active by exposing skin organ culture to an agonist and antagonist of the μ-opiate receptor system for 48 h. We observed a significant downregulation of the receptor with Naltrexone, the antagonist, and to a lesser extent with the agonist β-endorphin. It might be surprising that the agonist and the antagonist both downregulate the receptor; although several papers suggest that the antagonist of the opiate receptor system, like naltrexone, can behave as opioid agonist/antagonist (Krizanac-Bengez *et al*, 1995). Therefore some authors call the μ-opiate receptor antagonist an inverse opiate agonist (Cruz *et al*, 1996). Further studies have to reveal the influence of different concentrations of the agonist and antagonist on opiate receptor expression.

In recent years several investigators have studied neuropeptides and the expression of their receptors in human keratinocytes, including substance P (Eedy *et al*, 1991) and POMC (Slominski *et al*, 1993a, b). Various neurotransmitter receptors, such as those for substance P (Pincelli *et al*, 1993), adrenocorticotropin (Slominski *et al*, 1996), and α-MSH (Chakraborty and Pawelek, 1993) and nicotinic cholinergic receptors (Grando *et al*, 1995) are found in normal and pathologic human skin. Adrenocorticotropin and α-MSH are two of the biologically active molecules of POMC. POMC is also the precursor of β-endorphin, an opioid peptide. Our report is the first to show that a μ-opiate receptor, the receptor for the POMC product β-endorphin, exists in human skin. This is an important addition to the existing list of receptors of POMC molecules in human skin. It is also important evidence for the interaction between skin and the neural system.

The aggravation of skin diseases like atopic dermatitis and psoriasis by psychical stress is well known in clinical dermatology (Seville, 1989). It is known that skin and the immune system interact with each other closely; however, there is growing evidence that the neural system is also involved in this interaction. The μ-opiate receptor agonist β-endorphin is significantly elevated in sera of patients with severe atopic dermatitis (Glinski *et al*, 1995) and psoriasis (Glinski *et al*, 1994). The authors concluded in both papers that β-endorphin might be generated predominantly in lesioned skin. Recent research in other laboratories has indicated that human keratinocytes produce β-endorphin after stimulation by ultraviolet radiation, IL-1α, or phorbol ester (Wintzen *et al*, 1995, 1996). β-Endorphin is a specific ligand for μ-opiate receptor and its presence would account for the existence of μ-opiate receptor in human epidermis, which is exactly what we have discovered. Opioid peptides are probably involved in the elicitation of itching. Naloxone, an antagonist of the μ-opiate receptor system, has been found to relieve itching experienced by patients with chronic liver diseases (Bernstein and Swift, 1979) or uremia (Peer *et al*, 1996). Some of the itching is due to direct release of histamine by opioid peptides in skin. But not all of the itching can be relieved by antihistaminics, suggesting a direct action of opioid peptides modulating the perception of itching.

The expression of μ-opiate receptor in human epidermal skin and human keratinocytes suggests an important role of the opioids in the pathology of skin diseases, in wound healing and perception of itch. Further studies will open new prospects for skin physiology and pathology and bring about a better understanding of the mechanism of the interaction between the neural system and skin.

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