β-Endorphin Stimulates Cytokeratin 16 Expression and Downregulates μ-Opiate Receptor Expression in Human Epidermis

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It has been reported that opioid peptides modulate the differentiation of normal human keratinocytes and that μ-opiate receptors are expressed in human epidermis. The regulation of keratinocyte differentiation is particularly important in psoriasis, and one of the markers for hyperproliferative and differentiating skin diseases is cytokeratin 16. The finding that the endogenous μ-opiate receptor ligand β-endorphin is increased in serum of patients with psoriasis indicates that the μ-opiate system may play an important role in the pathophysiology of the skin. In this study, we addressed the question whether there is a link between μ-opiate receptor regulation and cytokeratin 16 expression in normal and psoriatic skin. Firstly, we demonstrate that β-endorphin concentrations between 16 and 1000 nM significantly downregulate μ-opiate receptor expression in epidermis of cultured human skin after 48 h. Secondly, we show that β-endorphin regulates cytokeratin 16 expression in the epidermis of skin organ cultures exposed to 41–125 nM β-endorphin for 48 h, leading to elevated cytokeratin 16 production. As expected, the expression of cytokeratin 16 was detected primarily in the suprabasal layer. The same pattern was observed in psoriatic lesional skin, i.e., μ-opiate receptor expression was significantly downregulated and cytokeratin 16 expression upregulated. These results suggest that the μ-opiate receptor system and its ligand β-endorphin are involved in the pathogenesis of psoriasis, especially in terms of differentiation. ***Key words: psoriasis/skin differentiation. J Invest Dermatol 114:527–532, 2000***

There is growing evidence that keratinocytes not only interact with cells of the immune system (Bos and De Rie, 1999) but also mediate signals between the skin and the nervous system through regulatory neuropeptides. Several authors have proposed an important pathogenetic role for neuropeptides in psoriasis (Dewing, 1971; Farber *et al*, 1986; Farber and Raychaudhuri, 1999). In particular, these peptides may be responsible for the psoriasis flare, especially with stress and for the maintenance of psoriatic lesions. One of the neuropeptides involved in this interaction is β-endorphin, an agonist of the μ-opiate receptor (Wintzen *et al*, 1996). β-Endorphin is produced by post-translational cleavage of the precursor proopiomelanocortin (POMC), from which other secreted neuropeptides such as adrenocorticotropic hormone (ACTH), α-melanocyte-stimulating hormone (α-MSH), and β-lipotropic hormone (β-LPH) are also derived (Eberle, 1988). Recently, β-endorphin and the other POMC peptides were shown to be produced by human keratinocytes (Köck *et al*, 1990; Wintzen *et al*, 1995). In addition, Glinski *et al* (1994) observed that the mean β-endorphin concentration in serum of patients with psoriasis was increased about 2-fold as compared with controls.

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Abbreviations: ACTH, adrenocorticotropic hormone; LPH, lipotropic hormone; MSH, melanocyte-stimulating hormone; POMC, proopiomelanocortin.

We recently demonstrated that human epidermal keratinocytes express μ-opiate receptors at both the mRNA and the protein level (Bigliardi *et al*, 1998). The μ-opiate receptor occurs in all layers of the epidermis. In the dermis it is expressed in the adnex structures, especially in the ducts of sweat glands and in the pilosebaceous unit of hair follicles. In this study we have investigated μ-opioid receptor expression after exposure to β-endorphin, one of the most important mediators of stress (Olson *et al*, 1986). As we intended to test μ-opiate receptor expression in a stress-dependent skin diseases such as psoriasis, we measured μ-opiate receptor expression in psoriatic lesional skin and compared it with that of nonlesional skin from the same patient. We also investigated the influence of β-endorphin on the expression level of cytokeratin 16, one of the most sensitive indicators of the psoriatic state of differentiation (Leigh *et al*, 1995), in human skin organ culture models. Cytokeratin 16 is not expressed in normal skin, but it is upregulated in the suprabasal, differentiating compartment of the epidermis during wound healing and hyperproliferative skin diseases such as psoriasis and skin cancer. The data found in this study suggest an important role of the μ-opiate receptor system in the pathogenesis of psoriasis.

MATERIALS AND METHODS

Preparation of human skin organ culture The method of functional assays with skin organ cultures was adapted from Paus *et al* (1994). Skin grafts of about 0.5 mm thickness were obtained by a dermatome from the upper leg. 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 placed in a Nunclon 24 well plate containing 2 ml

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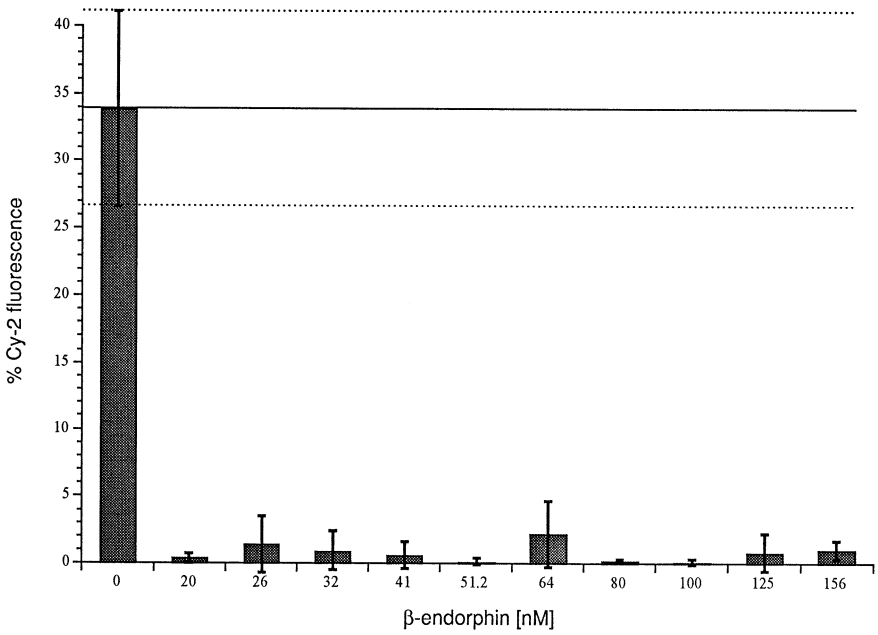

Figure 1. Quantification of the downregulation of μ-opiate receptor expression in human epidermis after exposure to β-endorphin. Skin culture samples from three different epidermal regions were exposed to 8–1000 nM β-endorphin for 48 h and μ-opiate receptors were quantified by determination of fluorescence intensities after incubation with polyclonal guinea pig anti-μ-opiate receptor antibodies followed by Cy2-fluorescence-labeled anti-guinea pig antibodies. The bars represent percentage of fluorescent area compared with the total epidermal area measured of three samples ± SD

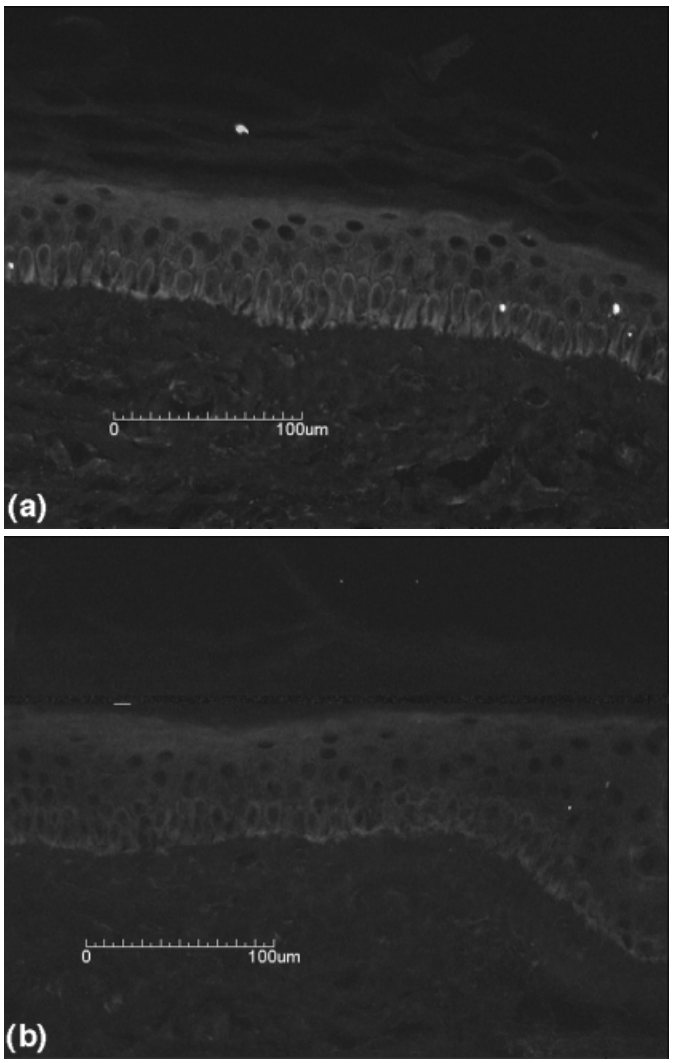

Figure 2. Downregulation of μ-opiate receptor expression in human skin organ cultures following incubation with 16 nM β-endorphin. The skin culture samples were incubated either without (*a*) or with (*b*) 16 nM β-endorphin for 48 h, stained with polyclonal rabbit anti-μ-opioid receptor antibody followed by incubation with Cy2-conjugated goat antirabbit IgG (details see *Materials and Methods*), and analyzed by immunofluorescence microscopy.

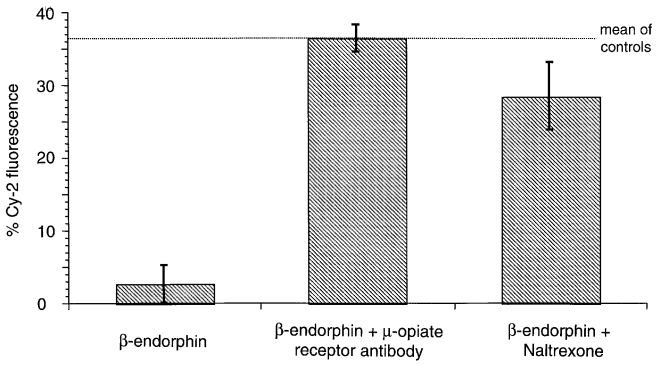

Figure 3. Blocking of the downregulation of μ-opiate receptor expression induced by β-endorphin in human skin organ. The skin organ cultures were incubated for 48 h with 60 nM β-endorphin in the presence or absence of 80 nM polyclonal guinea pig anti-μ-opiate receptor-antibody or 80 nM naltrexone. The bars represent percentage of fluorescent area compared with the total epidermal area measured of three samples ± SD

Dulbecco's modified Eagle's medium (DMEM with Glutamax-I; Life Technologies) supplemented with 10% fetal bovine serum and 50 μg gentamicin (Life Technologies) per ml. For the assessment of the μ-opiate receptor and cytokeratin 16 expression in psoriasis, we performed a punch biopsy of 3 mm diameter in lesional psoriatic skin and as a control, a similar biopsy of normal, nonlesional skin from the surrounding area. The biopsies were embedded in Tissue-Tek and immediately snap frozen in liquid nitrogen.

Functional experiments After addition of serial dilutions of β-endorphin (Sigma, St. Louis, MO), the organ cultures were incubated for 48 h at 37°C in 5% CO_2 and 100% humidity. Control skin organ cultures were incubated in the culture medium only. Blocking experiments were performed in the same way in the presence of 60 nM β-endorphin by adding either 80 nM polyclonal guinea pig anti-μ-opiate receptor antibodies (Gramsch, Schwabhausen, Germany) or 80 nM of the opiate receptor antagonist naltrexone. At the end of the incubation period the organ culture pieces were fixed in 4% formaldehyde and embedded in paraffin for use in immuno-histochemistry, or embedded in Tissue-Tek (Miles, Diagnostics Division, Elkhart, IN) and immediately snap frozen in liquid nitrogen for use in immunofluorescence experiments.

Immunofluorescence The frozen biopsies were cut into 6 μm sections and fixed in acetone and blocked with 5% normal goat serum and 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

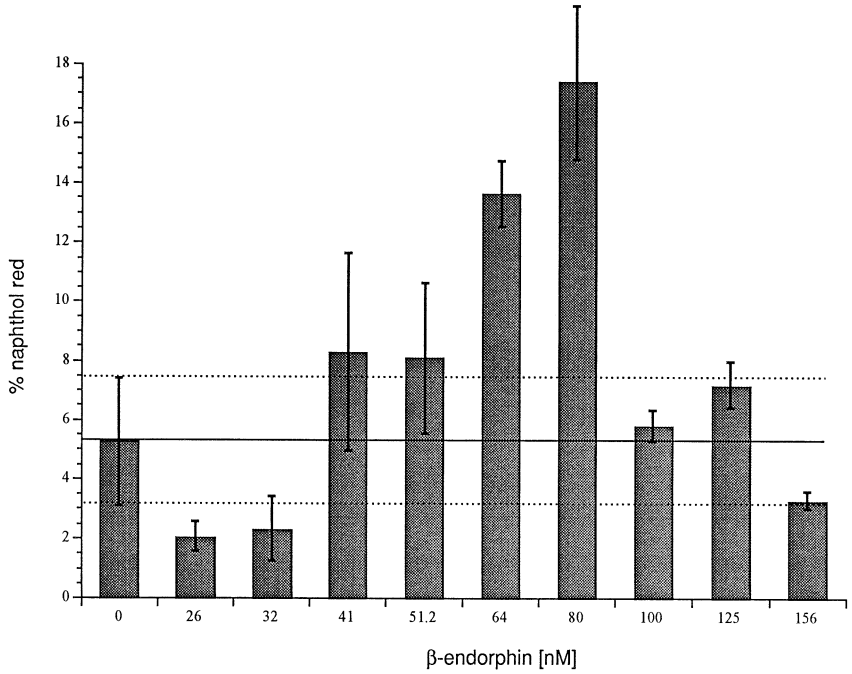

Figure 4. Upregulation of cytokeratin 16 in suprabasal keratinocytes. Skin culture samples from different epidermal regions were incubated with 26–156 nM β-endorphin for 48 h and cytokeratin 16 expression was quantified by measuring the intensity of naphthol red using triplicates of digital images from three different regions of the same skin culture sample. The bars represent percentage of fluorescent area compared with the total epidermal area measured of three samples ± SD.

receptor antibody (Pharmingen, Hamburg, Germany) specific for a 17-amino acid sequence that is identical in the human, mouse, and rat. The experiments were repeated using another commercially available polyclonal guinea pig anti-human-μ-opiate receptor antibody (Gramsch, Schwabhausen, Germany). Sections were incubated with the primary antibodies for 1 h at 37°C, whereas the controls were incubated in normal goat serum. After three washes with Tris buffer, all sections were all incubated with Cy2-conjugated goat anti-rabbit IgG or anti-guinea pig IgG (H + L) (Jackson Immuno Research Laboratories, West Grove, PA) at room temperature for 30 min. These secondary antibodies had been tested for minimal cross-reaction to human, mouse, and rat serum proteins. The sections were visualized using immunofluorescence microscopy (Diaphot 300; Nikon, Tokyo, Japan).

Immunohistochemistry The paraffin-imbedded skin culture punches were cut into 5 μm sections, dried overnight at 37°C, and deparaffinized with xylol, ethanol (100%, 96%, 90%, 80%, 70%, 50%), distilled H_2O, and phosphate-buffered saline (pH 7.0–7.2). The sections for cytokeratin 16 were then gently boiled in 0.01 M sodium citrate solution in a microwave oven for 10 min and cooled down to room temperature. All sections were preincubated in 0.1% albumin (fraction V) blocking solution for an hour. The primary antibodies against cytokeratin 16 (monoclonal antibody NCL-CK16; Novocastra Laboratories, Newcastle-upon-Tyne, U.K.) were diluted in common antibody diluent (BioGenex Laboratories, San Ramon, CA) and incubated with the sections for 1 h at room temperature. The binding was visualized using the StrAviGen Super Sensitive kit (BioGenex), which is based on biotinylated antibody containing alkaline phosphatase, naphthol red as substrate for the alkaline phosphatase, and Mayer's acid hematoxylin solution, containing sodium iodate, alaun, and hematoxylin, as counter-stain. In order to compare directly the skin samples exposed to serial dilutions of β-endorphin, the immunohistochemistry experiments in one series were performed simultaneously in the same manner. For immunohistochemical staining of cytokeratin 16 expresssion of lesional and nonlesional skin of patients with psoriasis, we used a peroxidase/AEC-substrate detection system (DAKO, Carpinteria, CA) instead of the alkaline phosphatase/naphthol red system.

Quantification of immunohistochemical staining and immunofluorescence The intensities of the expression of cytokeratin 16 and the μ-opiate receptor were quantified using a digital CCD color camera (CF 20 DXC air; Kappa Messtechnik, Gleichen, Germany) on an inverted microscope (Nikon Diaphot 300, Tokyo, Japan). All pictures were taken under the same conditions with 100–50× magnification. The area and intensities of the naphthol red signal and the green fluorescence were quantified on an image analysis system (PicEd Cora, Jomesa, Munich, Germany). The program parameters were set up so that the control sections without incubation with primary antibody had less than 1% staining. Measurements were taken from three different sections on the same slide. The expression level was estimated as the percentage of naphthol red stained area or green fluorescent area, compared with the total measured area in epidermis. The average values and standard deviations were taken from the triplicate readings.

RESULTS

β-Endorphin downregulates μ-opiate receptor expression in human skin organ cultures In order to test the regulation of the μ-opiate receptor in response to the specific endogenous ligand, we exposed the human skin organ cultures to different concentrations of β-endorphin. As shown in **Fig 1**, μ-opiate receptor expression in human skin organ cultures was significantly downregulated by incubation with β-endorphin. The two different antibodies gave similar results. μ-Opiate receptor expression was dramatically downregulated from a staining area of 35% in control skin, which was not exposed to β-endorphin, to less than 1% in skin culture samples exposed to 62 nM β-endorphin. **Figure 2**(***a***) shows a typical example of μ-opiate receptor expression in all layers of untreated epidermis, with a pronounced staining in the basal layer. After treatment with 16 nM β-endorphin specific staining was dramatically decreased and μ-opiate receptors had almost disappeared (**Fig 2*b***). This downregulation of the μ-opiate receptor could be blocked by incubating the skin organ cultures with anti-μ-opiate receptor antibody or 80 nM naltrexone. As shown in **Fig 3**, incubation with β-endorphin (60 nM) alone gave a staining area of 3%; addition of anti-μ-opiate receptor antibody or 80 nM naltrexone restored the expression of the μ-opiate receptor to normal levels, as shown by a staining area of 30%–35%. This indicates that the biological effect of β-endorphin indeed functions through the μ-opiate receptor.

β-Endorphin upregulates cytokeratin-16 expression in human skin organ cultures The expression of cytokeratin 16 in human skin organ cultures was upregulated after exposure to 41–125 nM β-endorphin, as shown in **Fig 4**. At β-endorphin concentrations of 80 nM, the cytokeratin 16 expression was almost 3-fold higher than that of control skin not exposed to β-endorphin. **Figure 5** compares the immunohistochemical staining of the human skin organ culture exposed to growth medium only and to 62 nM β-endorphin. In **Fig 5**(***b***) an intense red staining by

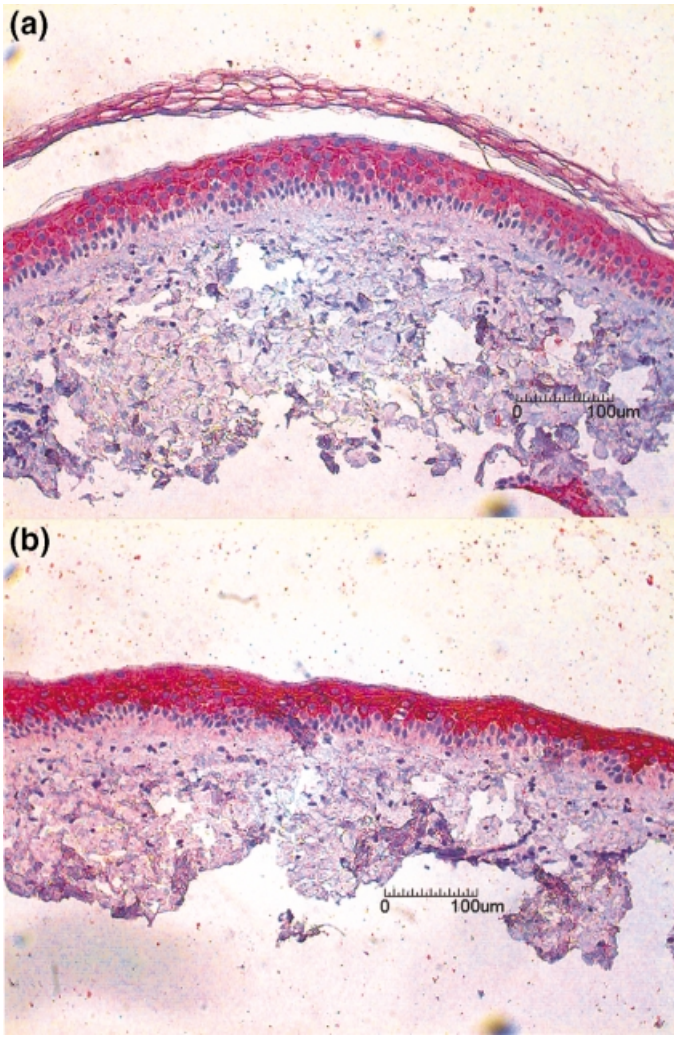

Figure 5. Upregulation of cytokeratin 16 expression in epidermis of human skin organ cultures following incubation with 62 nM β-endorphin. The skin culture samples were incubated without (*a*) or with (*b*) 62 nM β-endorphin for 48 h. The cytokeratin 16 expression was determined on deparaffinized sections using naphthol red as substrate of the alkaline phosphatase.

the cytokeratin 16 antibody was found in the expected regions in the differentiating compartment, i.e., the subcorneal and suprabasal layers.

μ-Opiate receptor expression is downregulated and cytokeratin 16 expression is upregulated in psoriatic lesional skin Analysis of μ-opiate receptor expression in the epidermis of eight different patients with psoriasis indicated receptor downregulation. **Figure 6** shows the results of a quantitative analysis of two typical patients: Immunohistochemical determination of μ-opiate receptor with either the rabbit or the guinea pig polyclonal antibodies demonstrated that receptor expression was downregulated to an average value of 10% compared with that of nonlesional skin. Whereas in normal skin the μ-opiate receptor occurs in relatively high density throughout the epidermis (**Fig 7*a***), receptor expression in psoriatic skin is almost completely downregulated except for the suprabasal layer where μ-opiate receptors occur, albeit at lower density (**Fig 7*b***). There was no cytokeratin 16 expression in nonlesional normal skin (**Fig 8*a***), whereas cytokeratin 16 was found throughout the epidermis of psoriatic skin biopsies, with elevated expression in the suprabasal layer from the same localization of the same patient (**Fig 8*b***).

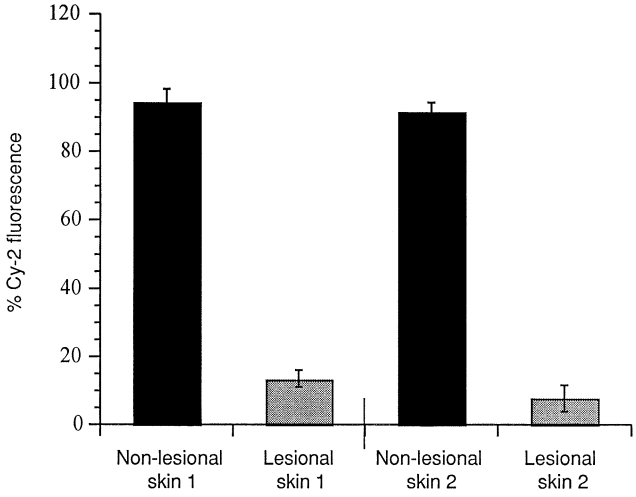

Figure 6. Downregulation of μ-opiate receptor expression in epidermis from lesional psoriatic skin. A punch biopsy of 3 mm diameter from lesional psoriatic skin and from normal skin of the surrounding area were taken from two patients. Fluorescence staining was conducted as described in **Fig 2**. The bars represent percentage of fluorescent area compared with the total epidermal area measured of three samples ± SD.

DISCUSSION

The nervous system plays a significant role in several skin diseases. Psoriasis provides one of the best examples of the function of the nervous system in skin pathology. Psychologic stress is often thought to trigger the onset or exacerbation of this disease (Farber *et al*, 1986; Seville, 1989). Several different neuropeptides, such as substance p (Misery, 1997), vasointestinal peptide (Pincelli, 1994), and also the POMC-derived products (Slominski *et al*, 1993), have been detected in psoriatic skin. The processing of POMC into its active peptides is tissue specific. For example, the anterior lobe of the pituitary gland contains mostly ACTH and β-LPH, whereas in the pars intermedia or in peripheral tissues such as skin POMC is processed to the shorter peptides, α-MSH, and endorphins (Eberle, 1988). Therefore in skin, α-MSH and endorphins appear to be important mediators, especially under stressful situations. It is not surprising that elevated levels of β-endorphin are found not only in sera of psoriasis patients (Glinski *et al*, 1994) but also in patients with vitiligo (Mozzanica *et al*, 1992), atopic dermatitis (Glinski *et al*, 1995), and other inflammatory skin disorders. It has been suggested that this β-endorphin is derived from inflammatory cells, peripheral nerves, and keratinocytes (Wintzen and Gilchrest, 1996). These observations are in accordance with our findings that the μ-opiate receptor in psoriatic lesional skin is dramatically downregulated compared with nonlesional skin and that the μ-opiate receptor in human skin organ cultures is downregulated after exposure to β-endorphin. The two different polyclonal antibodies from different host animals, raised against different regions of the μ-opiate receptor, gave similar results. The downregulation of the μ-opiate receptor by β-endorphin in human skin organ cultures could be reversed by blocking the receptor with anti-μ-opiate receptor antibodies or by addition of the antagonist naltrexone. This suggests that the μ-opiate receptor ligand β-endorphin indeed binds to the receptor and that the downregulation is driven by a specific receptor–ligand interaction.

It is known that cytokeratin 16 is an important factor in the differentiation of psoriasis. This is supported by our finding that cytokeratin 16 expression is significantly upregulated both in psoriatic lesional skin (compared with nonlesional skin) and in cultured human skin after exposure to β-endorphin. It has been reported that cytokeratin 16 is expressed at the suprabasal layer at

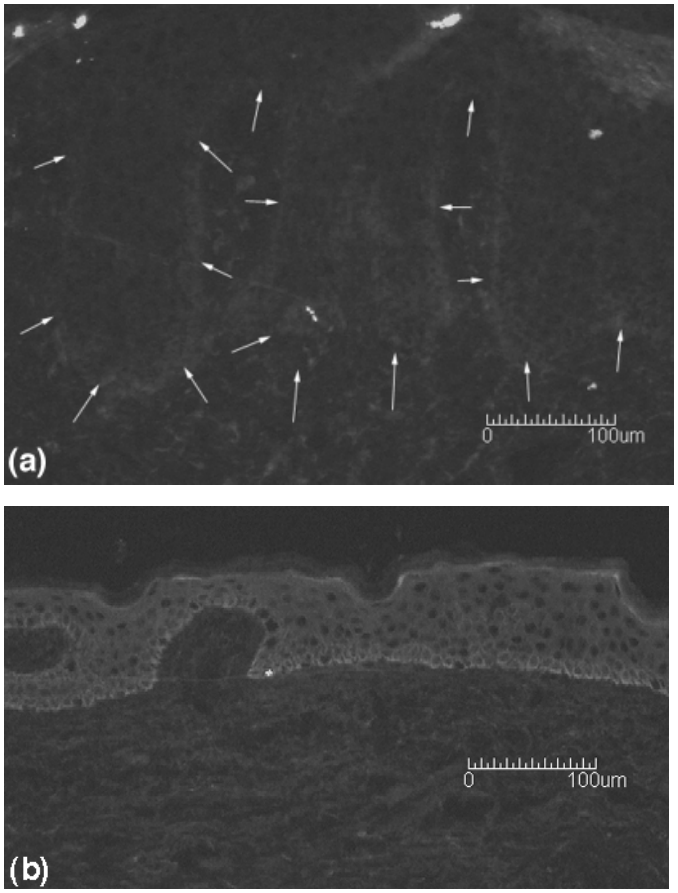

Figure 7. Downregulation of μ-opiate receptor expression in epidermis of psoriatic lesional skin. Biopsies from nonlesional (*a*) and lesional (*b*) skin were taken from the same localization of the same individual and μ-opiate receptor expression was measured by immunofluorescence as described in **Fig 2**. The arrows depict the border between the epidermis and dermis.

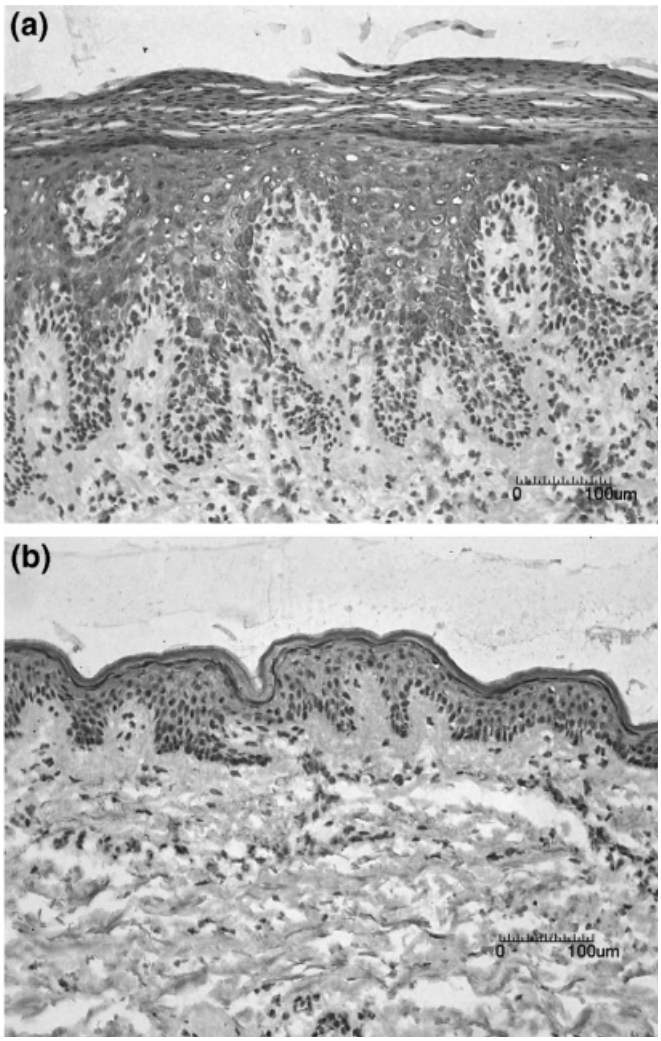

Figure 8. Upregulation of cytokeratin 16 expression in epidermis of psoriatic lesional skin. Biopsies from nonlesional (*a*) and lesional (*b*) skin were taken from the same localization of the same individual and cytokeratin 16 expression was determined by immunofluorescence as described in *Materials and Methods*.

the second stage of the development of the psoriatic lesion, when the inflammatory infiltrate forms and penetrates into the upper layers of the epidermis (Gerritsen, 1997). Although cytokeratin 16 is a marker for epidermal differentiation and hyperproliferation, we did not observe an increase in keratinocyte proliferation in the presence of β-endorphin (unpublished result) using a MTT-proliferation assay (Bigliardi *et al*, 1994). Our results agree with those of others (Nissen, 1997) who also did not find any effect of β-endorphin on keratinocyte proliferation; however, the δ-opiate receptor ligand methionine-enkephalin slightly inhibited the proliferation of keratinocytes. We hypothesize that the μ-opiate receptor system is actively involved in skin differentiation, but not in proliferation. The μ-opiate receptor ligand β-endorphin not only downregulates the μ-opiate receptor expression in human skin organ cultures, but also upregulates cytokeratin 16 expression. Time course experiments have shown that β-endorphin downregulates μ-opiate receptor expression as early as 24 h after the start of the incubation (unpublished data). Overexpression of cytokeratin 16 in human epidermis may therefore be initiated by an early signal originating from the initial ligand–receptor interaction. During the same period, downregulation of the μ-opiate receptor commences, representing a negative feed-back mechanism to compensate for the elevated concentrations of the ligand in the tissue. Despite the downregulation of the μ-opiate receptor in human epidermis, cytokeratin 16 synthesis is maintained at a high level because, after the initial signal of β-endorphin, expression appears to continue independently of the μ-opiate receptor system. An alternative explanation is that a continued signal through the μ-opiate receptor has to be present for normal cytokeratin 16 expression and once the μ-opiate receptor is downregulated, cytokeratin 16 expression is elevated. The upregulation of the cytokeratin 16 by β-endorphin clearly indicates, however, that the μ-opiate receptor may modulate keratinocyte differentiation in human skin *in vivo*. Our results suggest that the μ-opiate receptor system has an important functional role in skin physiology and pathogenesis of psoriasis.

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