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Mini-review

Immunomodulating effects of methionine enkephalin

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KEY WORDS neuroimmunomodulation; methionine enkephalin; naloxone; opioid receptors; interleukins; lipopolysaccharides; tumor necrosis factor; messenger RNA; gene expression

ABSTRACT Methionine enkephalin (Met-Enk), the endogenous neuropeptide, is suggested to be involved in the regulatory loop between immune and neuroendocrine systems. Our studies showed that Met-Enk over a wide range of concentrations increased interleukin-1 (IL-1) production from mouse peritoneal macrophages induced by lipopolysaccharides (LPS) and naloxone did not block the enhancing effect. Met-Enk promoted the proliferation of mouse splenocyte and the production of IL-2 and IL-6 in a dose-dependent manner. The up-regulating effects of IL-2 and IL-6 not only augmented their mRNA transcription but also increased their stability. Thus Met-Enk appears to be an important immunomodulatory signaling molecule to exert regulatory actions concerned with the expressing of pre-inflammatory cytokines.

The immune system is connected by numerous structural and functional processes with the nervous and endocrine systems, constituting a multisystem with convergence and homeostatic properties[1]. The immune microenvironment unifies cellular and extracellular elements from lymphoepithelial, nervous, and endocrine sources, represents a locus of integrative and specific/nonspecific activities, and provides a basis for interdisciplinary and multidirectional research undertaking.

Opioid peptides, the endogenic molecules that imitate pharmacologic effects of morphine, are widely distributed in the central nervous system and affect an array of biological functions[2-4]. Enkephalins, methionine enkephalin (Met-Enk) in particular, were suggested to be an important modulator in the regulatory loop between immune and neuroendocrine systems. The following experimental results studied in my laboratory showed the *in vitro* and *in vivo* activities of Met-Enk on humoral and cellular immune activities, on cytokines production and expression, in normal and immunodeficient conditions.

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Effects of Met-Enk on lymphocyte proliferation

There are opioid binding sites on many immune active cells[5]. On the basis of this idea, we investigated the effect of a specific opioid antagonist naloxone on the immunoregulating actions of Met-Enk.

Met-Enk 0.1 – 100 nmol·L^{-1} increased both Con A- and PHA-induced splenocyte proliferation[6]. Yet, Met-Enk alone had no effect on resting lymphocyte proliferation. Naloxone, which was generally considered as a μ opioid receptor antagonist, also enhanced lymphocyte proliferation.

That Met-Enk and naloxone had neither additive nor antagonistic effects on Con A-induced lymphocyte proliferation was similar to the observations that naloxone as an agonist acted on β-endorphin and Met-Enk in inhibiting T-lymphocyte chemostatic factor[7]. *In vivo* studies gave the same results. Met-Enk (0.1 mg·kg^{-1}·d^{-1} ip to C57BL mice for 4 d) markedly increased Con A-induced splenocyte blastogenesis tested by [^{3}H]TdR incorporation and reversed the immune suppressive actions caused by ciclosporin[6]. By using of flowcytometry and mouse monoclonal antibodies, it was found that Met-Enk markedly elevated the ratio of T_h/T_s of splenocytes, but not in thymocytes.

On the contrary, Met-Enk 0.1 – 100 nmol·L^{-1} inhibited LPS-induced B-lymphocyte proliferation *in vitro*. This effect was completely blocked by naloxone 50 nmol·L^{-1}. Naloxone *pers se* had no effect on LPS-induced lymphocyte blastogenesis[6].

Murine non-adherent splenocytes treated with Met-Enk exhibited a marked increase in natural killer cell (NK) activity. Met-Enk 0.01 – 10 μmol·L^{-1} directly augmented the NK activity *in vitro*[8]. The changes were correlated with an increased concentration of Met-Enk.

According to our studies[6] and other reports[9], the influences of opioid peptides on immune functions were possibly through different sites on different immune competent cells and the final outcome of immunomodulation by opioid peptides is by their binding to μ and κ receptors (downregulation) and/or to δ and ϵ receptors (upregulation)[10]. The conflicting results among investigators might be due to the different immune status of animals and different methods used. Our *in vivo* results indicated that the enhancement of T-lymphocyte proliferation by Met-Enk was stronger in ciclosporin-induced immunosuppressed mice than in the normal mice. This suggests that the immunomodulating effect of opioids is related to the immune status of the animals.

Effects on antibody formation

Endogeneous peptides, such as Met-Enk influence humoral immune responses *in vitro* and *in vivo*[1,11,12]. Our studies demonstrated that Met-Enk inhibited LPS-stimulated B-lymphocyte proliferation[6]. It was suggested that the inhibitory effect of Met-Enk was mediated through opioid receptors on B-cells, because the suppressive effect of the peptide was completely blocked by naloxone. After incubation with both splenocytes and SRBC, Met-Enk inhibited markedly plaque forming cells (PFC) formation *in vitro*. *In vivo* studies also showed that Met-Enk ($0.1\ mg \cdot kg^{-1} \cdot d^{-1}$ ip to SRBC-sensitized C57BL mice) suppressed markedly splenocyte PFC[6].

The 3F3 hybridoma tumor cells were cultured with Met-Enk in multiple replicate 24-well plates. The amount of anti-human growth hormone (hGH) in culture supernatants was assayed by ELISA. Met-Enk ($10\ fmol \cdot L^{-1} - 1\ \mu mol \cdot L^{-1}$) showed no significant effects on the synthesis or secretion of hGH. Moreover, the gene transcriptions of both antibody light chain and heavy chain were not changed by Met-Enk at a wide range of concentrations ($1\ pmol \cdot L^{-1} - 1\ \mu mol \cdot L^{-1}$)[13]. Met-Enk increased the capacity of mouse spleen cells to produce hemolytic antibody to sheep erythrocytes[11] and inhibited anti-tetanus toxoid antibody synthesis by human lymphocytes[12]. Met-Enk at lower doses enhanced antibody production and at higher doses inhibited it[11]. These actions were seen when cells were stimulated or induced by antigens. In the absence of mitogen or antigen, Met-Enk did not change immune functions in nonimmunized animals. Our results also indicated that Met-Enk only regulated the antibody production while B-cells were stimulated with antigens or mitogens, but not resting B-cells[13].

Effects of Met-Enk on cytokines

Interleukin-1 (IL-1) In view of the fact that IL-1 plays an important role in immune responses and nervous activities, such as increasing IL-2 production and inducing slow-wave-sleep (SWS), it was very interesting to investigate the effect of Met-Enk on monokines originating from macrophages. Met-Enk $1 - 100\ nmol \cdot L^{-1}$ inhibited phagocytosis of peritoneal macrophages and increased this activity at higher concentrations[14].

LPS is a well-known IL-1 triggering agent. When Met-Enk ($1\ pmol \cdot L^{-1} - 1\ \mu mol \cdot L^{-1}$) was added to LPS-stimulated peritoneal macrophages cultures for 24 h, IL-1 production, evaluated by thymocyte proliferation, was enhanced[15]. Met-Enk had no effect on IL-1 production from non-stimulated peritoneal macrophages.

To identify whether the observed enhancement of IL-1 by Met-Enk was due to the increase of synthesis or release of IL-1, after the supernatant (extracellular IL-1) of the incubated peritoneal macrophages was collected, the remaining adherent cells were frozen and thawed 3 times and the intracellular IL-1 levels were determined. Both extra- and intra-cellular IL-1 were increased proportionally by this pentapeptide. Naloxone 50 $nmol \cdot L^{-1}$ *per se* had no effect on IL-1 production, nor blocked the enhancing effect of Met-Enk, suggesting that the enhancing effect was not mediated via classical opioid receptors. In addition, in the absence of mitogen, Met-Enk alone did not stimulate IL-1 production[15]. This indicated that only activated macrophages expressed functional receptors for Met-Enk.

Studies were made in mice treated daily with ip Met-Enk 0.1 or 0.6 $mg \cdot kg^{-1}$ for 2 d. Extracellular IL-1 was markedly elevated from peritoneal macrophages induced by LPS 5 $mg \cdot L^{-1}$. Some stressors, such as treating animals with LPS[16], injuries of mammalian brain[17,18] induced IL-1 like factors from glia cells in neural tissues. No distinction was apparent when comparing macrophages derived IL-1 with the IL-1 like mediator produced from astrocytes[19]. These activities mediated by IL-1 are beneficial to the defense mechanisms against microbial invaders and repair from brain injuries. In view of the fact that the level of opioid peptides increased in the brain during stress, the observation that Met-Enk enhanced IL-1 production from LPS treated macrophages both *in vitro* and *in vivo* suggested that Met-Enk might contribute to both immunoprotective and immunopathological responses in the brain.

Interleukin-2 It is known that *in vitro* lymphocyte proliferation and NK cell activity are regulated by IL-2 and it has a synergetic effect on murine NK cells with interferon (IFN). Thus, examining whether Met-Enk increases IL-2 production may be helpful in elucidating the mechanism of Met-Enk on host defense and cytotoxic activity. Besides, IL-2 was also found to promote oligodendrocytes proliferation and differentiation[20].

IL-2 activity was determined by the ability of mouse splenocyte producing cytokine in supernatants to support the growth of IL-2 dependent cell line CTLL-2. Met-Enk ($10 - 1000\ nmol \cdot L^{-1}$, when cultured with splenocytes in the presence of Con A for 24 h), enhanced IL-2 production from mouse spleen cells in a concentration-dependent manner. The enhancement of IL-2 production requires the presence of Con A ($0.5\ mg \cdot L^{-1}$). C57BL mice ip with Met-Enk 0.01, 0.1, and 1 $mg \cdot kg^{-1}$ for 5 d, both splenocyte transformation and IL-2 production were increased[21].

Met-Enk 0.1, 10, and 1000 $nmol \cdot L^{-1}$ enhanced Con A-induced transcription of IL-2 mRNA and these effects were similar to the effects of Met-Enk on the production of IL-2[22]. Con A-induced murine splenocytes were treated with or without Met-Enk for 18 h, dactinomycin was added to inhibit further RNA synthesis, except in the control, then RNA was extracted 1 h later. RNA blots were hybridized with γ-^{32}P labeled probe. Met-Enk $10 - 1000\ nmol \cdot L^{-1}$ remarkably increased the stability of IL-2 mRNA.

These studies demonstrated the augmenting effects of Met-Enk on IL-2 production and expression. The action that

Met-Enk enhanced the IL-2 production from murine splenocytes involved the transcriptional and post-transcriptional mechanisms because it increased both the accumulation and stability of IL-2 mRNA. This may be achieved either by inhibiting the synthesis of RNAase, inactivating specific RNA binding proteins or by reducing the reiterated number of AUUUA instability sequences in the IL-2 mRNA[23,24].

When Met-Enk 10 nmol·L^{-1}, an effective concentration for IL-2 production, was incubated with lymphocytes from mouse thymus, spleen, and gut-associated lymph node, in the presence of Con A for 48 h, it did not show any effect on IL-2 receptor expression, the % of IL-2R positive cells remained unchanged[21]. It seems that IL-2 protein production and IL-2 gene transcription are far more sensitive to the modulatory effects of Met-Enk than that of its receptor expression.

Interleukin-6 production and gene expression IL-6 is a pluripotent cytokine that promotes B-cell to produce antibody, stimulates growth of hybridomas and plasmacytomas as well as the growth and differentiation of T-cells. Its spectrum of activity includes the hematologic and neurologic fields. IL-6 is similar, in structure and function, to a family of neuropoieitic cytokines capable of modulating the expression of a number of neurotransmitter synthetic enzymes and neuropeptide hormones in post-mitotic neurons[25,26].

The effects of Met-Enk on the production and gene expression of IL-6 from macrophages and L929 cells *in vitro* and mouse splenocytes *in vivo* were examined in my laboratory, to obtain a better understanding of its neuroimmunomodulating processes. Our results demonstrated that IL-6 production from murine peritoneal macrophages was enhanced after treatment with Met-Enk (0.001 – 10 μmol·L^{-1}, 20-h incubation). LPS, a potent inducer of IL-6 expression, enhanced Met-Enk stimulation of IL-6 synthesis and secretion from macrophages. IL-6 production from L929 cells was also markedly stimulated by Met-Enk. IL-6 gene expression in L929 cells was elevated with Met-Enk 0.1 – 1000 nmol·L^{-1} after 2-h and 6-h incubation. However, IL-6 mRNA did not increase further at higher concentrations of Met-Enk[22].

Serum IL-6 levels in mouse ip Met-Enk (0.1 or 1 mg·kg^{-1} ×6 d) were markedly higher than that of control-saline treated mice, and were comparable to that after 2-h treatment with ip LPS 5 mg·kg^{-1}. The increase in IL-6 mRNA caused by Met-Enk was consistent with an increased stabilization of IL-6 mRNA. The results after IL-6 mRNA hybridization and autoradiation showed that Met-Enk at 0.01 and 1 μmol·L^{-1} did increase the stability of IL-6 mRNA[22].

The involvement of Met-Enk in IL-6 production may be of physiologic relevance in stress and inflammatory responses and may play a role in the communication between neuroendocrine and immune systems. The physiologic source of the opioids may be the cells of the immune system and/or neuroendocrine origin[27,28]. But IL-6 can be generated not only by cells of the immunocompetent cells but also by astrocytes and fibroblast cells[29]. Therefore, Met-Enk may be one of the messengers mediating the primary or secondary responses when body is stimulated by antigens.

Tumor necrosis factor (TNF) The action of Met-Enk on TNF was examined on macrophages stimulated with or without LPS. The enhancement on TNF-α levels was found at the highest concentration (5 μmol·L^{-1}) of Met-Enk. However, this peptide had no effect on LPS-induced TNF-α production. It was very interesting that *in vivo* the lower dose (0.1 mg·kg^{-1} × 6 d) of Met-Enk potentiated the synthesis and secretion of TNF-α, but the higher one (1 mg·kg^{-1} × 6 d) did not bring about significant change[8].

TNF-α is a factor of inherent anti-neoplastic activity *in vivo*. That the *in vitro* low dose of Met-Enk increased the production of TNF-α suggested that *in vivo* this opioid peptide would contribute to host immune surveillance. As higher dose of Met-Enk did not exert any influence on TNF-α *in vivo*, it might be due to an involvement of other secondary messenger systems which abolished its initial effect on TNF-α production indirectly[30].

Summary

Opioid peptides, formerly thought of primarily as signal molecules confined to the nervous system, are now known to play multiple roles in the coordination of internal immunoregulatory processes. Met-Enk induced IL-1, IL-2 production and gene expression as well as IL-6 up-regulation via the increase in transcriptional activity and stability of these interleukins' mRNA. Thus, Met-Enk affects most immune competent cells and cytokines, especially the pre-inflammatory cytokines. In conclusion, Met-Enk is an important immunomodulatory signaling molecule and exerts important regulatory actions concerned with the expressing pre-inflammatory cytokines. In this regard it would be a critical signal in initiating inflammatory cascades.

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甲硫氨酸脑啡肽的免疫调节作用

R971.5

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关键词 神经免疫调节; 甲硫氨酸脑啡肽; 纳洛酮; 阿片受体; 白细胞介素; 脂多糖; 肿瘤坏死因子; 信使 RNA; 基因表达

摘要 甲硫氨酸脑啡肽(Met-Enk)在神经-免疫-内分泌调节网络中的作用引起广泛关注. 作者等实验证明它能使 LPS 刺激的小鼠腹腔巨噬细胞产生 IL-1, 纳洛酮不能阻止这种作用. Met-Enk 促进小鼠脾淋巴细胞增殖和 IL-2、IL-6 的产生, 这种上调作用与增强此二种白细胞介素 mRNA 转录并提高其稳定性有关. 因此 Met-Enk 在增强前炎性细胞因子表达方面是一个重要的免疫调节信号分子.