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Effect of enkephalins and endorphins on cytotoxic activity of natural killer cells and macrophages/monocytes in mice

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Abstract

The effect of [Met^5]enkephalin, [Leu^5]enkephalin, proenkephalin, dynorphin-(1–17) or β-endorphin on the cytotoxic (^{51}Cr release assay) activity of natural killer cells and macrophages/monocytes was studied in mice. It was found that a single i.p. injection of [Met^5]enkephalin, [Leu^5]enkephalin, β-endorphin, dynorphin or proenkephalin as well as repeated treatment with both enkephalins increased natural killer cell activity. In vitro only [Met^5]enkephalin stimulated natural killer cells. Opioid peptides did not affect the cytotoxic activity of macrophages/monocytes. In addition to functional alterations, both enkephalins and β-endorphin increased the percentage of cells with natural killer phenotype. The results of this study suggest that the increase in natural killer cytotoxicity after opioid peptides injected once or for 14 days may result from an increased number of natural killer cells in the spleen.

Keywords: Natural killer cell; Macrophage; Enkephalin; Endorphin; Cytotoxicity

1. Introduction

Natural killer cytotoxicity is an extracellular leukocyte-mediated killing mechanism directed against transformed cells. It occurs spontaneously without sensitization and in the absence of antibody and complement. Many research workers have reported an enhancing effect of opioid peptides on natural killer cells in humans (Faith et al., 1984; Kay et al., 1984; Mandler et al., 1986; Mathews et al., 1983; Wybran, 1985). Some authors reported that γ-endorphin, [Leu^5]enkephalin and morphine stimulated natural killer cells (Faith et al., 1984; Kay et al., 1984; Wybran, 1985). Others, however, found that α-endorphin, γ-endorphin, [Leu^5]enkephalin and morphine were unable to stimulate natural killer cells (Mandler et al., 1986; Mathews et al., 1983). Using the indium release method, one group of researchers found that preincubating effector cells with β-endorphin and dynorphin reduced indium release from target cells by up to 50% in comparison with controls (Prete et al., 1986). In rodents, low doses of opioids often stimulate the immune system. A single injection of morphine or D-Ala-[Met^5]β-endorphin enhanced the activity of splenic natural killer cells (Kraut and Greenberg, 1986). In a mouse model, electric electrode stimulation shock to the tail transiently suppressed the activity of splenic natural killer cells, an effect which could be prevented by naloxone (Kraut and Greenberg, 1986).

I was prompted to carry out this study mainly because previous reports on the effects of enkephalins, β-endorphin and dynorphin on the cytotoxic activity of mouse spleen lymphocytes and macrophage/monocytes varied greatly.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice aged 4–6 weeks were obtained from the Animal Farm of the Silesian University School of Medicine. They were kept 8 per cage at room temperature and under standard light conditions. They received standard mouse chow and water ad libitum.

2.2. Agents

[Met^5]Enkephalin, [Leu^5]enkephalin, proenkephalin, dynorphin-(1–17) and β-endorphin were purchased from Sigma. ^{51}Cr used in the cytotoxic assay was provided by DuPont NEN. FITC anti-mouse 5E6 (natural killer cell antigen) monoclonal antibody was obtained from Pharmingen.

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2.3. Culture medium

Culture medium RPMI 1640 (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 2 mM glutamine, 100 μg/ml streptomycin and 100 IU/ml penicillin, designated as complete medium, was used.

2.4. Cytotoxicity assay

Spleens were cut under sterile conditions. A single cell suspension of spleen cells was prepared in a Cell Dissociation Sieve-Tissue Grinder (Sigma). Mononuclear cells were separated via gradient centrifugation with the use of Lymphoprep (Nyegaard Co., Oslo, Norway). Then a splenocyte layer was removed, resuspended in complete medium, washed 4 times, placed in a 96-well microtiter plate (Flow-Linbro) and incubated for 2 h (37°C, 5% CO_2, 80% humidity). The adherent cell fraction consisted of 89–91% macrophages/monocytes, 8–9% lymphocytes and 1–2% granulocytes. The non-adherent fraction of lymphocytes was contaminated by 3–5% macrophages/monocytes. Adherent and non-adherent cells were stained by the May-Grünwald-Giemsa method. Non-adherent cells were transferred to a 96-well microtiter plate and assayed for natural killer cell activity. Adherent cells, mainly macrophages/monocytes, were gently washed and used for cytotoxic study. P815 cells and YAC-1 cells were used as target cells in the macrophage cytotoxicity assay and the natural killer activity assay, respectively. YAC-1 and P815 cells were incubated in complete medium for 2 h with 200 μCi [^{51}Cr]chromate, washed 4 times and mixed with effector cells at different ratios. Natural killer cell activity was measured after 4 h of incubation, while macrophage/monocyte activity was measured after 16 h. After incubation, experimental ^{51}Cr release (ER) was measured in 100 μl of supernatant. Maximal ^{51}Cr release (MR) was defined as the release after addition of 100 μl of 1% sodium dodecyl sulphate. Spontaneous release (SR) was measured after ^{51}Cr-labelled target cells had been incubated only in complete medium. Specific lysis was calculated as follows:

$$\text{specific lysis } \% = \frac{\text{ER} - \text{SR}}{\text{MR} - \text{SR}} \times 100$$

Differences between control and treatment groups were statistically analyzed by Student's *t*-test with $P < 0.01$ considered as significant. The results shown in the Tables are means of three experiments (three measurements in each experiment).

2.5. Culture and animal treatment

In the in vitro experiment opioid peptides were dissolved in complete RPMI 1640 medium. In the in vivo experiment they were dissolved in sterile phosphate-buffered saline, and 0.1 ml was injected per mouse. In the first in vivo experiment [Met^5]enkephalin, [Leu^5]enkephalin (each in doses of 200, 100 and 10 μg per mouse), β-endorphin, dynorphin and proenkephalin (each in doses of 20, 10 and 1 μg per mouse) were injected i.p. once. In the second experiment mice received [Met^5]enkephalin or [Leu^5]enkephalin (10 or 1 μg/mouse) once or for 14 consecutive days. 24 h later mice were killed and splenectomized in order to determine the cytotoxic activity of natural killer cells and macrophages/monocytes.

In the in vitro experiment adherent or non-adherent spleen cells were placed in a 96-well microtiter plate and exposed to [Met^5]enkephalin, [Leu^5]enkephalin, β-endorphin, dynorphin, or proenkephalin at concentrations of 1 μM, 10 nM and 100 pM for 24 h. Then ^{51}Cr-labelled YAC-1 cells or P815 cells were added and cytotoxic activity was measured as described in Section 2.4.

2.6. Determination of percentage of natural killer (5E6 antigen-positive) cells

Mononuclear cells were separated using Lymphoprep gradient centrifugation, washed with phosphate-buffered saline and diluted in RPMI 1640 medium supplemented with 5% fetal calf serum and 0.1% sodium azide. Cells (1×10^6) were incubated with 10 μl of monoclonal antibodies conjugated with fluorescein isothiocyanate in a total volume of 100 μl for 30 min (4°C). This antibody binds to the 5E6 antigen, which is present on approximately 50% of natural killer-1.1$^+$ cells. After incubation, the cells were twice washed with RPMI 1640 medium supplemented with 5% fetal calf serum and 0.1% sodium azide and then resuspended in 50 μl of the same medium. At least 400 cells in each smear were counted under a fluorescence microscope (Nikon, Japan) and the percentage of cells with green fluorescence was calculated.

3. Results

3.1. Augmentation of natural killer cell activity

In the in vivo experiment, [Met^5]enkephalin and [Leu^5]enkephalin at doses of 200 and 10 μg, β-endorphin, dynorphin, proenkephalin at a dose of 1 μg and proenkephalin at a dose of 20 μg increased natural killer activity. No changes were observed after 20 μg of dynorphin or β-endorphin. Except for proenkephalin, the increase in natural killer activity was greater after the lower doses of the peptides than after the higher doses (Table 1). The effect of a single injection of 10 μg and 1 μg [Met^5]enkephalin or [Leu^5]enkephalin on natural killer cell activity was similar to that observed after 14-day administration of these enkephalins. However, 10 μg of [Met^5]enkephalin given for 14 days augmented natural killer activity to a greater degree in comparison with the effect

Table 1
Effect of opioid peptides (expressed as % cytotoxicity ± S.E.) on natural killer cell activation in vivo

Agent	Dose	Effector/target cell ratio		
	(μg/mouse)	100:1	50:1	25:1
Control	–	12 ± 0.1	8 ± 0.1	4 ± 0.1
[Met^5]Enkephalin	200	16 ± 0.4 [a]	10 ± 0.1	6 ± 0.1
[Met^5]Enkephalin	10	19 ± 0.3 [a]	11 ± 0.3	7 ± 0.4
[Leu^5]Enkephalin	200	17 ± 0.3 [a]	13 ± 0.3 [a]	7 ± 0.2
[Leu^5]Enkephalin	10	22 ± 0.3 [a]	13 ± 0.2 [a]	7 ± 0.3
β-Endorphin	20	13 ± 0.3	11 ± 0.2	6 ± 0.2
β-Endorphin	1	20 ± 0.5 [a]	11 ± 0.3	8 ± 0.3
Dynorphin-(1–17)	20	15 ± 0.3	12 ± 0.4	6 ± 0.3
Dynorphin-(1–17)	1	19 ± 0.4 [a]	11 ± 0.3	7 ± 0.2
Proenkephalin	20	16 ± 0.4 [a]	14 ± 0.2 [a]	7 ± 0.3
Proenkephalin	1	16 ± 0.4 [a]	12 ± 0.2	5 ± 0.1

Mice (3/group) were injected with different doses of opioid peptides dissolved in phosphate-buffered saline 1 day before splenic natural killer cell activity was assessed (see Section 2). Results are expressed as the means ± S.E. of three experiments.
[a] Significant increase in comparison with control ($P < 0.01$).

Table 2
Effect of enkephalins given once or for 14 days (expressed as % Cytotoxicity ± S.E.) on natural killer cell activation in vivo

Agent	Dose	Effector/target cell ratio		
	(μg/mouse)	100:1	50:1	25:1 [b]
1 injection				
Control	–	14 ± 0.5	11 ± 0.4	8 ± 0.2
[Met^5]Enkephalin	10	21 ± 0.9 [a]	18 ± 0.6 [a]	10 ± 0.4
[Met^5]Enkephalin	1	19 ± 0.8 [a]	13 ± 0.7	8 ± 0.3
[Leu^5]Enkephalin	10	21 ± 0.4 [a]	14 ± 0.3	10 ± 0.6
[Leu^5]Enkephalin	1	19 ± 0.9 [a]	14 ± 0.6	8 ± 0.4
14 injections				
Control	–	15 ± 0.6	14 ± 0.6	9 ± 0.2
[Met^5]Enkephalin	10	27 ± 0.6 [a]	19 ± 0.4 [a]	14 ± 0.6
[Met^5]Enkephalin	1	21 ± 0.8 [a]	17 ± 0.6	13 ± 0.4
[Leu^5]Enkephalin	10	21 ± 1.2 [a]	20 ± 0.5 [a]	9 ± 0.4
[Leu^5]Enkephalin	1	21 ± 0.6 [a]	17 ± 0.3	11 ± 0.3

Mice (3/group) were injected i.p. with enkephalins dissolved in phosphate-buffered saline (once a day). Natural killer cell activity was assessed 1 day after the last injection (see Section 2). Results are expressed as the means ± S.E. of three experiments.
[a] Significant increase in comparison with control ($P < 0.01$).

Table 3
The effect of methionine-enkephalin on natural killer cell activation in vitro

Agent	Concentration	% Cytotoxicity ± S.E.
Control	–	5 ± 0.2
[Met^5]Enkephalin	1 μM	20 ± 1.8 [a]
[Met^5]Enkephalin	10 nM	31 ± 2.7 [a]
[Met^5]Enkephalin	100 pM	22 ± 1.5 [a]

Spleen lymphocytes from C57BL/6 mice were incubated in vitro 24 h with complete medium or various doses of methionine-enkephalin and examined as described in Section 2. Each point is a mean of triplicates and expressed as a percentage.
[a] Significant increase in natural killer cell activity ($P < 0.01$).

Table 4
Effect of opioid peptides on natural killer cell frequency

Agent	Dose	Percentage of natural killer cells ± S.E.	
	(μg/mouse)	1 injection	14 injections
Control	–	3.5 ± 0.1	3.4 ± 0.1
[Met^5]Enkephalin	10	9.4 ± 0.5 [a]	9.9 ± 0.7 [a]
[Leu^5]Enkephalin	10	9.0 ± 0.6 [a]	10.0 ± 0.9 [a]
β-Endorphin	1	9.1 ± 0.4 [a]	–

Mice (3/group) were injected with enkephalins once or for 14 days (1 inj./day). Frequency of natural killer phenotype cells was determined 1 day after the last injection (see Section 2). Results are expressed as the means ± S.E. of three experiments (in each experiment at least 400 cells were counted in 3 samples).
[a] Significant increase in comparison with control ($P < 0.01$).

induced by 10 μg of [Met^5]enkephalin given once (Table 2).

In the in vitro experiment, only 1 μM, 10 nM, 100 pM [Met^5]enkephalin significantly augmented natural killer cell activity. Natural killer cells were maximally stimulated by 10 nM [Met^5]enkephalin (Table 3). None of the concentrations of β-endorphin, dynorphin, proenkephalin and [Leu^5]enkephalin stimulated natural killer cell activity in vitro (results not presented).

3.2. Macrophage / monocyte activation

In the in vivo experiment, [Met^5]enkephalin, [Leu^5]enkephalin, β-endorphin, dynorphin and proenkephalin did not stimulate macrophage/monocyte activity. In the in vitro experiment, none of the concentrations of [Met^5]enkephalin, [Leu^5]enkephalin, β-endorphin, dynorphin and proenkephalin (1 μM, 10 nM, 100 pM) affected macrophage/monocyte cytotoxic activity (results not presented).

3.3. Modulation of percentage of natural killer (5E6-positive) cells by opioid peptides

The percentage of 5E6-positive cells was determined in mice treated with enkephalins for 1 and 14 days, and with β-endorphin for 1 day. Natural killer phenotype-positive cells increased in the spleens of mice treated with [Met^5]enkephalin, [Leu^5]enkephalin and β-endorphin (Table 4).

4. Discussion

It has been suggested that enkephalins and β-endorphin may modulate the functions of both the immune and the nervous system. This study supports this hypothesis. Some authors state that endogenous opioid peptides inhibit natural killer cell activity. This study, however, has shown that exogenous enkephalins and endorphins enhance natural

killer cell activity. Perhaps the reason for this discrepancy is that the effects of enkephalins and endorphins may depend on the baseline activity of lymphocytes. As a result, opioid peptides could increase the cytotoxic activity of unstimulated lymphocytes, while they decrease the activity of strongly stimulated lymphocytes (Fiatarone et al., 1988; Oleson and Johnson, 1988; Rowland et al., 1987). In cell cultures only [Met^5]enkephalin increased natural killer cell activity. This finding suggests that [Met^5]enkephalin may directly act upon the opioid receptors of natural killer cells, while β-endorphin, dynorphin, [Leu^5]enkephalin and proenkephalin probably need to interact with other cells of the immune system or with stimulating factors in order to increase natural killer cell activity (Bessler et al., 1990; Gilmore and Weiner, 1988). To test whether the observed modulation of natural killer cell activity depends on the number of natural killer cells, I analyzed the expression of 5E6 antigen – a marker of the mouse natural killer cells – in mice treated with opioid peptides. Both enkephalins and β-endorphin increased the percentage of spleen cells presenting 5E6 antigen, which may explain the augmented cytotoxicity. Alterations in perforin activation and of other known killing mechanisms of neoplastic transformed cells by natural killer cells should also be taken into consideration.

Single injections of enkephalins and endorphins, 14-day enkephalin treatment and in vitro exposure of spleen monocytes/macrophages to opioid peptides did not affect the cytotoxic activity of natural killer cells. It is not clear why opioid peptides stimulate natural killer cells and do not enable macrophages to kill neoplastic cells. It is possible that an immunomodulator may stimulate one cell type and not affect the function of other cells. For example, Talmadge et al. (1986) have shown that bestatin stimulates the tumoricidal activity of macrophages but does not affect the cytotoxic activity of natural killer cells. In contrast, Kowalski et al. (1995) have found that bestatin augments the activity of natural killer cells but not of macrophages. Radulovic et al. (1995) have shown that the potential stimulation of macrophages by opioids depends on the animal strain. It cannot be excluded that the macrophages used in this study showed a very weak response to opioid peptide modulation. Enkephalins and endorphins can directly increase such parameters as H_2O_2 release (Radulovic et al., 1995) and chemotaxis (Van Epps and Saland, 1984). However, Foster and Moore (1987) and Hagi et al. (1994) showed that opioid peptides did not increase the activity of unstimulated peritoneal macrophages, but increased the activity of macrophages stimulated by γ-interferon and lipopolysaccharide. In the present study I measured ^{51}Cr release from P815 neoplastic cells killed by macrophages. The killing of neoplastic cells requires the activation of many intracellular processes via antigenic and receptor stimulation. Thus stimulation of macrophage opioid receptors only may be insufficient for triggering killing mechanisms in vitro.

The issue of [Met^5]enkephalin treatment of immunological deficiency is still discussed. Recent studies indicate that up to 0.5% of the mRNA made by activated helper T cells encodes preproenkephalin, and that activated helper T cells secrete immunoreactive [Met^5]enkephalin (Zurawski et al., 1986). Therefore, enkephalins may function as 'primitive cytokines', which are able to augment the activity of immunocompetent cells. Inhibition of the activity of enkephalinase present on human CD10$^+$ polymorphonuclear leukocytes dramatically potentiates the stimulatory effect of [Met^5]enkephalin on these cells (Shipp et al., 1991), suggesting an important immunostimulatory role of endogenous enkephalins. In newly diagnosed patients, single administration of [Met^5]enkephalin had a salutary effect on a number of immune parameters (Plotnikoff et al., 1986; Wybran and Schandene, 1986). This study and other reports concern only murine spleen, while the activity of human natural killer cells is studied in blood. It is assumed that there is a certain correlation between the activity of spleen natural killer and blood lymphocytes because it has been shown that their activity in the spleen and blood increases similarly (Budzynski et al., 1987).

Long-term administration of natural killer stimulators such as interferons decreases their stimulatory effect on natural killer cells (Bruley-Rosset and Rappaport, 1983; Brunda and Rosenbaum, 1984). The fact that the number of cytotoxically active natural killer cells increased to a similar level both after a single and 14 injections of [Met^5]enkephalin may be interesting in the context of discussion concerning the use of [Met^5]enkephalin as an immunostimulating drug.

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