ACTA MEDICA LITUANICA. 2008. Vol. 15. No. 4. P. 206–210

Human neutrophil peptide 2 impairs wound-induced migration of cultured pulmonary artery endothelial cells

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Introduction. Human neutrophil peptides (HNP) or α-defensins are cysteine-rich cationic polypeptides that possess a wide range of antimicrobial and cytotoxic capabilities. Inflammatory processes, such as neutrophil infiltration and HNP release, are present in the patient lungs associated with chronic obstructive pulmonary disease (COPD). We sought to determine whether presence of HNP impairs wound-promoted migration of lung endothelial cells *in vitro*.

Materials and methods. Cultured porcine pulmonary artery endothelial cells (PAEC) were used as cell models to investigate the effects of HNP on wound-induced migration. Cells were HNP-treated and their metabolic activity was assessed using WST-1 assay. Wounds were induced in the cell monolayer incubated in the medium containing 0–100 nM HNP-2. Cell morphology and migration were examined under a confocal microscope. In parallel, fluorescein-labelled HNP-2 was applied and its internalization into PAEC was assessed.

Results. HNP-2 attenuated *in vitro* wound-induced endothelial migration in dose-dependent manner. HNP-2 at the concentrations of 15 to 100 nM did not affect the cell viability. Labelled HNP-2 was internalized by PAEC.

Conclusions. Our results suggest that HNP can attenuate wound-induced endothelial migration, which may be associated with the internalization of the peptide. HNP may contribute to the loss of lung vasculature in the development of COPD and other inflammatory conditions.

Key words: neutrophil degranulation products, defensins, human neutrophils peptides, cationic peptides, endothelial permeability

INTRODUCTION

Vascular damage contributes to the development of various inflammatory diseases, such as chronic obstructive pulmonary disease (COPD) and pulmonary fibrosis (1–3). Neutrophil infiltration and release of their granule content may damage endothelium and cause vascular abnormalities. Human neutrophil peptides (HNP), i. e. α-defensins, released together with other pro-inflammatory molecules and enzymes contribute to the endothelial dysfunction and can play a critical role in the lung destruction seen in the COPD patients. Defensins promote adhesion molecule expression in and stimulate the binding of lipoprotein to endothelial cells (4, 5). It has already been reported that defensins cause endothelial dysfunction (6, 7). Furthermore, genetic variants of human defensins were correlated with the risk of COPD development (8). The levels of defensins in the circulation were assessed and reported recently. For instance, the levels of α-defensins in the lungs of individuals with alpha-1-antitrypsin deficiency and with moderate to severe lung disease were compared to those of healthy donors (9). It is generally accepted, that major antimicrobial processes primarily take place within neutrophil vacuoles after phagocytosis. Still, activated human neutrophils also release up to 10% of their defensin content extracellularly (10).

HNP or α-defensins are diverse members of a large family of cationic host defence peptides and widely distributed throughout the plant and animal kingdoms. These cysteine-rich peptides vary in their length, the spacing of their cysteine residues, and their disulfide connections. The mammalian HNP are generally small (3–6 kDa, 29- to 35-amino acid) peptides, containing six conserved cysteine residues. They were originally isolated from rabbit neutrophil granules (11). The family consists of the originally isolated α-defensins, i. e. human neutrophil peptides 1–4 and human defensins 5 and 6, the β-defensins (1–4), and the more recently identified theta-defensins or retrocyclins (12).

HNP 1–4 are abundant in the granules of human inflammatory cells, neutrophils. They constitute 5% of all neutrophil proteins and 30–50% of the total protein content of the azurophilic granules. HNP are accepted as classic antimicrobial peptides. However, other functions of HNP have been also reported. Recent work has demonstrated that defensins have immuno-modulatory effect and potentially provide a link between innate and adaptive immunity, being chemotactic for $CD4^+$ T cells and immature dendritic cells (13).

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We have investigated the effects of HNP-2, as a dominant neutrophil peptide, on wound-stimulated migration of pulmonary artery endothelial cells (PAEC), cell morphology and viability. The internalization of HNP-2 into the endothelial cells was examined. Our data will open new insights into understanding of endothelial permeability processes occurring in neutrophil-associated inflammatory conditions.

MATERIALS AND METHODS

Materials. Unless specified, reagents were obtained from Sigma Chemical (St Louis, MO, USA). HNP-2 was purchased from Bachem (USA). The peptide was fluorescein tagged and purified at Mass Spectrometry & Protein Characterization Division at Interdisciplinary Centre for Biotechnology Research, University of Florida, USA.

Cell culture. Primary porcine PAEC were obtained from the main pulmonary arteries of 6-month-old pigs from a local slaughterhouse on a weekly basis and were grown in monolayer cultures. Fifth to seventh passage cells in confluent monolayers maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY, USA) with 5% foetal bovine serum (HyClone Laboratories, Logan, UT, USA) and antibiotics, i. e. 10 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Grand Island, NY, USA), were used for all the experiments. Cells were incubated in serum free medium 12 hours prior to and during the experiments.

Cell viability measures. The viability of the HNP-treated cells was assessed using the WST-1 cell proliferation kit (Roche, Switzerland). The colorimetric change due to cleavage of tetrazolium salts by mitochondrial dehydrogenase was determined following the manufacturer's instructions. The amount of formazan dye produced is directly proportional to the number of metabolically active cells. Metabolic activity measured gives a view of cell viability and HNP cytotoxicity.

Immunocytochemistry. Control and fluorescent HNP-treated cells (for 15 min and 2 hours) in monolayers were washed, fixed in 4% paraformaldehyde, and mounted using Vectashield mounting medium (Vector Laboratories, USA) that has been optimized to preserve fluorescence and counterstained for DNA with DAPI (blue).

Wound healing assay. Cells were treated with HNP-2 at indicated concentrations for total of 22 hours. Four hours post seeding, *in vitro* wounds were induced on the cell monolayers. The scratch was created by manually scratching the cell layer with a disposable sterile pipette tip. The wounded monolayers were incubated for 18 hours, fixed in 4% paraformaldehyde, and stained using FITC-phalloidin (Sigma, USA). Vectashield mounting medium with DAPI was used. Confocal microscopy images were taken and evaluated visually.

Confocal microscopy. Control and HNP-treated cells were examined under a Zeiss LSM 510 laser scanning confocal microscope. Visualization was performed using a laser scanning confocal microscope with excitation and emission maxima at 495 and 515 nm, respectively. LSM 510 (version 3.0 SP3) software for the Carl Zeiss Laser Scanning Microscope was used. The representative images of at least 3 independent experiments are shown.

Statistics. In each experiment, control and experimental PAEC were matched for cell line, age, density, number of passages, and state of confluence. Results are shown as mean ± SD. The differences in the means of experimental results were analyzed for their statistical significance by two sided t-test and $p < 0.05$ was taken as significant. Sigma Stat software was used for the calculations.

RESULTS

Up to 100 nM of HNP-2 does not change cell viability

PAEC were incubated in the medium containing 0–100 nM of HNP-2 for 2 hours. The viability of HNP-treated and control cells was measured using the WST-1 assay. The relative levels of metabolized WST-1 in the 0–100 nM HNP-2-treated cells were comparable, indicating that HNP-2 had not altered cell viability (Fig. 1).

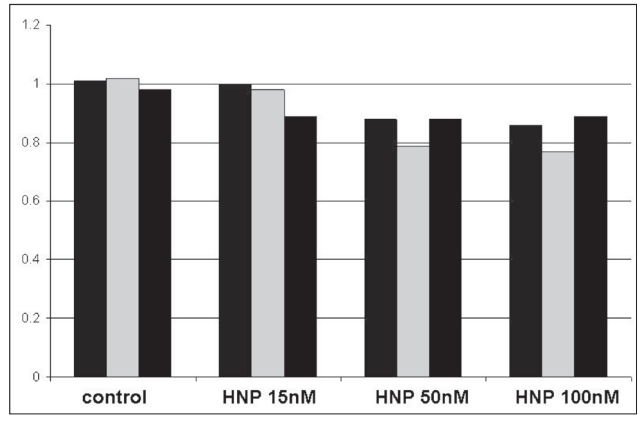

Fig. 1. Effects of HNP-2 on cell metabolic activity.
PAEC were incubated in the medium containing 0–100 nM of HNP-2 and subjected to WST-1 assay to assess their metabolic activity. Amount of formazan dye produced was measured (presented as optical density, OD) to evaluate the number of metabolically active cells. Three bars for the each treatment group represent three different experiments. Significance of differences was calculated (n = 3). No significant differences were found between control and HNP-treated cells

Dysfunction of HNP-treated endothelium: impaired cell migration and disorganized cytoskeleton

PAEC were incubated in the medium containing 0–100 nM of HNP-2. This range is still way below the concentrations detected in COPD patients with alfa-1-antitrypsin deficiency and inflammation (14). We show that HNP-2 attenuates wound-stimulated cell migration in dose-dependent manner (Fig. 1). As low as 15 nM of HNP-2 impairs cell ability to migrate and covers the scratched area. To determine the effects of HNP-2 on the cell structure, cellular cytoskeleton and nuclei were stained using FITC-phalloidin (green) for polymerized actin and with DAPI for DNA (blue). HNP-2 treatment resulted in cell rounding, loss of stress fibres, and cortical organization of actin. In addition, cell-cell contact was lost, and fine granular structures became evident in cells exposed to higher HNP concentrations (Fig. 2, insets).

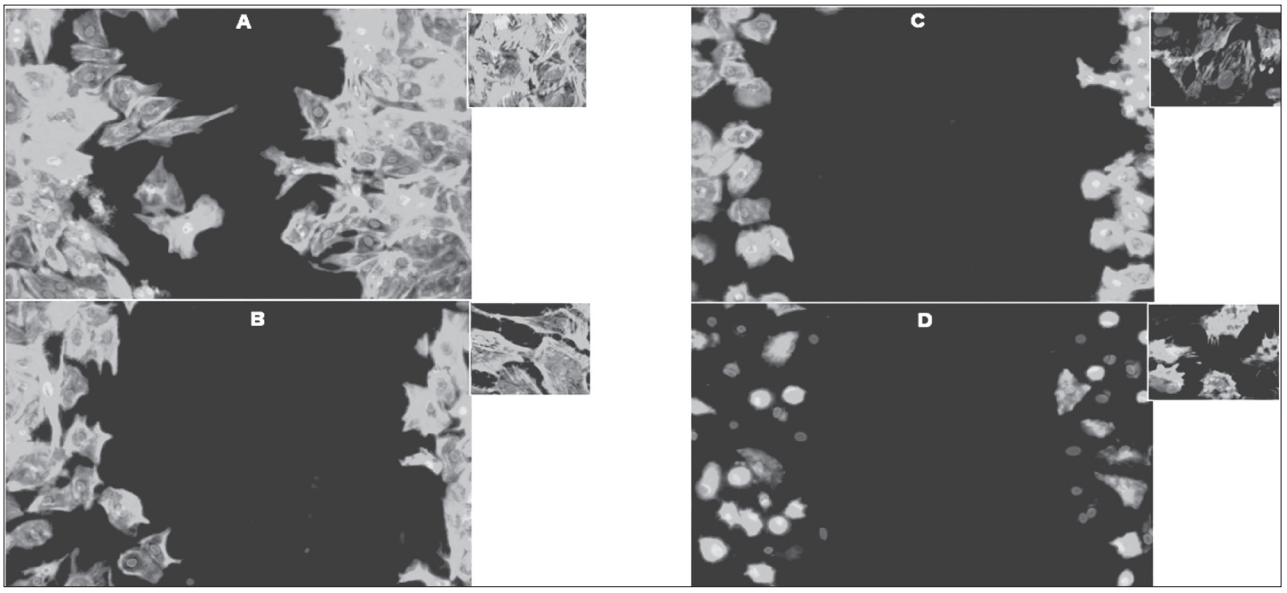

Fig. 2. Effects of HNP-2 on endothelial cell migration and cytoskeleton.
PAEC monolayers were incubated in the medium containing 0–100 nM of HNP-2 and wounded using a pipette tip. Following the 18-hours incubation cells were fixed, labelled, and examined under a confocal microscope as described in Materials and Methods. Panel A: control cells; panel B: cells treated with 15 nM HNP-2; panel C: cells treated with 50 nM HNP-2; and panel D: cells treated with 100 nM HNP-2. Images represent at least 3 independent experiments. Magnification – 400×. Closer view of cell cytoskeleton is presented in the insets next to the each panel (inset magnification 1000×)

Uptake of HNP-2 by PAEC

PAEC monolayers were incubated in the medium containing fluorescein-labelled HNP-2 for 15 min and 2 hours. The treated cells were fixed and mounted using DAPI containing mounting medium. As shown in Fig. 3, PAEC uptake HNP-2 in a time-dependent manner. After 15 min incubation peptides are localized mainly at the cell membrane, probably adhering to the F-actin and participating in focal adhesion disassembly. This is in agreement with the observations related to the internalization of other cationic peptides (15). Interestingly, 2-hour incubation leads to different distribution of intracellular HNP-2. Peri-membrane staining becomes weak, while the intensities of labelled HNP-2 in the peri-nuclear region and granular structures are increased (Fig. 3).

DISCUSSION

To study the possible interaction of endothelial cells and neutrophils defensins we have assessed the abilities of injured endothelial layer to cover the wounded area in the presence of HNP-2. Cell migration assay performed suggests that HNP-2 changes endothelial cell cytoskeleton and migration at even

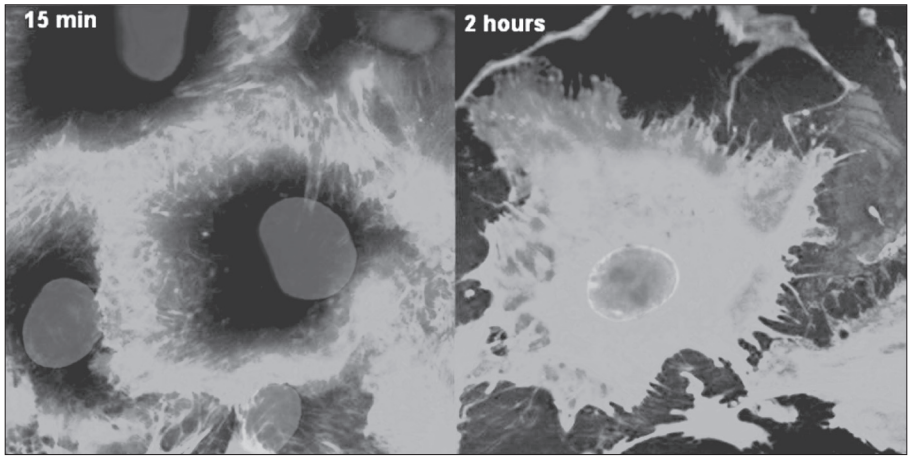

Fig. 3. HNP-2 is up-taken by PAEC.
PAEC were incubated in the medium containing 50 nM of fluorescein-labeled HNP-2 for 15 minutes and 2 hours. Nucleic DNA was stained with DAPI. The fluorescent intensities were examined under a confocal microscope. The representative images of at least 3 experiments are shown. Magnification – 1000×

sub-inflammatory concentrations (Fig. 2). HNP-2 treated cells have lost their intercellular contacts, stress fibres have disappeared, and actin has reorganized into cortical type. Moreover, fine granular structures have been formed (panel B, Fig. 2) and cell shape has become rounded (panel D, Fig. 2). We speculate that granular structures with the actin involved might be the porous structures that defensins form on their target cells. Endothelial cells might be able to counteract some HNP induced citotoxicity since we do not detect significant cell metabolism changes in our model (Fig 1). However, it is obvious that cell functional activities, e. g. their ability to migrate and cover scratched areas of the monolayer, are affected by even relatively low doses of HNP.

Our study also shows that HNP-2 is up-taken by endothelial cells (Fig. 3). As reported previously, HNP are able to adhere to the negatively charged cell plasma membrane via their cationic residues. Then several molecules organize into a specific structure, and pore is formed. Subsequently, cytoplasm leakage may be initiated and peptides may penetrate the cell. Our finding that HNP-2 localizes at the cell plasma membrane immediately after the application supports this notion further. The specific staining pattern may be related to the HNP ability to stick to actin fibrils. Longer incubation causes drastic changes in the localization of intracellular HNP. 2 hours later peptide is found in the peri-nuclear structures and fine granules. The latter may actually represent porous structures formed by HNP molecules. Further staining experiments are needed to clarify this issue.

It is generally accepted that HNP exert their killer properties on invading micro organisms. However, the growing body of evidence provides new insights into HNP role in inflammatory processes. Some diseases are associated with enhanced amounts and activation of human neutrophils, i. e. chronic obstructive pulmonary disease. In such cases neutrophil degranulation products, including HNP, are delivered not only to foreign cells, but also to the host cells. Injury induced by neutrophil activation products leads to apoptosis of tissue structural cells, extracellular matrix degradation, promotion of chronic unregulated inflammatory process, continuous recruitment of inflammatory cell and subsequent tissue loss. Since vascular endothelium is among the major possible targets for defensins, we have studied NHP / endothelium interaction in our model. Our results presented here suggest the novel role of HNP in neutrophil-mediated inflammatory processes.

CONCLUSIONS

We demonstrate that human neutrophil-released cationic peptides are able to enter vascular endothelial cells and affect the barrier function of endothelial monolayer. Even at low sub-inflammatory concentrations, without significant cytotoxicity induced, HNP-2 has initiated changes in the cell shape, cytoskeleton organization and intercellular connections. HNP-2 has also demonstrated distinct uptake pattern. Peri-membrane localization found immediately after the treatment was followed by pronounced peri-nuclear and some granular staining 2 hours later. Such endothelial / HNP interactions elucidate a complex mechanism of neutrophil-mediated damage of the host tissues. It is evident that even slight neutrophils degranulation if not enclosed within vacuoles may induce vascular dysfunction and contribute to the tissue damage.

ACKNOWLEDGEMENTS

This work was partially suported by the Lithuanian State Science and Studies Foundation, grants No. B-25/2008 and No. C-04/2008.

Received 28 October 2008
Accepted 28 November 2008

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ŽMOGAUS NEUTROFILŲ PEPTIDAS (HNP-2) SILPNINA PLAUTINĖS ARTERIJOS ENDOTELIO LĄSTELIŲ GEBEJIMĄ MIGRUOTI PAŽEIDIMO SRITYJE

S a n t r a u k a

Įvadas. Žmogaus neutrofilų peptidai, arba α-defensinai, tai – antimikrobiniai katijoniniai peptidai, turintys daug cisteino. Audinių infiltracija neutrofilais ir jų peptidų išsiskyrimas iš granulių būdingas daugeliui uždegiminių ligų. Vienas iš galimų šių peptidų veikimo taikinių yra kraujagyslių endotelis. Mes tyrėme, ar defensinų šeimos atstovas HNP-2 gali pakeisti plaučio arterijos endotelio ląstelių gebėjimą migruoti ir padengti monosluoksnio pažeidimus *in vitro*.

Metodai. Įbrėžę kiaulės plaučių arterijos endotelio ląstelių monosluoksnį pipete, konfokaliniu mikroskopu stebėjome, kaip ląstelės padengia pažeidimą aplinkoje didinant HNP-2 koncentraciją (0–100 nM). Taip pat stebėjome, ar žymėtas HNP-2 patenka į ląstelės vidų. Be to, tyrėme, kaip HNP-2 keičia ląstelės metabolinį aktyvumą.

Rezultatai. HNP-2 (0–100 nM) nesukėlė didesnių ląstelės metabolizmo pakitimų, tačiau akivaizdžiai sumažino ląstelių pajėgumą užpildyti monosluoksnio pažeidimus. Šis efektas stiprėjo didinant HNP-2 koncentraciją ląstelių aplinkoje. Be to, įsitikinome, kad žymėtas HNP-2 patenka į ląstelės vidų.

Išvados. Mūsų rezultatai leidžia manyti, kad net ir nedidelės HNP-2 koncentracijos, nepažeisdamos endotelinių ląstelių gyvybingumo, mažina jų gebėjimą migruoti ir užpildyti pažeidimus, taip pat keičia ląstelių morfologiją. Šie efektai, ko gero, yra susiję su HNP-2 patekimu ir susikaupimu ląstelėje. Taigi neutrofilų peptidai gali dalyvauti pažeidžiant kraujagyslių endotelio barjerinę funkciją uždegimo srityse.

Raktažodžiai: neutrofilų degranuliacijos produktai, defensinai, žmogaus neutrofilų peptidai, katijoniniai peptidai, endotelio vientisumas