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The Effect of Autologous and Heterologous Antimicrobial Peptides Extract on Rabbit and Ovine Leukocytes Evaluated During Repair Process

Joanna Wessely-Szponder[a], Joanna Zdziennicka[a], Tomasz Szponder[b], Michał Latalski[c], Beata Żylińska[b] and Aleksandra Sobczyńska-Rak[b]

[a]Department of Pathophysiology, Institute of Preclinical Veterinary Sciences, Faculty of Veterinary Medicine, University of Life Sciences, Akademicka 12, 20-033 Lublin, Poland, joanna.wessely@up.lublin.pl, joanna.michalska15@gmail.com
[b]Department and Clinic of Animal Surgery, Faculty of Veterinary Medicine, University of Life Sciences, Głęboka 30, 20-612 Lublin, Poland, tomszpon@op.pl, beatazyl@wp.pl, olsob2@gmail.com
[c]Department of Pediatric Orthopedics, Medical University, Gębali 6, 20-093 Lublin, Poland, michallatalski@umlub.pl

Abstract Antimicrobial peptides (AMPs) appeared to be a new approach both against microorganisms and for regulation of inflammatory and repair process. To evaluate their potential usefulness in regenerative medicine we prepared different extracts of neutrophil-derived AMPs, from rabbit, ovine or porcine blood, which contained AMPs of different composition, mainly defensins, cathelicidins and the fragments thereof. Then, we assessed the influence of different AMPs extracts on activity of neutrophils and monocyte-derived macrophages (MDM) *in vitro*. For this purpose these cells were obtained from experimental animals, rabbits or sheep submitted to different orthopedic procedures such as insertion of titanium implant into the tibial defect, implantation of biomaterials into osteochondral or bone defect or osteochondral transplantation. Stimulation of cultured cells was autologous or heterologous dependently on origin of AMPs extract and the species of experimental animal. The neutrophil activity was assessed on the basis of release of enzymes from azurophilic and secondary granules and free radicals generation. MDM functional assessment was done on the basis of NO and superoxide generation and arginase activity, additionally morphological changes were evaluated in these cell cultures. Our results indicated that the origin of AMPs extract is crucial for its activity, autologous extracts stimulated antiinflammatory responses, whereas heterologous extracts acted pro-inflammatory on neutrophils and macrophages *in vitro*. These results could be considered during introduction of new preparations in regenerative medicine.

Keywords: cathelicidins; (neutrophil extract), (implantation of biomaterial), (osteochondral graft), neutrophils; (monocyte-derived macrophages), (natural antimicrobial peptides)

Introduction

Antimicrobial peptides are widely recognized for their multifunctional roles regarding both antimicrobial and immunomodulatory activities. These functions refer to modulation of pro- and antiinflammatory responses including among other macrophage differentiation and modulation of wound healing. AMPs can suppress proinflammatory responses as in case of human cathelicidin LL-37 by reduction of the release of proinflammatory mediators and LPS neutralization [1]. Similar

effect was seen in porcine PR-39 [2]. It was estimated that AMPs induce macrophage differentiation towards an intermediate phenotype between proinflammatory M1 and antiinflammatory M2 macrophages. Furthermore, AMPs also influence functions of neutrophils, the major innate immune effector cells of the early-phase response to injury. Neutrophils are both producers and receivers of AMPs, and are a source of both defensins and cathelicidins, which are stored and released from granules during neutrophil degranulation. AMPs can enhance influx of neutrophils both by direct chemotactic function and indirectly by promoting the secretion of these neutrophil products [3].

In our experiment we evaluated the influence of different AMPs extracts on activity of neutrophils and monocyte-derived macrophages (MDM) obtained from experimental animals, rabbits or sheep submitted to different orthopedic procedures.

Materials and methods

The study was conducted on rabbit and ovine model for biomaterial implantation. After implant insertion the response of neutrophils and MDM was evaluated *in vitro*. Neutrophil activity was assessed on the basis of enzymes release, reactive oxygen species-ROS and reactive nitrogen intermediates-RNI generation. MDM response was assessed on nitric oxide (NO) and superoxide generation, arginase activity and morphological changes of these cells. Before the experiment AMPs extracts of autologous or heterologous origin were prepared, additionally some components of AMPs extracts were separated using gel filtration chromatography and also tested for their activity.

Preparation of autologous (rabbit) AMPs and heterologous (porcine) AMPs extract and its isolated components (PR-39, protegrin mixture), neutrophils isolation and stimulation

Porcine neutrophils for AMPs products were isolated from blood collected at an abattoir. After the red blood cells were lysed by the addition of 0.83% ammonium chloride to the blood sample and centrifuged, the remaining pellet was washed twice with phosphate-buffered saline (PBS). The final cells were homogenized to release the neutrophil granules. These granules were collected (25 000 x g, 40 min, 4°C), suspended in 10% acetic acid and stirred overnight at 4°C to extract the antimicrobial peptides. The solution containing the peptides was separated from the granules (25 000 x g, 20 min, 4°C) and obtained extract was considered as AMPs neutrophil extract. Then, the peptides were isolated according to their molecular mass using gel filtration chromatography. Obtained products, namely crude extract, PR-39 and protegrins were used for stimulation of cultures of neutrophils or macrophages. Rabbit AMPs were prepared similarly from rabbit blood.

Rabbit neutrophils for preparation of rabbit AMPs extract and cell culture were isolated from blood collected from the ear vein. The cell suspensions were supplemented as follows: the control group with PBS (marked as unstimulated), other groups were stimulated with crude porcine antimicrobial extract, PR-39, protegrins or crude rabbit antimicrobial extract. Then, cultures were incubated for 30 min and for 22 hrs at 37°C in the presence of 5% CO_2. Next, the activity of neutrophils was assessed. Enzyme release from azurophilic granules was assayed on the basis of elastase release and compared to maximal enzyme content obtained after treatment of the cells with 0.5% Triton X-100. The assay of elastase activity was based on the cleavage of azocasein as a substrate at 25°C for 10 min; thereafter absorbance was assessed at 490 nm. Alkaline phosphatase (ALP) release, constituting a marker of specific granule response, was estimated after 10 min incubation at 25°C with an equal volume of 4-nitrophenyl phosphate disodium salt hexahydrate, after which absorbance was measured at 405 nm. Nitric oxide level was determined by means of the Griess reaction [4]. Briefly, equal volumes of the culture supernatant and Griess reagent (0.1% N-[1-naphtyl] ethylendiamine dihydrochloride 1% sulphanilamide and 2.5% H_3PO_4) were mixed and incubated at room temperature for 10 min and absorbance was measured. The values obtained were expressed as nitrite concentration. Superoxide anion generation was measured by incubating neutrophils with 0.1%

nitroblue tetrazolium solution at room temperature for 10 min and reading absorbance at 545 nm. Generation of superoxide was assessed using the extinction coefficient 21.1 nM [4].

Evaluation of the influence of rabbit AMPs extract on rabbit MDM

The influence of rabbit AMPs extract on MDM was assessed on the basis of morphological and functional changes. Blood for leukocytes isolation was obtained before and after Ti implant insertion into the tibial defect. Mononuclear cells were isolated from whole blood by gradient centrifugation over Histopaque-1077 and immediately cultured at a concentration of 1.0×10^6 cells/mL into 96-well flat-bottomed tissue culture plates at 37°C and 5% CO_2 for 72 hrs in Dulbecco's Modified Eagle's Medium (DMEM) with 10% bovine calf serum (BCS) to obtain MDM [4]. The cultures described as BCS were left without additional stimulation. Other cultures were stimulated with 40 μg/mL of AMPs and marked as the rANE group. All these cultures were incubated for 3 days at 37°C and 5% CO_2, then the functional analysis was done on the basis of superoxide and NO generation and arginase activity of cultured MDM [5]. Microscopic analysis of the morphology was conducted using a reversed phase microscope (Olympus).

Evaluation of ovine macrophages response to AMPs extract

Blood for MDM culture was obtained from each sheep before implantation of Ti plate into proximal tibia and 5 months after. AMPs extracts were prepared previously as described, and stored for use. To obtain MDM mononuclear cells fraction (MNC) was isolated by gradient centrifugation over Histopaque-1077 as in case of rabbit MNC. Then, cultured MDM were stimulated with ovine or porcine AMPs extract or left without additional stimulation as an unstimulated group. MDM morphology and function was then assessed as described in rabbits.

Results

Rabbit neutrophil response to autologous AMPs and heterologous (porcine) AMPs extract and its isolated components (PR-39, protegrin mixture).

After stimulation of rabbit neutrophils with autologous AMPs extract their response was diminished in comparison with unstimulated cells. Contrary to this heterologous AMPs extract acted proinflammatory on neutrophils obtained before and after implantation. However, some components of porcine extract, namely PR-39 and protegrins diminished neutrophil activity (Figure 1).

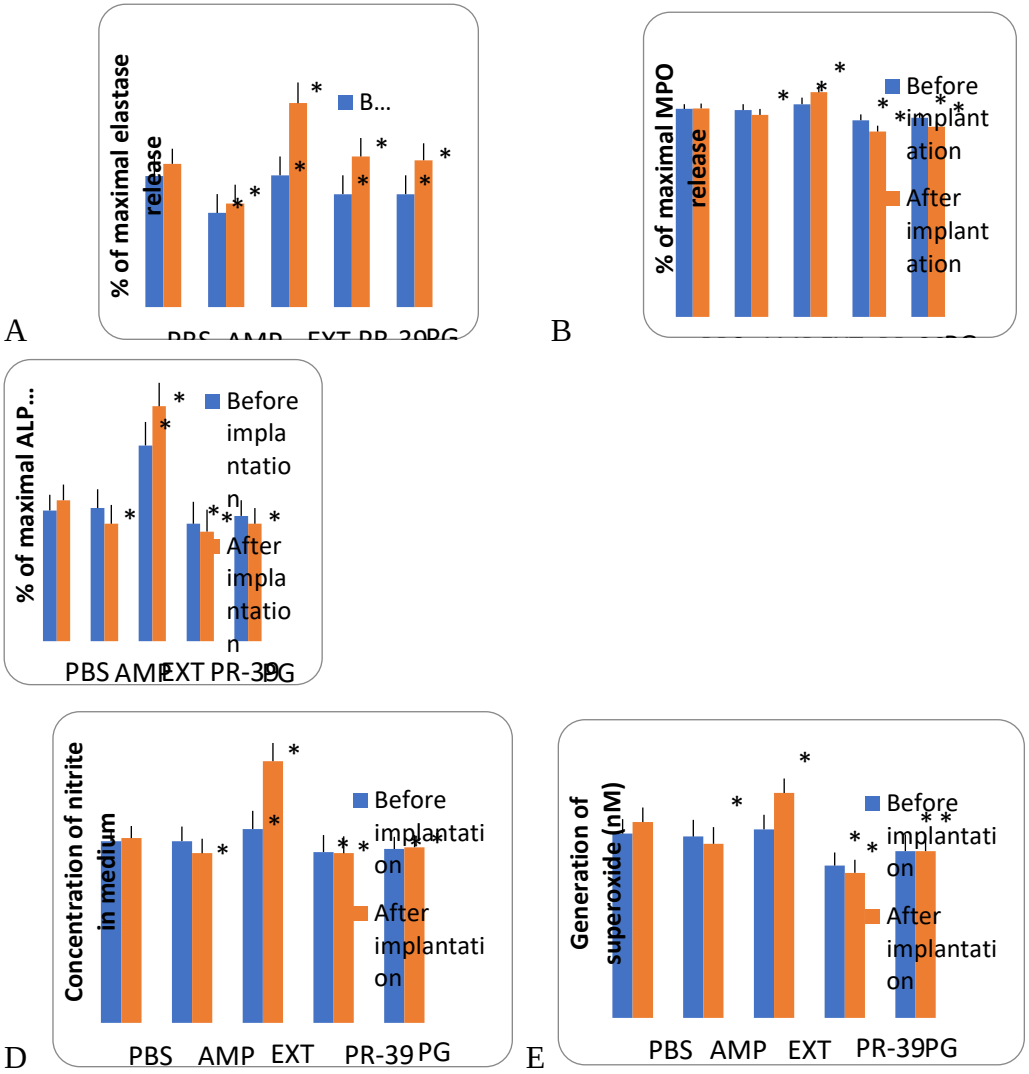

Figure 1. Response of rabbit neutrophils to autologous rabbit AMPs extract (AMP), heterologous porcine extract (EXT) and isolated products of porcine extract (PR-39, protegrin-PG) before and after implantation. (A) Elastase release, (B) myeloperoxidase release, (C) alkaline phosphatase release, (D) nitric oxide generation, (E) generation of superoxide.*$p<0.05$.

The influence of rabbit AMPs extract on morphology and function of rabbit MDM obtained before and after implantation of biomaterial

We estimated that rabbit macrophages treated with autologous AMPs extract showed mixed partially antiinflammatory features with unchanged arginase activity (Figure 2).

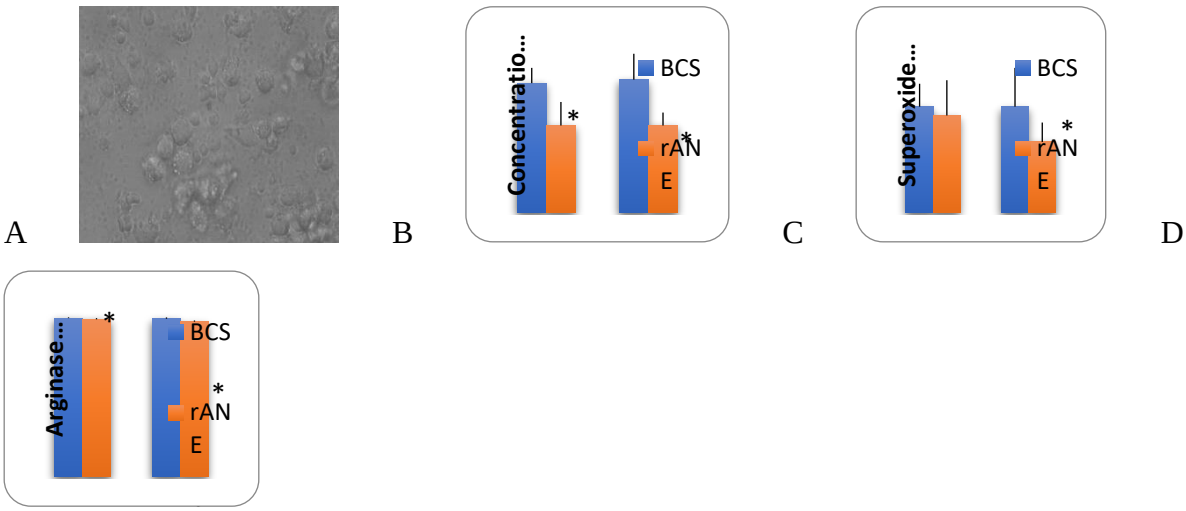

Figure 2. The influence of rabbit AMPs extract (rANE) on morphological and functional changes MDM before and after Ti implant insertion. (A) Morphology of MDM after treatment with rANE, (B) nitric oxide generation, (C) superoxide generation, (D) arginase activity .*p<0.05.

The influence of ovine and porcine AMPs extract on morphology and function of rabbit MDM obtained before and after implantation of biomaterial

In the experiment on MDM stimulation with autologous (ovine) and heterologous (porcine) AMPs neutrophil extract we estimated that MDM response was different and related to the origin of extract. Autologous extract causes decrease of NO and superoxide generation, whereas arginase activity remained unchanged. Conversely, stimulation with heterologous extract showed increase in free radical generation and arginase activity (Figure 3).

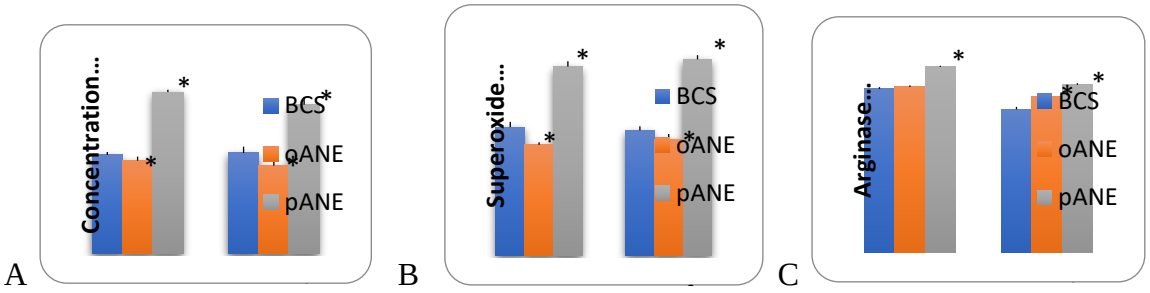

Figure 3. The influence of autologous (oANE) and heterologous (pANE) extract on MDM function before and after Ti implant insertion in ovine model (A) nitric oxide generation, (B) superoxide generation, (C) arginase activity. *p<0.05.

Discussion

Apart from microbial killing AMPs are regulatory molecules that limit enhanced inflammation. Therefore, they appeared the molecules that can balance inflammation to promote immune homeostasis. The antiinflammatory function of AMPs was previously confirmed by several studies on animal models which have demonstrated that deficiency of these peptides results in overexpressed inflammatory responses; i.e. cathelicidin- deficient mice exhibited a more severe inflammatory phenotype compared with wild-type mice. Similarly, reduced expression of β-defensin in enterocytes of humans has been noted in Crohn's disease. Moreover, the critical role of defensins in maintaining the integrity of intestinal mucosa and immune homeostasis is well established. Exogenous application of AMPs such as human cathelicidin LL-37, CATH-2, BMAP-28 or HBD2, and synthetic peptides (for example, IDR-1 and IDR-1002) has been shown to control inflammation in various animal models of infection and sepsis. Similarly, LL-37-derived peptide controlled the

disease process in a mouse model of inflammatory arthritis and IDR-1002 effectively suppressed airway inflammation *in vivo* [3]. In the light of these results we conducted the study on the influence of autologous and heterologous preparations of AMPs on some components of white blood cells system (WBC). Our study revealed that the response depended on origin of extract, animal species, the cell type and animal status (before and after implantation). In the study of neutrophils we estimated that autologous AMPs decreased their activity in respect of enzymes release and free radicals generation. Contrary to this, heterologous AMPs extract increased secretory activity of these cells.

After implant insertion MDM are among the first cells at the implant site and they are considered as key regulators of both the initiation and the resolution of inflammation [6]. Therefore, in our experiment we also evaluated MDM response to AMPs extracts. MDM after stimulation with autologous extract showed decreased ROS and NO generation with intact arginase activity in comparison with cultures stimulated only with BCS. After stimulation with heterologus AMPs extract, in turn, these cells generated higher amounts of superoxide and NO together with higher arginase activity. These results indicated mixed subpopulation of both pro- and antiinflammatory features in which unchanged arginase activity ensures undisturbed healing process. Similar intermediate state was described in response to synthetic IDR-1018 peptide, when macrophages developed a unique capability to maintain particular proinflammatory activities, while producing antiinflammatory and regulatory mediators [1].

Conclusion

The inflammatory response can be modulated using blood derived products, since these products apart from their antimicrobial activity can regulate inflammation dependently on current organism needs. Therefore, different AMPs extracts could be used for enhancement or suppression of inflammatory response.

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