

Effect of Intradermal Injection of Methionine-enkephalin on Human Skin

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Methionine-enkephalin (met-enk) detected in monocytes in psoriatic skin can modulate inflammatory processes and keratinocyte differentiation/proliferation *in vitro*. The purpose of the present study was to determine the effect of intradermal injection of met-enk on normal human skin and on the development of a delayed type skin hypersensitivity reaction. In 6 healthy volunteers, 50 µl of met-enk (16, 30, and 45 nmol) was injected once in the forearm and the reaction was evaluated clinically and by video-optical recording for 120 min. Compared to vehicle (0.9% saline), met-enk induced a time- and dose-dependent flare reaction, but no significant stimulation of a weal reaction. The flare reaction was maximal after 1 min and disappeared within 45 min. Pre-treatment with the antihistamine cetirizine reduced the flare reaction. Furthermore, the effect of met-enk on lymphocyte/monocyte infiltration and epidermal proliferation in normal skin and on a delayed type skin hypersensitivity reaction was assessed. Met-enk (45 nmol/50 µl) was injected at 0, 24 and 48 h. In normal skin, met-enk increased the number of dermal lymphocytes/monocytes (CD3/CD68 positive cells) and the degree of epidermal proliferation (MIB1-Ki67). In a delayed type hypersensitivity reaction induced by tuberculin (PPD), the degree of epidermal proliferation and the number of infiltrating lymphocytes/monocytes were reduced compared to PPD alone. Our study suggests that intradermal injection of met-enk in normal human skin induces an inflammatory reaction that may involve the release of histamine. In contrast, met-enk seems to down-regulate the development of a delayed type skin hypersensitivity reaction. These results may indicate that the direction of the effect of the opioid peptide met-enk on human skin depends on the rate of epidermal proliferation and the activity of immunocompetent cells. **Key words: inflammation; epidermal proliferation; flare reaction; neurogenic inflammation.**

(Accepted June 18, 1998.)

Acta Derm Venereol (Stockh) 1999; 79: 23–26.

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Opioid peptides including enkephalins can be detected in peripheral tissues. Thus, in human skin, enkephalin-like immunoreactivity has been visualized in dermal CD68-positive cells (1). Opioid peptides can modulate inflammatory reactions and cell growth. Thus, it has been suggested that opioid peptides work as fine-tuners or biological response modifiers of the immune system (2, 3) both to suppress a strong immune response and to reverse an immunosuppression, depending on the activity of the cell or the immune response (4, 5).

It has been shown that a single injection of dynorphin, beta-endorphin, DADL (D-ala²-D-leu⁵-enkephalin), morphine and morphine-related substances is able to induce a weal and flare

reaction after injection in normal human skin (6, 7). The purpose of the present study was to determine the capacity of the naturally occurring opioid peptide methionine-enkephalin (met-enk) to induce or modulate skin inflammation. The effect on normal human skin was evaluated both clinically and quantitatively by video-optical recording. Furthermore, immunohistochemical analysis of the epidermis and the lymphocyte/monocyte infiltration was conducted.

MATERIALS AND METHODS

Materials

The following were used: lyophilized met-enk (Neosystem Laboratoire, France), tuberculin (PPD RT 23) (0.1 U/ml) (the Serum Institute, Denmark), monoclonal mouse anti-human NCL-CD3 (Novocastra), monoclonal mouse anti-human MIB1-Ki67 (Immunotech), monoclonal mouse anti-human CD68-PGM1, biotinylated goat anti-mouse, streptavidin/HRP, antibody diluent, glycerol, 3,3-diaminobenzidine tetrahydrochlorid (DAB) (Dako, Denmark), Tris-HCl and paraformaldehyde (Sigma, USA). The retrieval buffers (citrate pH 6.0, EDTA disodium pH 8.0 and TEG buffer pH 9.0) were prepared by the local Department of Pharmacy.

Study design

Six healthy persons (4 males, 2 females, age range 19–49 years) were injected intradermally in the volar aspect of their left and right forearms with three dosages (16 nmol, 30 nmol, 45 nmol) of the pentapeptide met-enk and vehicle (0.9% saline). Injections were performed randomly, with 2 injections on each arm using an Insupak 0.5-ml syringe and a 0.3-mm needle. The peptide was solubilized in 0.9% saline to a stock solution (8.7×10^{-4} M) which was stored in aliquots of 200 µl in sterile, capped vials at -20°C . On the day of injection, one aliquot was thawed and the peptide solution was diluted. The final injection volume was 50 µl. Furthermore, 3 of the 6 volunteers were given 10 mg of the antihistamine cetirizine orally 1 h before intradermal injection of 45 nmol met-enk.

In another experiment, 8 healthy volunteers (5 males, 3 females, age range 29–45 years) were injected with met-enk (45 nmol, 50 µl) and vehicle (0.9% saline) at 0, 24 and 48 h. Four of the 8 volunteers were also injected with tuberculin (PPD, 0.1 U/ml, 100 µl) 30 min prior to the first injection of met-enk (45 nmol, 50 µl). These 4 persons had all been previously vaccinated against tuberculosis. After 4 quadrant injections of 1% lidocaine, 3-mm punch biopsies were obtained at 48 h from areas injected with PPD or PPD combined with met-enk. At 72 h, additional punch biopsies were obtained from areas injected with met-enk or vehicle. The biopsies were fixed in 4% paraformaldehyde/0.1 M sodium biphosphate pH 7.2 for 3 h.

The study was approved by the local ethics committee, and the volunteers gave informed consent.

Recording of weal and flare reaction

Video-optical pictures consisting of 512×476 pixels in 32,000 colour shades were generated by a Sony DXC-151P video camera (Sony Corporation, Japan). Each picture was captured and stored in a PC and the area of the flare and weal reaction in cm^2 was subsequently analysed by dedicated software. The time course of each weal and flare reaction was monitored by making 10 recordings beginning at zero and then record-

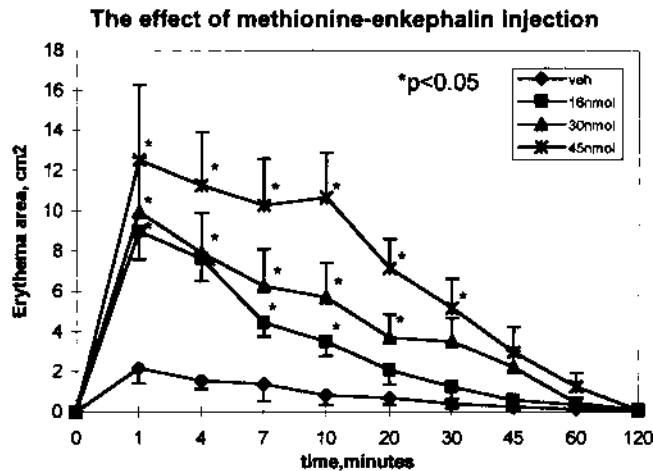


Fig. 1. The time- and concentration-dependent flare reaction after injection of met-enk and saline in normal human skin. The skin surface areas were measured in cm^2 from video-optical pictures. * $p < 0.05$ compared to control levels. Results are expressed as mean \pm SEM ($n = 6$).

ing again 1, 4, 7, 10, 20, 30, 45, 60 and 120 min after the injections. All analyses were performed blinded.

Immunohistochemical analysis

The paraffin-embedded paraformaldehyde-fixed biopsies were sectioned (2–3 μm), treated with xylene and hydrated in decreasing concentrations of ethanol and water. After boiling in a retrieval buffer 3×5 min at 750 W in a microwave, the sections were rinsed in a tris buffer (Tris-HCl 50 mM, triton X-100 0.05%, pH 7.6) and incubated with monoclonal antibodies directed towards CD3 (NCL-CD3 1:50), CD68 (PGM1-CD68, 1:1000) and MIB1 (MIB1-Ki67, 1:100) for 30 min at room temperature. The endogenous peroxidase activity was blocked by incubation in methanol/ H_2O_2 (60:1). Biotinylated goat anti-mouse (1:250) and streptavidin/HRP (1:250) were applied for 30 min each. Finally, the colour reaction was developed in DAB (0.1%) and the sections were counter-stained with haematoxylin and mounted in a glycerol-gelatine solution.

Statistical analysis

The statistical analysis was based on parametric (Student's *t*-test) and non-parametric (Wilcoxon) tests for paired data. A *p*-value < 0.05 was accepted as statistically significant.

The number of CD3/CD68 and MIB1 positive cells was estimated semiquantitatively. No statistical analysis was performed on these data.

RESULTS

Injection of met-enk in normal human skin induced a weal and flare reaction. Clinically, there was an erythematous flare spreading centrifugally from the injection centre, and a small weal reaction. None of the volunteers experienced any itch. Measurement of the flare areas by video imaging showed a time- and dose-dependent change (Fig. 1). The maximal flare reaction developed within the first minute. Three subjects received cetirizine 10 mg orally 1 h before injection with 45 nmol met-enk. After treatment with cetirizine, the flare reaction was reduced at all time points (Fig. 2).

The weal reaction also showed a time-dependent change (Fig. 3). The maximal response was seen after 7 min. The

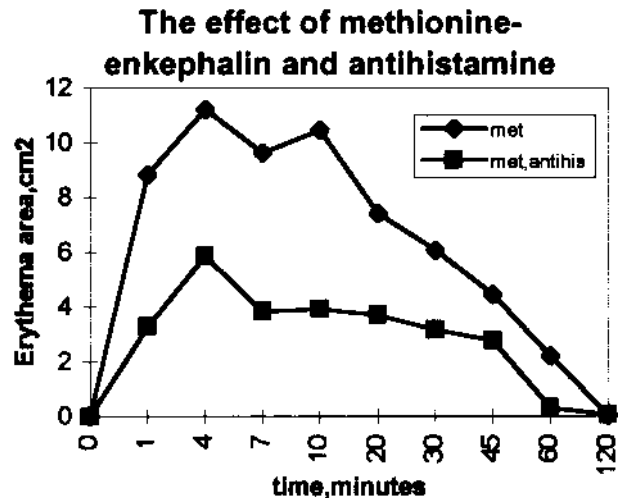


Fig. 2. The effect of cetirizine on the met-enk induced flare reaction in 3 volunteers. Results are expressed as mean \pm SEM.

increase was most pronounced for the high doses, but it was not statistically significant.

Another set of experiments assessed the effect of repeated intradermal injections of met-enk on lymphocyte/monocyte infiltration and epidermal proliferation. In normal human skin, each injection induced a short-lived flare reaction. Histologically, met-enk induced an increase in perivascular dermal lymphocyte/monocytes and epidermal proliferation (MIB1-Ki67) (data not shown). These increases were strong in 5 subjects and weak in the remaining 3 subjects. The CD68-positive cells, and particularly the CD3-positive cells, were increased in number.

In contrast, repeated injections of met-enk did not alter the intense erythema and induration seen after tuberculin (PPD) injections. However, the PPD-induced massive dermal infiltration of CD3- and CD68-positive cells was reduced in 3 of 4 subjects. Likewise, the PPD-induced increase in basal keratinocyte proliferation (MIB1-Ki67) was reduced strongly in 2 subjects, but only slightly in the 2 other subjects (data not shown).

DISCUSSION

The present study demonstrated a time- and dose-dependent induction of a flare reaction in normal skin after injection with the naturally occurring opioid peptide, met-enk. We chose to examine the effect of met-enk because it is released from proenkephalin A in the highest ratio (8), and although the half-life of naturally occurring opioids is very short (9), natural met-enk was used because stabilization of enkephalin by amino acid substitution (e.g. DADL) might result in different effects (10). Furthermore, we showed that the effect of met-enk was partly mediated through histamine release. Opioid peptides are known to degranulate mast cells (6), and because the flare reaction is thought to be neurogenically mediated and largely dependent on the activation and degranulation of mast cells (6, 11), a similar mechanism may be suggested for met-enk.

In determining the flare area, video-optical monitoring may be superior to previously applied methods, such as measurement of the maximal diameter, which may be inaccurate because of the great variability in shape. Likewise, the use of cellophane for subsequent planimetry has been suggested as

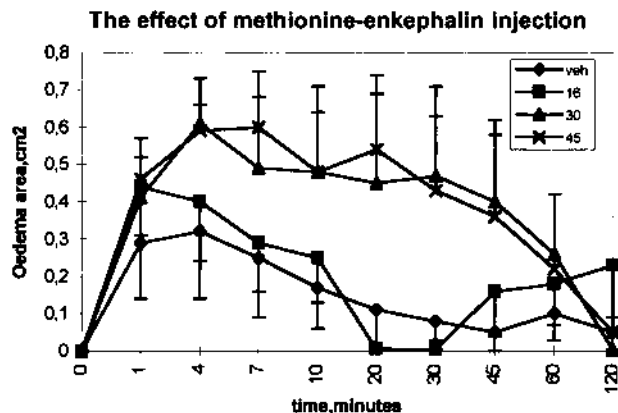


Fig. 3. The weal reaction after injection of met-enk and saline in 6 volunteers. The areas were measured from video-optical pictures. Results are expressed as mean \pm SEM.

unsuitable for time-course measurements because the flare reaction is sensitive even to slight mechanical manipulations of the skin (12). In addition, planimetry may be inaccurate for measurement of the weal reaction because the measured areas are often small (12). In the present study, video-optical measurements of the weal reaction showed no difference between the weal reactions induced by met-enk and saline. This result differs from other studies where both dynorphin and morphine have been shown to induce distinct weal reactions (6, 7). Although we used a high concentration of met-enk in the present study, met-enk might have been degraded too rapidly, probably by mast cell-derived enzymes (13) or epidermal peptidases (14). Alternatively, the relatively large injection volume might have concealed a minor increase in water content (6, 7).

Repeated injections of met-enk increased the number of CD3- and CD68-positive cells in normal human skin, whereas the cell number was reduced in a tuberculin (PPD)-induced skin reaction. These results are compatible with the idea that opioid peptides work as fine-tuners of the immune system (2, 3) to suppress a strong immune response and reverse an immunosuppression (4, 5). These effects may be induced directly via opioid receptors (15, 16) or indirectly by release of mediators from mast cells. While met-enk increased epidermal proliferation in normal skin, it reduced the increased proliferation in a delayed type skin hypersensitivity reaction. A similar differential effect on keratinocyte proliferation is known for other substances, including vitamin D (17). The effect of met-enk on epidermal proliferation might be secondary to the inflammatory reaction (18, 19). However, naltrexone-sensitive effects on keratinocyte proliferation demonstrated *in vitro* (20) indicate that the met-enk *in vivo* may directly effect keratinocytes.

The applied concentration of met-enk was high compared to physiological blood concentration (21). Because enkephalins are present in, and probably released from, immunocompetent cells infiltrating human skin, the local concentration may be much higher, thus enabling the described modulations of cell infiltration and proliferation.

In conclusion, intradermal injection of met-enk induces a histamine-dependent flare reaction and stimulates epidermal proliferation and the infiltration of lymphocytes/monocytes in normal human skin. In contrast, met-enk down-regulates

the infiltration of lymphocytes/monocytes and the enhanced epidermal proliferation in a PPD-induced delayed type skin hypersensitivity reaction. These results suggest that the direction of the effect of the opioid peptide met-enk depends on the rate of epidermal proliferation and the activity of the immunocompetent cells.

ACKNOWLEDGEMENTS

This work was kindly supported by the Danish Medical Research Council and The Danish Psoriasis Foundation. Special thanks to the laboratory technicians at the Dept of Pathology, Aarhus University Hospital, Aarhus for excellent help with immunohistochemical staining.

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