BIBLID: ISSN 0253-9756 中国药理学报 *Acta Pharmacologica Sinica* 1992 Mar; **13** (2) · 131-136

Influence of methionine-enkephalin on interleukin-2 production and interleukin-2 receptor expression

YANG Si-Xun[1], LI Xiao-Yu
(*Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 200031, China*)

ABSTRACT Influences of methionine enkephalin (met-enk) on delayed hypersensitivity (DH) against 2,4-dinitrofluoro-benzene (DNFB) and interleukin-2 (IL-2) production of mouse lymphocytes were examined. Met-enk enhanced the DH response to ear challenge when mice were treated with met-enk beginning at the same time as epicutaneous sensitization with DNFB but not after being sensitized. When met-enk (10 nmol · L^{-1}-100 μmol · L^{-1}) was included in Con A-stimulated lymphocyte cultures, the IL-2 production increased in a concentration-dependent manner. Furthermore, *in vivo* treatment with met-enk also increased IL-2 production of splenocytes, and the enhancement of IL-2 production was parallel to that of lymphocyte proliferation. However, met-enk 10 nmol · L^{-1} had no effect on IL-2 receptor expression on thymocytes, splenocytes, and gut-associated lymphocytes. The data suggested that the stimulative effect of met-enk on lymphocytes may be mediated through the increase of IL-2, but not through the IL-2 receptor expression.

KEY WORDS methionine enkephalin; interleukin-2; interleukin-2 receptors; delayed hypersensitivity, lymphocyte transformation

The discovery of opioid receptors on lymphocytes[1,2] and The finding that leukocytes also produce opioid peptides[3] suggest that these peptides are the neuroimmuno-modulators and they may regulate leukocyte functions by autocrine. The opioid peptides enhanced the natural killer (NK) cell activity through increasing effector-tumor cell conjugated formation and accelerating the kinetics of lysis[4]. β-Endorphin and met-enk stimulated T cytotoxic cells[5], lymphocyte proliferation[6,7], but β-endorphin also suppressed the PHA-stimulated mitogenic response of human peripheral mononuclear cells[8]. Furthermore, met-enk and β-endorphin were found to enhance the γ-interferon (γ-IFN) production by concanavalin A (Con A)-induced human mononuclear cells[9], but inhibit the T cell chemotactic factor production from T-lymphocytes. Recently, we demonstrated that met-enk increased IL-1 production both *in vitro* and *in vivo*[10].

It was known that the *in vitro* lymphocyte proliferation and NK cell activity is regulated by IL-2 and it has a synergetic effect on murine NK cells with IFN. Thus, to examine whether met-enk could increase IL-2 production may be helpful in elucidating the mechanism of the effects of met-enk on host defense and cytotoxic activity. Besides, IL-2 was also found to promote oligodendrocytes to proliferate and differentiate[11]. The importance of IL-2 in homeostasis encouraged us to investigate met-enk on IL-2 production from mouse lymphocytes.

MATERIALS AND METHODS

Three-month-old BALB / cA and C57BL mice, obtained from Shanghai Animal Center, Chinese Academy of Sciences, were housed under standard conditions (light : dark = 12 h : 12 h) in groups of 5-6.

RPMI 1640 medium (Gibco Laboratories) was supplemented with 10% newborn bovine serum (NBS, Shanghai Gulu Biological Products Factory, China), HEPES 10 mmol · L^{-1} (Fluka AG, Buchs, Switzerland), *L*-glutamine 2 mmol · L^{-1}, 2-mercaptoethanol 50 μmol · L^{-1} (Fluka AG, Buchs, Swi-

Received 1990 Dec 19 Accepted 1991 Nov 29

[1]Now in *Department of Immunology, Beijing Medical University, Beijing 100083, China*

tzerland), penicillin 100 IU · ml^{-1} (Suzhou Second Pharmaceutical Factory, Suzhou, China) and streptomycin 100 μg · ml^{-1} (Shanghai Fourth Pharmaceutical Factory, China). Met–enk was obtained from Sigma Chemical Co(St Louis MO, USA).

IL–2 dependent cell line, CTLL–2, kindly provided by Shanghai Medical University, was grown in complete RPMI 1640 medium containing 1:5 dilution of IL–2–riched culture supernatants derived from rat spleen cells stimulated with Con A 10 μg · ml^{-1} (Sigma Chemical Co(St Louis MO, USA) for 24 h.

Delayed hypersensitivity(DH) DH response was performed using the method of Ref 12. C57BL mice were sensitized on the skin with 0.05 ml of 1% 2,4–dinitrofluorobenzene (DNFB, Xingta Chemical Industrial Factory, Shanghai, China) in oil–acetone (1:1) solution to their shaved abdominal skin (3×3 cm^2) on d 0. Right and left ears were challenged with 1% DNFB 10 μl oil–acetone solution and 10 μl oil–acetone solution respectively 4 d later. Ear pinnae ($\Phi = 9$ mm) were weighed 24 h after challenge. DH responses were expressed as the net ear swelling in mg, which was calculated as the differences of ear weights between right (challenged) and left (unchallenged) ears.

Cell preparation and lymphocyte proliferation assay Spleens were removed aseptically from intact healthy mice or met–enk–treated mice and placed in a complete RPMI 1640 medium. Single cell suspensions and lymphocyte proliferation assay were performed as described previously[7].

Interleukin–2 (IL–2) production and assay For testing the IL–2 production *in vivo*, splenic cells from met–enk–treated mice were adjusted to 2×10^6 · ml^{-1} in medium and added in triplicate to wells of 24–well plates (Costar, Cambridge MA, USA) containing Con A (5 μg · ml^{-1}) in total volume of 1 ml. Supernatants were removed after a 24 h incubation in a humidified atmosphere at 37℃ and 5% CO_2 + 95% air.

For IL–2 production assay *in vitro*, single splenic cell suspensions were prepared and diluted to 2×10^6 · ml^{-1} in medium, then distributed in 1 ml total volume to the wells of 24–well plates. The cells were cultured in the presence of various concentrations of Con A and met–enk in a humidified atmosphere at 37℃ and 5% CO_2. Supernatants were obtained at desired incubation times and tested for IL–2 activity.

IL–2 activity was determined by the ability of the supernatants to support the growth of IL–2–dependent cell line(CTLL–2) according to Ref 13 with some modifications. Briefly, CTLL–2 cells were washed 3 times and then resuspended at 1×10^5 · ml^{-1} in medium. The cell suspensions (100 μl) were added to the wells of 96–well plates containing serial dilutions of the supernatants (100 μl). The plates were incubated for 24 h at 37℃ in 5% CO_2, accompanied by [*methyl*–^{3}H]thymidine 148 kBq / well during the last 6 h. The cultures were then harvested and the incorporated radioactivity was determined by liquid scintillation counter. The data, collected as dpm, were then analyzed by probit analysis as Ref 13. Results were expressed as units per milliliter (U · ml^{-1}) *vs* the standard IL–2 preparation (Human ultrapure interleukin–2, Genzyme Corporation, Boston MA, USA).

Detection of IL–2 receptors by fluorescence–activated cell sorter (FACS) The fluorescence analysis was done as Ref 14. The splenocytes were cultured in 24–well plates at 37℃ in 5% CO_2 for 48 h at 2×10^6 · ml^{-1} in 1 ml of complete medium with or without Con A (5 μg · ml^{-1}) and / or met–enk 10 nmol · L^{-1}. At the end of incubation, the cells were collected and washed twice in phosphate–buffered solution (PBS, pH 7.2) containing 1% NBS, and stained with the

monoclonal rat anti–mouse IL–2 receptor (Boehringer Mannheim Biochemica, Germany) and in succession with FITC–conjugated rabbit anti–rat IgG (ICN Immunobiologicals, Liele IL, USA). The cells were washed completely and fixed with 1% formaldehyde. To detect cell surface–associated antigens recognized by anti–mouse IL–2, we used 10 000 cells for flow cytofluorometry by FACStar PLUS (Becton–Dickinson).

Statistical analysis Evaluation for statistical significance was accomplished by using *t*(two tail) test, comparing with the data ($\bar{x} \pm s$) obtained in the absence or presence of met–enk.

RESULTS

Effect of met–enk on DNFB–induced DH response to epicutaneous sensitization To determine whether met–enk affected induction phase and / or effector phase, C57BL mice were injected ip with met–enk either before or after epicutaneous sensitization. Met–enk significantly enhanced DH response given to mice from sensitization day to challenge day (Tab 1, $P<0.05$). No effect of met–enk on

Tab 1. Effect of ip methionine enkephalin (met–enk) on 2,4–dinitrofluorobenzene (DNFB)–induced (challenged on d 4) delayed hypersensitivity (DH) in C57BL mice. $^{*}P>0.05$, $^{**}P<0.05$ *vs* control. NS = normal saline.

	Dose / $mg \cdot kg^{-1} \cdot d^{-1}$	*n*	Net ear swelling / mg
Control	NS × 5 d	6	7.8 ± 1.3
	0.01 × 5 d	6	9.8 ± 1.7*
Met–enk	0.1 × 5 d	6	12.3 ± 4.3**
	1 × 5 d	6	10.2 ± 3.5*
Mice were injected ip met–enk or NS immediately before and 12 h after challenge.			
Control	NS	5	8.2 ± 2.9
	0.01	5	9.5 ± 3.6*
Met–enk	0.1	6	8.9 ± 4.6*
	1	6	9.5 ± 3.5*

DH response was observed when met–enk was given beginning on the challenge day.

Effect of met–enk on Con A–stimulated IL–2 production in mouse splenocytes Met–enk 10–1000 $nmol \cdot L^{-1}$, when cultured with splenocytes in the presence of Con A (5 $\mu g \cdot ml^{-1}$) for 24 h, significantly enhanced Con A–stimulated IL–2 production from spleen cells in concentration–dependent manner. The IL–2 activities were 24.5 ± 1.5, 26.2 ± 3.1 ($P>0.05$), 29.8 ± 2.5 ($P<0.01$), and 32.2 ± 3.5 ($P<0.01$) $U \cdot ml^{-1}$ in met–enk 0, 0.1, 10, 1000 $nmol \cdot L^{-1}$ groups, respectively. Further studies indicate that enhancement of IL–2 production by met–enk may require suitable concentrations of Con A. That is, both at a lower Con A concentration (0.5 $\mu g \cdot ml^{-1}$) and at a higher Con A concentration (10 $\mu g \cdot ml^{-1}$), the increased IL–2 production by this peptide did not appear any more (Fig 1).

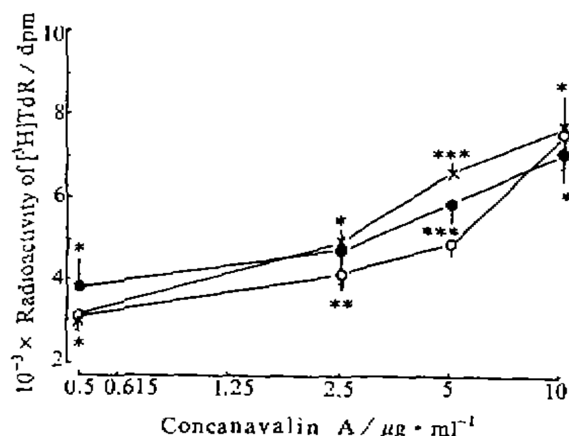

Fig 1. Influence of methionine–enkephalin on Con A–stimulated interleukin–2 production from mouse splenocytes *in vitro*. $n=5$, $\bar{x} \pm s$. $^{*}P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$ *vs* control. (○) control. (●) met–enk 10 $nmol \cdot L^{-1}$, (×) met–enk 1 $\mu mol \cdot L^{-1}$.

In order to confirm if met–enk also has stimulating effect on IL–2 production *in vivo*,

we injected C57BL mice ip with metenk 0.01, 0.1, and 1 mg · kg^{-1} for 5 d, and the spleens removed on d 6 for lymphocyte proliferation and IL–2 production tests. Both lymphocyte transformation and IL–2 production were significantly increased by met–enk. Furthermore, the enhancement of IL–2 by met–enk was parallel to that of lymphocyte proliferation by the peptide.

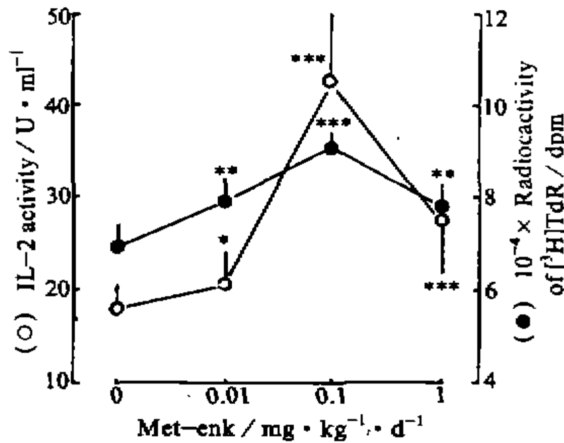

Fig 2. Effects of methionine–enkephalin on lymphocyte proliferation and interleukin–2 production *in vivo*. $n=6$, *$P>0.05$, **$P<0.05$, ***$P<0.01$ *vs* **control.**

Effect of met–enk on IL–2 receptor expression on lymphocytes When met–enk 10 nmol · L^{-1}, an effective concentration for IL–2 production, incubated with lymphocytes from different immune organs in the presence of Con A (5 μg · ml^{-1}) for 48 h, the peptide did not show any effect on IL–2 receptor expression on splenocytes (percent of IL–2 receptor positive cells was 57 ± 6 in Con A control, and 58 ± 7 in Con A+met–enk treated group, $P>0.05$); thymocytes (percent of IL–2 receptor positive cells was 60 ± 7 in Con A control, and 61 ± 8 in Con A+met–enk group, $P>0.05$), and gut–associated lymphocytes (percent of IL–2 receptor positive cells is 46 ± 7 in Con A control, and 44 ± 9 in Con A+met–enk group, $P>0.05$). Met–enk 10 nmol · L^{-1} had no effect on spontaneous IL–2 receptor expression either. After lymphocytes cultured with met–enk 10 nmol · L^{-1} for 48 h, the spontaneous IL–2 receptor positive cells in splenocytes (control: 7 ± 0.5%; met–enk group: 6.8 ± 1.1%, $P>0.05$), thymocytes (control: 4.5 ± 0.7%; met–enk group: 4.7 ± 1.2%, $P>0.05$) and gut–associated lymph node cells (control: 5.2 ± 0.8%; met–enk group: 5 ± 1%, $P>0.05$) did not show any significant alteration.

DISCUSSION

Our results revealed that met–enk significantly augmented IL–2 production with concentration dependent fashion from sub–optimal concentrations of Con A–activated splenocytes. Mice treated with met–enk *in vivo* also showed significant enhancement of IL–2 production. These results did not agree with that of Ref 15, which showed that met–enk did not increase IL–2 production from LBRM–33, the mouse T cell lymphoma. The difference may be explained as follows: there were some differences between normal and tumor cells, eg, transformed human lymphocytes had high and low affinity special binding sites for β–endorphin[1], but EL4 cells had only moderate and low affinity ones[2]. In addition, lymphocytes from different individuals did not all respond to one opioid and *vice versa*.

This explanation was consistent with studies by Brown and Van Epp[9] who demonstrated that an enhanced mitogen–stimulated IFN production in response to β–endorphin did not always correspond with an enhanced response to met–enk. The reverse was also observed that the individual responded to met–enk but not to β–endorphin.

The effective dose of met–enk *in vitro* was higher than the physiological level in blood.

This may be due to the rapid decomposition of met-enk during experiment. The possibility that there were high concentrations of opioids in the local environment, in which opioid peptides may exert their immunomodulation in a paracrine and / or autocrine manner, can not be excluded. In fact, lymphocytes stimulated by antigens or mitogens expressed POMC and preproenkephalin mRNA, and then produced opioid peptides in the local[3]. These may indicate that opioids could regulate immune responses at least during diseases, such as infections and tumors.

In view that the IL-2 production and IL-2 receptor expression are two closely linked events during T lymphocyte activation, we investigated whether met-enk also altered the IL-2 receptor expression by T cells using FACS. When cultured with lymphocytes from thymuses, spleens and gut-associated lymph nodes respectively for 48 h in the presence or absence of suboptimal concentration of Con A, the effective concentration of met-enk did not show any effect on spontaneous nor Con A-activated IL-2 receptor expression. Perhaps, the sensitivity to the same signal in the initiation of IL-2 production and IL-2 receptor expression by lymphocytes was different.

In light of the observations that IL-2 promoted the oligodendrocytes to proliferate and differentiate[11] and enhanced the POMC gene expression in pituitary cells, and that lymphocytes produced opioid substances[3], our data provided a further evidence that a regulatory loop existed between the immune and neuroendocrine systems by which they modulated each other effectively.

AKNOWLEDGEMENTS We are grateful to Ms XU Wei-Min, Shanghai Medical University, for providing us CTLL-2 cell line; to Dr WU Zhi-Jiang and Ms LENG Yong, Shanghai Institute of Cell Biology, Chinese Academy of Sciences, for performing the FACS analysis.

REFERENCES

1 Hazum E, Chang K-J, Cuatrecasas P. Specific nonopiate receptors for β-endorphin. *Science* 1979; **205** : 1033-5.

2 Schweigerer L, Schmidt W, Teschemacher H, Wilhelm S. β-endorphin: interaction with specific nonopioid binding sites on EL4 thymoma cells. *Neuropeptides* 1985; **6** : 445-52.

3 Zurawski G, Benedik M, Kamb BJ, Abrams JS, Zurawski SM, Lee FD. Activation of mouse T-helper cells induces abundant preproenkephalin mRNA synthesis. *Science* 1986; **232** : 772-5.

4 Froelich CJ, Bankhurst AD. The effect of β-endorphin on natural cytotoxicity and antibody dependent cellular cytotoxicity. *Life Sci* 1984; **35** : 261-5.

5 Carr DJJ, Klimpel GR. Enhancement of the generation of cytotoxic T cells by endogenous opiates. *J Neuroimmunol* 1986; **12** : 75-87.

6 Plotnikoff NP, Miller GC. Enkephalins as immunomodulators. *Int J Immunopharmacol* 1983; **5** : 437-41.

7 Yang SX, Li XY. Enhancement of T lymphocyte proliferation and suppression of antibody producing cell formation by methionine-enkephalin. *Acta Pharmacol Sin* 1990; **11** : 355-9.

8 Puppo F, Corsini G, Mangini P, Bottaro L, Barreca T. Influence of β-endorphin on phytohemagglutinin-induced lymphocyte proliferation and on the expression of mononuclear cell surface antigens in vitro. *Immunopharmacology* 1985; **10** : 119-25.

9 Brown SL, Van Epps DE. Opioid peptides modulate production of interferon by human mononuclear cells. *Cell Immunol* 1986; **103** : 19-26.

10 Yang SX, Li XY. Enhancement of interleukin-1 production in mouse peritoneal macrophages by methionine-enkephalin. *Acta Pharmacol Sin* 1989; **10** : 266-70.

11 Merrill JE. Macroglia: neural cells responsive to lymphokines and growth factors. *Immunol Today* 1987; **8** : 146-50.

12 Asherson GL, Ptak W. Contact and delayed hypersensitivity in the mouse. I. Active sensitization and passive transfer. *Immunology* 1968; **15** : 405-16.

13 Gillis S, Fern MM, OU W, Smith KA. T cell growth factor: parameters of production and a

quantitative microassay for activity. *J Immunol* 1978; **120** : 2027–32.

14 Diamantstein T, Osawa H, Graf L, Schirrmacher V. Studies on interleukin 2 receptor expression and IL–2 production by murine T cell lymphomas. *Br J Cancer* 1985; **51** : 23–30.

15 Gilmore W, Weiner LP. β–Endorphin enhances interleukin–2 (IL–2) production in murine lymphocytes. *J Neuroimmunol* 1988; **18** : 125–38.

131-136

甲硫氨酸脑啡肽对白细胞介素 2 产生及其受体表达的影响

杨四句、李晓玉 (中国科学院上海药物研究所，上海 200031，中国)

R967

提要 甲硫氨酸脑啡肽(met–enk)在 10 nmol · L⁻¹–100 μmol · L⁻¹ 浓度下与 Con A 能协同增强淋巴细胞产生 IL–2，并呈剂量依赖关系：ip 0.01、0.1 及 1 mg · kg⁻¹ 后，IL–2 产生与脾淋巴细胞增殖平行加强；但 met–enk 10 nmol · L⁻¹ 对胸腺细胞、脾细胞及肠淋巴结细胞上 IL–2 受体表达无影响，提示 met–enk 的免疫调节作用是通过促进 IL–2 产生而不是增加其受体表达而实现的.

关键词 甲硫氨酸脑啡肽；白细胞介素 2；白细胞介素 2 受体；迟发型超敏感性；淋巴细胞转化

BIBLID: ISSN 0253–9756 中国药理学报 *Acta Pharmacologica Sinica* 1992 Mar; **13** (2) : 136–139

Effects of copper and selenium on electric parameters of cultured myocardial cells damaged by xanthine–xanthine oxidase

ZHONG Guo–Gan, JIANG Yan[1], YUE Gang, LI Yun–Yi, SUN Xiao–Xia, ZHANG Wen–Jie (*Department of Physiology, Norman Bethune University of Medical Sciences, Changchun 130021, China*)

ABSTRACT Addition of xanthine 0.42 mmol · L^{-1} and xanthine oxidase 5.3 nmol · L^{-1} (X–XO) to the culture medium increased the amplitude of ESR spectra of myocardial cells, demonstrating an increase in free radical contents; diminished the action potential parameters significantly and reduced the input impedances from 0.34 ± 0.11 to 0.24 ± 0.1 MΩ, expressing a typical electrical appearance of membrance damage. Supplying Cu 62.5 ng · ml^{-1} and / or Se 173 ng · ml^{-1} to the medium brought all of the electric parameters and the free radical content of myocardial cells back to normal. The results indicate that both the two trace elements are able to scavenge free radicals, thus antagonizing X–XO, which induces damage to myocardial cells.

KEY WORDS cultured cells; myocardium; copper; selenium; free radicals; action potentials; xanthine oxidase

Received 1990 Nov 19 Accepted 1991 Nov 29

[1]*Central Laboratory of Physiology, Norman Bethune University of Medical Sciences, Changchun 130021, China*

Cu and Se are the active centers of superoxide dismutase (SOD)[1] and glutathione peroxidase (GP)[2] respectively, which are powerful scavengers of free radicals in the body. In this experiment, we studied the antioxidative action of the two trace elements, taking the action potential, input impedance, and free radical content of cultured myocardial cells as indices.

METHODS

Cardiomyocyte culture[3] Dispersed cardiomyocytes from neonatal Wistar rats were cultured in a carbon dioxide incubator (36.5℃, pH 7.2), and divided into 5 groups, according to the different compositions of the culture media: