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Endogenous Opioids Modulate Immunosuppressive Effect of Exogenous Corticosteroids in Mice

Henryk Lach[1], Krystyna Pierzchała-Koziec[2*], Joanna Zubel-Łojek[2], Zbigniew Doniec[3], Małgorzata Plackowska[4], Ewa Ocłoń[2], Anna Latacz[2]

[1]*Joseph Dietl Malopolska Higher School in Cracow, Rynek Główny 34, Cracow, Poland,*
[2]*University of Agriculture, Al. Mickiewicza 24/28, Cracow, Poland,*
[3]*Department of Pneumonology, Institute for Tuberculosis and Lung Diseases, Rabka-Zdroj Branch, Prof. J. Rudnika 3b, Rabka-Zdroj, Poland*
[4]*G. Narutowicz Municipal Specialist Hospital in Cracow, Prądnicka 35/37, Cracow, Poland*

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Endogenous opioid peptides are involved in modulation of pain, immune system regulation and activity of corticosteroids. However, in spite of many experiments, the direct effect of opioids on the corticosteroid-induced immunosuppression was not clearly proved. The aim of the study was to compare endogenous opioid peptide responses to different corticosteroid treatments during chronic inflammation in mice. Chronic inflammation was induced by inhalation of ovoalbumin with aluminium ammonium sulfatum complex (mice asthma model). Budesonide (corticosteroid GI) was given by inhalation for 34 days, prednisolone (GII) was injected every 7 days (5 injections in total). After 48 days of the experiment, blood, spleen and bone marrow were removed, and white blood cells were counted using a hemocytometer, Met-enkephalin concentrations were measured in the bone marrow and spleen by radioimmunoassay method. The total number of leukocytes was increased by chronic inflammation, but both corticosteroids decreased the number of white blood cells ($P<0.01$). Met-enkephalin concentration in the bone marrow was significantly decreased in all the experimental groups ($P<0.001$). On the other hand, in the spleen the opioid concentration was increased by inflammation and this effect was attenuated only by prednisone immunosuppression. The obtained results clearly showed the involvement of opioid peptides in the modulation of the immunosuppressive effect of corticosteroids in mice.

Key words: opioids, inflammation, corticosteroids, immunosuppression, mice

INTRODUCTION

Endogenous opioid peptides (EOP) belong to three families: endorphins, enkephalins and dynorphins and are released from their precursors by enzymatic hydrolysis. They act through the specific G protein-coupled receptors named μ (MOR), δ (DOR) and κ (KOR). Endogenous opioids are widely distributed in the central nervous system and in the peripheral tissues. They are also released from immune cells which express

*e-mail rzkoziec@cyf-kr.edu.pl

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opioid receptors. Opioid receptor transcripts have been found mainly in lymphocytes, macrophages and dendritic cells but their distribution and activity are different for each subclass (Benard et al., 2008). Earlier studies showed that the expression of opioid receptors changed under cytokine action and immune cell activity (Sharp, 2004; Kraus et al., 2006).

An inflamed tissue is a target for neutrophils, lymphocytes and monocytes, however, the recruitment of immune cells is controlled by chemokines and additionally by neuropeptides, such as opioids released from peripheral sensory nerve terminals. EOP elicit analgesia and modulate the locomotion of immune cells at the site of inflammation. Pello et al., (2006) showed that under *in vitro* conditions opioids increased integrin-stimulated adhesion and migration of all types of leukocytes.

The study of Jaume et al., (2007) and Benard et al., (2008) showed that opioids locally produced by leukocytes at the site of inflammation reduced the intensity of migration of mature dendritic cells to lymphatic vessels. While the opioid-mediated modulation of the migration of monocytes and lymphocytes has been extensively studied in the peripheral and inflamed tissues, changes in opioid peptides in the major lymphatic organs, such as bone marrow and spleen, are still unknown.

Chronic inflammation often accompanies different diseases, such as diabetes, obesity, allergy or asthma. The main pharmacological treatment is based on exogenous corticosteroids applied systematically by injection or by inhalation. Long-term treatment with corticosteroids may cause side effects manifested by immunosuppression, perturbation of glucose and lipid metabolism, and growth retardation. Inflammation also affects endogenous opioid peptides which modulate side effects of this pathological situation – pain, stress reaction caused by catecholamines, and corticosteroid release (Dandona et al., 2014).

However, in spite of many experiments, the direct effect of opioids on the corticosteroid-induced immunosuppression was not clearly proved. Thus, the aims of the study were: 1. induction of the chronic inflammation state; 2. measurement of changes in blood leukocytes; 3.comparison of endogenous opioid peptide responses in the bone marrow and spleen to different corticosteroid treatments during chronic inflammation in mice.

MATERIALS AND METHODS

The experiment was carried out on 40 male Swiss mice, weighing 24-26 g, kept under controlled environmental conditions (light 12L:12D, temperature 20ºC), with *ad libitum* access to food and water. The animals were divided into 5 groups (n=8): control CI (intact), control CII (nebulized with physiological saline) and experimental – chronic inflammation (asthmatic, A), chronic inflammation (asthmatic) treated with nebulized corticosteroid GI (A+GI), chronic inflammation (asthmatic) treated with corticosteroid injection (i.p.) GII (A+GII).

Experimental asthma (chronic inflammation) was induced in three experimental groups according to the model described by Henderson et al., (2002) and Kumar and Foster (2002) in which some modifications were introduced. Briefly, the mice received injections of 100 μg of complex ovoalbumin (OVA) with aluminium ammonium sulfatum (AAS) on days 0 and 14. After induction of acute inflammation, the mice were kept individually in the chamber and nebulized for 10 min (inhalator DeVilbis, volume of 3500 ml) with 500 μg of OVA+AAS complex on days 14, 27, 28, 29 and 47. The control CII mice were injected and nebulized with 0.9% NaCl in the same manner as that used for the experimental animals.

Corticosteroid I (Budesonide) was given by inhalation once a day (5 min, 0.5 mg) starting from day 14 until the end of the experiment (day 48). Injections of corticosteroid II (GII, prednisolone, methylprednisolone acetate, 5 μg/mouse) were given every 7 days starting on day 14.

On day 48 the animals were decapitated, their blood, bone marrow and spleen were removed, and the tissues were immediately frozen and kept at -70ºC. Leukocytes were counted using a hemocytometer and calculated as counts per one liter of blood.

The bone marrow and spleen were homogenized in phosphate buffer, pH=6.5 (1:10 v/v or 1:10 w/v for spleen), centrifuged for 20 min at 2500 rpm at 4ºC, and the supernatant was used for Met-enkephalin estimation according to the radioimmunoassay method described by Pierzchała and Van Loon, (1990). Briefly, 100 μl of supernatant was loaded on the Porapak Q chromatography column (Waters), cleaned with

6 ml of double distilled water and eluted with 4 ml of absolute ethanol. Samples were lyophilized, dissolved in 100 µl of phosphate buffer, pH=6.5, and then 100 µl of ^{125}I-Met-enkephalin (3000 cpm) and 50 µl of Met-enkephalin antibody (rabbit, 1:8000) were added. The samples were incubated at 4° C for 18-20 hours and next treated with 0.1% gamma-globulin and 20% polyethylene glycol 8000, and centrifuged for 20 min at 2000 rpm. The antibody/opioid/^{125}I-Met-enkephalin complex was counted using a gamma counter.

The results were presented as $\bar{x}$+SEM and statistically calculated using analysis of variance (test F).

RESULTS

Total number of leukocytes (Fig.1). The total number of leukocytes was significantly increased in the control mice (CII) nebulized with physiological saline compared with intact mice (CI) [from $4.3\times10^9\pm0.4\times10^9$/l to $6.5\times10^9\pm0.5\times10^9$/l (P <0.01)]. The number of leukocytes in the mice with chronic inflammation (induced asthma) was much higher than in the CII group mice [$9.2\pm0.6\times10^9$/l (P<0.01)]. The treatment with Budesonide decreased the total number of leukocytes to $6.2\times10^9\pm0.4\times10^9$/l (P<0.01), but the injections of prednisolone caused a smaller decline in the number of leukocytes, to $7.1\times10^9\pm0.5\times10^9$/l (P<0.01).

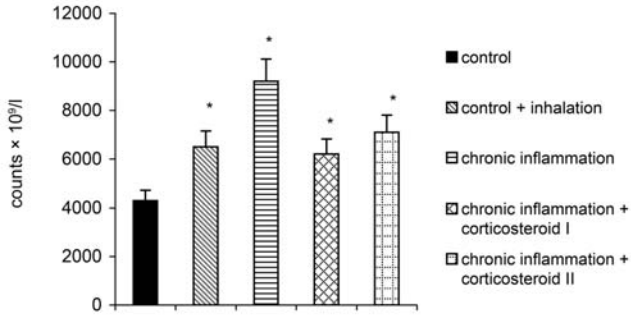

Fig. 1. Total number of white blood cells (counts × 10^9/l, $\bar{x}$±SEM), *P<0.01-0.001 compared with the control.

Met-enkephalin concentration in the bone marrow (Fig. 2). The concentration of Met-enkephalin in the bone marrow of control mice was 98.0±11.3 fmol/10 µl and gradually decreased in the CII group [(to 42.7±3.2 fmol/10 µl, P<0.001)] and in the group with chronic inflammation [to 22.2±1.6 fmol/10 µl (P<0.001)]. The treatment with corticosteroids caused a further decrease in the opioid concentration – to 18.1±0.9 fmol/10 µl after Budesonide and to 12.9±1.1 fmol/10 µl after prednisolone injections (P<0.001).

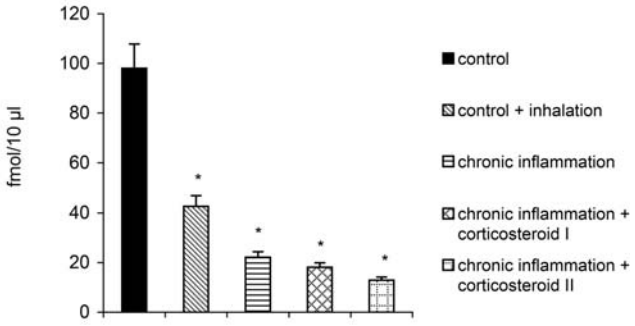

Fig. 2. Met-enkephalin concentration in the bone marrow (fmol/10 µl, $\bar{x}$±SEM), *P<0.01-0.001 compared with the control.

Met-enkephalin concentration in the spleen (Fig. 3). The Met-enkephalin concentration in the spleen of CII mice was increased to 13.7±1.1 pmol/g w.t. (P<0.01) compared with the value of 7.2±0.8 pmol/g w.t. observed in intact mice. Chronic inflammation increased the opioid concentration in the spleen of asthmatic mice to 18.0±1.4 pmol/g w.t. (P<0.01). Unexpectedly, the treatment with Budesonide did not change the Met-enkephalin concentration (18.0±1.7 pmol/g w.t.). In contrast, injections of prednisolone decreased the opioid concentration to the value of 14.7± 1.6 pmol/g w.t. which was similar to the level observed in the CII group but significantly higher than the concentration in intact mice (P<0.01).

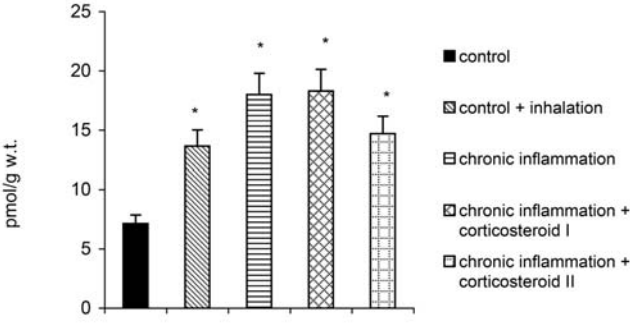

Fig. 3. Met-enkephalin concentration in the spleen (pmol/g w.t., $\bar{x}$±SEM), *P<0.01-0.001 compared with the control.

DISCUSSION

The studies using murine experimental models of chronic inflammation have been mostly directed to induce allergic asthma. Mice exhibit a specific chronic inflammation mechanism involving not only airway wall remodeling but inducing changes in nervous, humoral and immune systems. Due to these changes, murine models are used for the investigation of phenotypic and functional analysis of the cellular and mediator response (Wills-Karp, 2000; Oh et al., 2002).

We have demonstrated that our model of chronic inflammation exhibits many of the features of chronic asthma, including: 1. an increase in the number of leukocytes; 2. remodeling changes; 3. increase in the plasma cytokine levels; 4. changes in plasma cortisol, leptin and ghrelin (data not shown). Changes in the number of leukocytes suggest increasing migration of white blood cells from their sources (bone marrow, spleen) to the site of inflammation. It is interesting that inhalation with physiological saline (CII group) increased the number of leukocytes in the blood, which might suggest that NaCl started the sensitization of airways and also should be considered as an allergen under specific conditions. Inhalation of allergen (OVA+AAS) potentiated this effect and induced chronic inflammation. Kumar et al. (2008) reviewed OVA challenge models showing that ovoalbumin derived from chicken eggs is a frequently used allergen that induces robust, allergic pulmonary inflammation in rats and mice (Nials and Uddin, 2008).

The chronic exposure model also induces the production and release from leukocytes of various cytokines. Pro-inflammatory interleukins affect the remodeling process and migration of eosinophils and dendritic cells. Studies focusing on painful inflammatory models in which local opioid release may affect pain perception and immune response highlighted a crosstalk between the immune and opioid systems. A high level of cytokines also decreases the opioid chemotactic activity. Endogenous opioid peptides are locally produced at the inflammatory site by leukocytes and other cells. In the inflamed tissue antigens are captured and processed by dendritic cells which are released from the bone marrow. Dendritic cells are the most potent antigen-presenting cells generating an adaptive immune response. Maturing dendritic cells migrate towards draining lymph nodes to initiate a T-cell response (Lee and Iwasaki, 2007).

Benard et al., (2008) showed that only DOR mRNA was expressed in bone marrow-derived immature dendritic cells from BALB/C mice. However, the up-regulation of DOR mRNA was associated with lipopolysaccharide-induced maturation of dendritic cells.

Chronic inflammation in our experimental animals significantly decreased the Met-enkephalin concentration in bone marrow, which may suggest that: 1. opioids stimulated maturation of dendritic cells migrating to the inflammation site; 2. opioids were released in order to modulate the reaction to pain; 3. bone marrow is only a storehouse for opioids and not a place of proenkephalin synthesis.

Kumar and Foster, (2002) showed that in many murine models of chronic inflammation (asthma) researchers also showed a decrease in bone marrow eosinophil precursors.

In contrast, the Met-enkephalin concentration was increased in the spleen of all experimental mice. The spleen is one of the lymphatic system major organs, considered as a center of activity of the mononuclear phagocyte system, responsible for storage of monocytes, synthesis of antibodies and maturation of mononuclear blood cells (Swirski et al., 2009).

It was previously found that opioids are synthesized in the spleen and are released in response to stress or immune disorders. Met-enkephalin, one of the most widely expressed opioid peptides, has been recognized as a modulator of macrophage physiology acting in an autocrine or paracrine fashion to influence macrophage activation, phenotype polarization and production of cytokines that would enhance lymphocyte activation at early stages of an immune response (Valdes-Tovar et al., 2014).

In contrast to the present data, Van Loon et al., (1991) showed that in rats subacute nicotine administration decreased splenic concentrations of native (free form) Met-enkephalin, paralelly with increased release of Met-enkephalin from the spleen and decreased synthesis of proenkephalin or inadequate processing of larger peptides into enkephalin pentapeptides in the spleen

to compensate for the increased release during this period.

This comparison clearly showed the difference between Met-enkephalin activity in modulating acute and chronic/prolonged inflammation. During chronic inflammation the spleen is responsible for the synthesis of antibodies by lymphocytes and enhanced activation of macrophages, which results in increased synthesis and processing of proenkephalin into free Met-enkephalin.

Corticosteroids are the most effective anti-inflammatory therapy for many chronic inflammatory diseases, however their effectiveness depends on the type of tissue and on the dose or route of application. Chronic inflammation is characterized by increased expression of multiple inflammatory genes that are regulated by proinflammatory transcription factors. Corticosteroids are able to suppress the multiple inflammatory genes that are activated in chronic inflammatory diseases mainly through binding of glucocorticoid receptors to coactivators (Barnes, 2006). Mice with induced chronic inflammation treated with corticosteroids showed a significant decrease in the total number of leukocytes but the immunosuppression response was stronger after inhaled Budesonide. Corticosteroids decreased the bone marrow Met-enkephalin concentration in asthmatic mice, which confirmed the depletion of white blood cells in this tissue in response to inflammation. Surprisingly, inhaled Budesonide did not change the enkephalin concentration in the spleen of experimental mice, which may suggest that its effect is quick and not able to affect the activity/number of up-regulated lymphocytes. Probably, the synthesis and processing of the enkephalin precursor have been constantly activated by the inhaled allergen. On the other hand, systematically injected high doses of long-acting prednisolone decreased the splenic Met-enkephalin concentration, which may be the effect of down-regulation of the chemotactic activity of DOR by corticosteroids.

Our results highlight the importance of the mouse strain used for the development of an experimental model. Most of the research works have been done on the BALB/C strain in which the Met-enkephalin concentration in the tested tissues is much lower. The present study, however, was performed on the Swiss strain. Our results have proven the usefulness of Swiss mice for the murine model of asthma.

In this study we showed that Met-enkephalin originating from the bone marrow and spleen displayed strong activity regarding the immunosuppressive effect of corticosteroids; it depended, however, on the route of steroid administration. Another interesting observation emerging from our investigation is that in contrast to the response to short-term high-level of corticosteroids, prolonged exposure of low level corticosteroids decreased Met-enkephalin synthesis and processing.

It may be suggested that animals that have not been systematically sensitized but have been subjected to chronic inhalational challenge with an antigen turn out not to be a zero response control, in contrast to the situation with short models because of eventual development of sensitization via the airways.

To conclude, our results indicate that Met-enkephalin synthesized at the lymphatic organs might regulate the migration of immune cells towards inflammation sites and might modulate the mechanism of the adaptive immune response as well as regulate the immunosuppressive effect of corticosteroids.

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