



Effect of Exogenous Opioid Peptides on TNF- α -Induced Human Neutrophil Apoptosis *in Vitro*

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Abstract. Polymorphonuclear leukocyte (neutrophil) apoptosis is an important mechanism regulating the life span and some functions of neutrophils at inflamed sites. Opioid peptides are present in the peripheral circulation and their concentrations rapidly increase as a result of stress and inflammation. The effect of opioid peptides such as met-enkephalin (M-ENK) and β -endorphin (β -END) on tumor necrosis factor (TNF)- α -induced apoptosis in human neutrophils *in vitro* was investigated. Neutrophils isolated from peripheral blood were cultured in the absence or presence of 10^{-6} - 10^{-10} M of opioid peptides for 8, 12 and 18 h. Features of apoptotic neutrophils were measured by a flow cytometric method based on analysis of the apoptotic nuclei (DNA content). We found that M-ENK and β -END enhanced both uninduced and TNF- α -induced neutrophil apoptosis *in vitro* in a dose-dependent manner. The effect of opioid peptides on the modulation of neutrophil apoptosis was not reversed by the opioid-receptor antagonist naloxone. The results suggest that M-ENK and β -END can regulate neutrophil life span via apoptosis and in this way may participate in the resolution of inflammation.

Key words: apoptosis; opioid peptides; human polymorphonuclear leukocytes (neutrophils); flow cytometry; TNF- α .

Introduction

Bi-directional communication occurs between the neuroendocrine and immune systems, and these neuroimmunological interactions involve a variety of molecules and cells in these systems^{1, 19, 20, 35, 41}. Opioid peptides, such as endorphins and enkephalins share a common N-terminal repeated sequence which consists of five amino acids Tyr-Gly-Gly-Phe-Met or Leu, and this is responsible for their biological activity. The smallest molecule, called met-enkephalin (M-ENK), is

a pentapeptide; the largest, β -endorphin (β -END), is composed of 31 amino acids.

Stress reaction and inflammation are associated with the release of high levels of neuroendocrine mediators, such as catecholamines and opioid peptides, into systemic circulation²¹. It has been found that cells of the immune system are the source of different neuropeptides, including opioid peptides^{16, 27, 30, 42}. VINDROLA et al.⁴⁰ have demonstrated that human peripheral polymorphonuclear cells contain and release proenkephalin-derived peptides. Receptors for the naturally occurring opioids

Abbreviations used: β -END – β -endorphin, M-ENK – met-enkephalin, PMNs – polymorphonuclear leukocytes, TNF- α – tumor necrosis factor α , PBS – phosphate-buffered saline, PI – propidium iodide.

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have been found and characterized on human leukocytes^{2, 3, 10, 24, 34}. Therefore, endogenously released or exogenously administered opioids may exhibit their biological activity and exert influence on inflammatory and immune cells and participate in the inflammatory process.

Normal human polymorphonuclear leukocytes (PMNs, neutrophils) are short-lived cells, with a half-life oscillating between 8 to 20 h. Mature neutrophils spontaneously undergo apoptosis *in vitro* and *in vivo*. The life span and functional activity of mature PMNs can be extended and modified *in vivo* and *in vitro* by numerous agents, such as proinflammatory cytokines, including granulocyte colony-stimulating factor (G-CSF), interleukin (IL)-1, IL-8, lipopolysaccharide (LPS), and tumor necrosis factor (TNF)- α ^{4, 6, 7, 22, 23, 25}. The aim of this study was to examine the effect of M-ENK and β -END on the course of TNF- α -mediated apoptosis in normal human neutrophils *in vitro*.

Materials and Methods

Cell preparation. PMNs were isolated from heparinized human peripheral blood collected from healthy donors using one-step density-gradient centrifugation on GradiSol G (Polfa, Kutno, Poland) at 400 g at room temperature for 30 min. The residual erythrocytes were removed from the cell population by hypotonic lysis. The neutrophils were washed twice and resuspended in phosphate-buffered saline (PBS, Biomed, Lublin, Poland)⁴⁴. The cell suspensions were stored in PBS at 4°C and used within 2 h. Cell purity (>98%) and viability (>98%) were determined by May-Grünwald-Giemsa (AQUA-MED, Łódź, Poland) staining of cytocentrifuged samples and by Trypan blue (Sigma, USA) exclusion, respectively.

Informed consent was obtained from all donors. The Local Ethical Committee accepted this study.

Culture conditions of normal human neutrophils. Neutrophils were cultured in RPMI 1640 supplemented with 10% fetal calf serum and antibiotics: 100 U of penicillin and 100 μ g/ml of streptomycin (Sigma, USA). Either 2 ml or 200 μ l of cell suspension (1×10^6 /ml) were placed in 24- or 96-well plates (NUNC, Denmark), respectively, and were cultured – under duplicate conditions in humidified atmosphere at 37°C with 5% CO₂. Neutrophil cultures were incubated in the presence or absence of 10^{-6} – 10^{-10} M of M-ENK and β -END (Sigma, USA) for 8, 12 and 18 h and then washed once in PBS and used in assays of neutrophil apoptosis. In the set of experiments with recombinant

human TNF- α (Sigma, USA), cells were first incubated in the presence of opioid peptides or PBS (control sample) at 37°C for 30 min and TNF- α (10.0 ng/ml, Sigma, USA) was then added. The cells were harvested after 8, 12, and 18 h. Aliquots were removed and washed once in PBS before use in assays of neutrophil apoptosis. In a separate set of experiments with the opioid receptor antagonist, naloxone, neutrophils were incubated with opioid peptides alone or together with naloxone (10^{-6} M/ml, Sigma, USA) at the indicated concentrations²⁴.

Measurement of PMN apoptosis by flow cytometry. Apoptotic cell death was assessed by flow cytometric analysis of DNA content using propidium iodide (PI) staining. DNA content was analyzed by flow cytometry as previously described by NICOLETTI et al.²⁶ with a slight modification by KETTRITZ et al.²². Briefly, PMNs (10^6 /200 μ l), after washing with PBS supplemented with 0.5 mM EDTA (Sigma, USA), were resuspended in ice-cold 70% ethanol and stored at –20°C for one to two days. Then the neutrophils were treated with ribonuclease A (10 μ g/ml, Sigma, USA) and stained with 20 μ g/ml of PI (Sigma, USA). The cells were kept in the dark at room temperature for 15 min and then stored at 4°C until flow cytometric analysis (FACScan flow cytometer, Becton Dickinson, USA). The fluorescence of individual nuclei was measured using Cell Quest software for cell acquisition and data analysis. The percentage of cells with apoptotic nuclei (located as a hypodiploid DNA peak in the DNA fluorescence histogram) was calculated^{8, 9, 11}.

Statistical analysis. The results are expressed as the mean $\pm$ SD of at least five independent experiments. Evaluation of statistical significance was performed by Wilcoxon's signed rank test. A value of $p < 0.05$ was considered significant.

Results

Apoptosis of neutrophils was determined *in vitro* by flow cytometric analysis of DNA content using PI staining (Fig. 1).

To determine the effect of TNF- α on the course of neutrophil apoptosis *in vitro*, PMNs were incubated in RPMI 1640 with TNF- α (10.0 ng/ml) or without the cytokine. The data are presented as the mean percentage of cells with apoptotic nuclei (Table 1). These results show that the TNF- α -accelerated apoptosis effect was at the 8-hour time point. Longer incubation of neutrophils with TNF- α had no such effect. At 12 h and 18 h, the apoptosis of TNF- α -treated neutrophils was not significantly different from that of the untreated

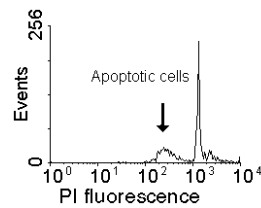


Fig. 1. An example of the quantitation of apoptosis using flow cytometry. Histogram of cells analyzed for propidium iodide fluorescence (DNA content). Histogram shows the appearance of the apoptotic region (indicated by arrow) of cells undergoing apoptosis

Table 1. Time course of the effect of TNF- α on human neutrophil apoptosis *in vitro*

Stimulator	DNA content – apoptotic nuclei (%)			
	0 h of culture	8 h of culture	12 h of culture	18 h of culture
Medium alone	3.2 $\pm$ 1.4	15.2 $\pm$ 3.5	55.0 $\pm$ 10.4	81.2 $\pm$ 5.6
TNF- α	4.1 $\pm$ 2.0	21.8 $\pm$ 3.0*	49.2 $\pm$ 5.5	76.0 $\pm$ 8.2

Statistical significance: * $p < 0.05$ vs. medium alone.

Table 2. The effect of opioid peptides on neutrophil apoptosis *in vitro* measured by DNA content

Stimulator	DNA content – apoptotic nuclei (%)		
	8 h of culture	12 h of culture	18 h of culture
M-ENK			
0 M	15.2 $\pm$ 3.5	41.0 $\pm$ 15.0	68.0 $\pm$ 10.0
10^{-6} M	15.6 $\pm$ 4.0	44.0 $\pm$ 16.0	80.0 $\pm$ 7.0*
10^{-8} M	20.8 $\pm$ 5.0*	53.0 $\pm$ 14.0	82.0 $\pm$ 7.0*
10^{-10} M	18.6 $\pm$ 6.8	53.0 $\pm$ 15.0	68.0 $\pm$ 19.0
β -END			
0 M	13.6 $\pm$ 2.1	50.1 $\pm$ 17.0	75.0 $\pm$ 9.0
10^{-6} M	14.2 $\pm$ 5.5	47.1 $\pm$ 18.0	69.0 $\pm$ 8.0
10^{-8} M	23.6 $\pm$ 5.1*	58.4 $\pm$ 17.6	67.0 $\pm$ 15.0
10^{-10} M	20.4 $\pm$ 4.1*	58.0 $\pm$ 9.0	75.0 $\pm$ 10.0

The data are given as the mean and SD of the percentage of cells with hypodiploid DNA.

* Values statistically significant: * ($p < 0.05$) vs. control (0 M).

Table 4. The effect of the opioid-receptor antagonist naloxone on neutrophil apoptosis *in vitro*

Stimulator	Apoptotic nuclei (%)	
	naloxone ⁻	naloxone ⁺
Medium alone	56.1 $\pm$ 4.2	55.2 $\pm$ 8.7
M-ENK 10^{-8} M	64.4 $\pm$ 9.9*	65.6 $\pm$ 6.7*
β -END 10^{-8} M	68.8 $\pm$ 8.6*	67.2 $\pm$ 6.6*

Values statistically significant: * $p < 0.05$, compared with medium alone.

control cells. On the basis of these data we assessed the next set of experiments at the 8-hour and 12-hour time points. The effects of M-ENK and β -END on neutrophil apoptosis were dependent on the time of culture and dose of opioids (Table 2). The presence of M-ENK at the concentrations of 10^{-6} and 10^{-8} M in medium caused a significant increase in apoptotic cells after 18 h of culture as compared with the control cell samples. The percentages of apoptotic cells in β -END-treated (10^{-8} and 10^{-10} M) and control samples differed statistically after 12 h of culture.

The effect of M-ENK and β -END on TNF- α -induced human neutrophil apoptosis is shown in Table 3. A significant enhancement of TNF- α -mediated neutrophil apoptosis was observed after preincubation of the cells with M-ENK and β -END in 8 h of culture. After 12 h, TNF- α had no significant effect on neutrophil apoptosis. However, the ability of TNF- α to enhance the apoptosis of neutrophils was maintained if neutrophils were preactivated with M-ENK at concentrations of 10^{-8} M and 10^{-10} M and β -END at 10^{-6} M and 10^{-8} M for 30 min before TNF- α addition.

We have examined whether the effects of both M-ENK and β -END on neutrophil apoptosis are mediated via a specific opioid receptor. As is shown in Table 4, incubation of neutrophils together with M-ENK, β -END and the opioid-receptor antagonist naloxone (10^{-6} M/ml, Sigma, USA) for 18 h at 37°C did not reveal any differences in their effect on cell apoptosis. These results

Table 3. Effect of met-enkephalin (M-ENK) and β -endorphin (β -END) on TNF- α -mediated human neutrophil apoptosis

Opioid peptides	DNA content – apoptotic nuclei (%)			
	8 h of culture		12 h of culture	
	TNF- α ⁻	TNF- α ⁺	TNF- α ⁻	TNF- α ⁺
Medium alone	13.6 $\pm$ 2.1	22.8 $\pm$ 3.7*	60.4 $\pm$ 8.7	65.4 $\pm$ 5.5
M-ENK 10^{-6} M	15.6 $\pm$ 4.0	34.2 $\pm$ 12.6**	59.0 $\pm$ 13.8	73.8 $\pm$ 6.9
M-ENK 10^{-8} M	21.8 $\pm$ 3.0*	38.8 $\pm$ 9.2**	72.8 $\pm$ 8.1	87.2 $\pm$ 3.4**
M-ENK 10^{-10} M	18.6 $\pm$ 6.8	37.8 $\pm$ 11.3**	62.8 $\pm$ 11.0	80.4 $\pm$ 3.5**
β -END 10^{-6} M	14.2 $\pm$ 5.5	44.8 $\pm$ 21.5	65.4 $\pm$ 4.6	81.0 $\pm$ 2.6**
β -END 10^{-8} M	23.6 $\pm$ 5.1	47.5 $\pm$ 12.0**	71.6 $\pm$ 6.2*	76.0 $\pm$ 6.4**
β -END 10^{-10} M	20.4 $\pm$ 4.1*	51.0 $\pm$ 18.0**	72.0 $\pm$ 8.0*	77.0 $\pm$ 9.5

Values statistically significant: * $p < 0.05$, compared with medium alone; ** $p < 0.05$, compared with TNF- α -mediated apoptosis.

show that naloxone did not prevent the enhancement of apoptosis induced by opioid peptides. Naloxone administered alone or 30 min before addition of the opioid peptides had no effect on neutrophil survival.

Discussion

Neutrophil apoptosis as an *in vitro* model to examine the effect of exogenous opioid peptides on neutrophil survival was used in this study. In response to tissue inflammation, PMNs migrate to the inflamed site, where they are exposed to numerous environmental signals, such as soluble substances, including cytokines and neuroendocrine mediators (opioid peptides), as well as cell-cell and cell-matrix interactions. These inflammatory agents may function as regulators of neutrophil activity. The concentrations of opioid peptides in the peripheral circulation rapidly increase during acute inflammation^{16, 21}. There are controversial data concerning the effect of opioid peptides on some functions of human neutrophils. HABERSTOCK et al.¹³ have shown that human neutrophils released increased amounts of superoxide anion when they were treated with 10^{-8} M M-ENK, whereas other concentrations caused the stimulation in different ways. It has been also shown that M-ENK or β -END might, like inflammatory cytokines, prime neutrophils to enhanced response after subsequent challenge with specific activation^{12, 13, 28, 29, 39}. The ability of opioid peptides to stimulate or inhibit the migration and adhesion of inflammatory cells suggests that these molecules may contribute to the regulation of the inflammatory response by modulation of cell infiltrate^{14, 43}. Neutrophil apoptosis provides a signal for PMN removal from inflammatory sites by macrophages and also results in the loss of functional PMN reactivity³⁷. The modulation of this process is very important for maintaining the balance between defense and injury^{32, 33}. The *in vitro* effect of M-ENK and β -END varies greatly, depending on the time of exposure of cells to their action and the doses used; some concentrations have no effect on apoptosis in neutrophils, and others decrease neutrophil survival by enhancing their apoptosis.

The mechanisms by which the tested opioids affect neutrophil apoptosis are unclear. We would like to suggest some of them. The priming phenomenon is referred to as a process wherein the response of neutrophils to an activating stimulus is enhanced considerably by pre-exposure of cells to the priming agents¹⁵. Some authors have considered that most neutrophil priming agents delay apoptosis and hence increase the survival of neutrophils at the inflamed site^{5, 25}. PAŚNIK et al.²⁹

and HABERSTOCK and MAROTTI¹² have shown that these opioid peptides, like inflammatory cytokines, might prime the neutrophil function *in vitro*. This might suggest the delay of apoptosis in the presence of opioid peptides. MURRAY et al.²⁵ have shown that TNF- α contrary to other priming agents can promote neutrophil apoptosis, but this effect was restricted to the 8-hour incubation period studied. Our data revealed the pro-apoptotic effect of opioid peptides and also showed that the pro-apoptotic effect of TNF- α on neutrophils was extended to up to 12 h after incubation with opioid peptides. In addition, a slight synergistic effect of opioid peptides and TNF- α on apoptosis induction was observed.

A number of studies have provided evidence for the presence of δ , μ and κ opioid receptors and their subtypes on human inflammatory cells^{24, 36, 38}. Different classes of opioid receptors can be present on the same cell. It has been proposed that granulocytes contain two possibly related receptors mediating opposing effects: a stimulatory, opioid peptide-sensitive δ_2 -receptor and an inhibitory, opioid peptide-insensitive μ_3 -receptor²⁴. To determine the effect of the opioid receptor on neutrophil apoptosis we used its antagonist, naloxone. Naloxone is known as a "non-selective" antagonist since it interacts not only with μ - but also with δ - and κ -receptors^{18, 31}. We reported here that naloxone was ineffective in delaying neutrophil apoptosis and did not prevent the enhancement of apoptosis induced by opioid peptides. This may suggest that this effect of β -END and M-ENK is not mediated by an opiate receptor of the classical type, but perhaps by a non-opiate-specific receptor¹⁷. This might imply the involvement of another system mediating the opioid effect.

In summary, opioid peptides such as M-ENK and β -END enhance apoptosis in untreated neutrophils and in PMNs treated with TNF- α *in vitro* in a dose-dependent manner. We have shown that opioid peptides can regulate neutrophil functions via apoptosis and this way may participate in the resolution of inflammation. Our results indicate, that depending on the balance of inflammatory mediators present at an inflamed side, the longevity of neutrophils can be enhanced or decreased. When neutrophil apoptosis is augmented by the synergistic effect of opioid peptides and TNF- α , then the removal of activated neutrophils from the inflamed site is also accelerated and facilitated. M-ENK and β -END may contribute to the regulation of the inflammatory response by modulation not only of the inflammatory cell infiltrate, but also by a modification of the life span of resting or activated cells.

Acknowledgment. We are grateful to K. Krawczyk, M.S., for technical assistance.

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Received in January 2003

Accepted in April 2003