Life Sciences 157 (2016) 152–157

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Critical role of neutrophil alkaline phosphatase in the antimicrobial function of neutrophils

Haining Li [a,b], Yao Zhao [a], Wei Li [a], Jin Yang [a], Huiyi Wu [a,*]

[a] Center of Research Laboratory, Lianyungang Hospital Affiliated to Xuzhou Medical College, 182 Tongguan Road, Lianyungang 222001, China
[b] Deparment of Clinical Laboratory Diagnostics,Kangda College of Nanjing Medical University, Lianyungang 222000, China

ARTICLE INFO

Article history:
Received 25 February 2016
Received in revised form 2 June 2016
Accepted 6 June 2016
Available online 7 June 2016

Keywords:
Neutrophil
Alkaline phosphatase
Migration
Reactive oxygen species
Apoptosis

ABSTRACT

Aims: To investigate the roles of neutrophil alkaline phosphatase (NAP) in the migration, reactive oxygen species (ROS) generation and apoptosis of neutrophils.
Main methods: NAP was overexpressed in neutrophil-like differentiated HL-60 cells via transfecting coding sequence of NAP by lentivirus. NAP overexpression in HL-60 cells was confirmed by the methods of quantitative RT-PCR and Western blotting. HL-60 cells were induced to differentiate into neutrophil-like cells by exposure to 1.5% dimethylsulfoxide (DMSO). The migration of neutrophil-like cells were detected by Transwell migration assay. ROS generation of neutrophil-like cells were determined by flow cytometry. Neutrophil-like cells continued to be cultured for 24 h, and were then harvested for apoptosis and Western blotting.
Key finding: After GFP-NAP infection by lentivirus, the expression of NAP was up-regulated in HL-60 cells. HL-60 cells were allowed to differentiate into neutrophil-like cells after 5-day exposure to 1.5% DMSO. Overexpression of NAP in neutrophil-like cells resulted in an increase in the number of migrated cells, intracellular ROS and cell apoptosis followed by a rise in the expression of Caspase 3, Caspase 9 and Bax, while those results were reversed in the NEG and CON group.
Significance: NAP might play a critical role in the anti-microbial function of neutrophils by promoting its migration and ROS generation, as well as accelerating apoptosis in neutrophils.

1. Introduction

As a key factor in the first line of defense against infection, neutrophils play a central role in the innate immune system. Upon infection, bacterial- or host- derived inflammatory signals, such as lipopolysaccharide (LPS) and formyl-Met-Leu-Phe (f-MLP), could promote neutrophils to migrate from circulation into inflammatory sites. Then neutrophils begin to implement their regime of microbial killing by executing the programs of phagocytosis, degranulation and neutrophils extracellular traps (NETs) [1].When the number and function of neutrophils are deficient, the body will certainly be attacked by pathogenic microorganisms [2].

Except of transphosphorylation, alkaline phosphatase is also known to catalyze phosphoprotein phosphatase activities. However, the biological significances of this enzyme has not been well established [3,4]. As a maker of mature neutrophils, neutrophil alkaline phosphatase (NAP) is mainly localized in the secretory vesicles or on the plasma membrane of neutrophils. NAP genes, which belong to the tissue nonspecific alkaline phosphatase or liver/bone/kidney (L/B/K) alkaline phosphatase, map to the distal short arm of human chromosome 1, bands p34-p36.1 [5]. Currently, what NAP acts in the biological function of the human body has not yet been clear. Utilization of the substrate 4-MUP has been recommended as a sensitive, repeatable and reliable method of neutrophil adherence determination in vitro by Bednarska et al. [6]. Other studies reported that NAP localized in the secretory vesicles and organelles of neutrophils could be detected after being translocated onto the plasma membrane by f-MLP stimulation [7–9]. Those suggest that NAP might play an important role in the development of inflammation by enhancing the functions of neutrophils.

The promyelocytic leukemia cell line HL-60 contains cells with heterogeneous morphology resembling myebloasts and promyelocytes. However, they could be differentiated into cells resembling neutrophils both in their morphology and function by DMSO or retinoic acid [10–13]. Differentiated HL-60 (dHL-60) cells could then be used as a simple model for the investigation of neutrophil functions. Since low level of NAP expression has been confirmed to be presented in HL-60 cells before or after differentiation [14], NAP was over-expressed by transfecting coding sequence of NAP gene via lentivirus to investigate its role in the functions of neutrophils in our study.

This study aims to investigate the effect of NAP on migration, ROS generation and apoptosis of neutrophils. We found that NAP enhanced

* Corresponding author at: Central Laboratory, Center of Research Laboratory, Lianyungang Hospital Affiliated to Xuzhou Medical College, 182 Tongguan Road, Lianyungang 222001, China.
E-mail address: 18961326009@163.com (H. Wu).

http://dx.doi.org/10.1016/j.lfs.2016.06.005
0024-3205/

the anti-microbial function of neutrophils and played an important role in the development of inflammation, suggesting NAP as a potential therapeutic target for patients in inflammatory conditions.

2. Materials and methods

2.1. Cell culture

HL-60 cells (accquired from Shanghai Institute of Hematology) were maintained in Iscoves modified Dulbecco medium (IMDM) medium (Gibco; Carlsbad, CA, USA), supplemented with 20% heat-inactivated fetal calf serum (FBS; Gibco) in 5% CO_2 humidified air at 37 °C. For differentiation into a neutrophilic phenotype, HL-60 cells (1×10^5/ml) were induced by 1.5% DMSO (Sigma; St. Louis, MO, USA) for 5 days in the medium. The viability and proliferation of differentiated cells was determined by Vitality Test in Muse™ smart touch cell analyzer (Merk Millipore; Boston,,MA, USA) and MTT.

2.2. Lentiviral transduction

To over-express NAP gene, lentivirus with coding sequence of NAP (Shanghai Genechem Co; Shanghai, China) were transfected into HL-60 cells. Lentivirus with vector was used as the negative control. HL-60 cells were added into a 6-well culture plate at 5×10^4 cells/well. Subsequently, 50 μl lentivirus with coding sequence of NAP or vector (1×10^8 TU/ml) was added into the cells. After 8–12 h culture, the supernatant was replaced by the grow medium and the expression of GFP protein was observed with a fluorescence inverted microscope (Olympus; Tokyo, Japan) after 3–4 days. Positive clones were selected with 300 ng/ml puromycin.

2.3. Quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Life Technologies, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions (Roche Diagnostics; Indianapolis, IN, USA). A NanoDrop-2000 spectrophotometer (Thermo Scientific; Waltham, MA, USA) was used to determine the yield and purity of total RNA. Following the manufacturer's instructions, 1 μg RNA was processed for cDNA synthesis with Superscript II reverse transcriptase (Roche Diagnostics; Indianapolis, IN, USA) in a 20 μl reaction volume. The quantitative RT-PCR, containing target genes and SYBR Green PCR mix (Roche Diagnostics; Indianapolis, IN, USA), was performed on the Veriti 96 Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA)according to manufacturer's instructions. Relative mRNA levels were calculated by using the $2^{-\Delta\Delta Ct}$ method and normalized by β-actin. The primers used were: NAP 5′-CGCAGGATTGGAACATCAGT-3′ (forward) and 5′-TGTCTCTTGCGCTTGGTCTC-3′ (reverse); β-actin 5′-TGGACTTCGAGCAAGAGATG-3′ (forward) and 5′-GGATGTCCACGTCACACTTC-3′ (reverse).

2.4. Western blotting analysis

Briefly, total cells were lysed with a lysis buffer containing a protease inhibitor. Total protein were quantified by a NanoDrop-2000 spectrophotometer. Then the proteins were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene fluoride (PVDF) membrane. After washed with TBST for 30 min, the membranes were blocked with milk for 60 min. This was followed by incubation with appropriate primary antibodies against ALPL (1:300), Bax (1:2000), pro-caspase-9 (1:200), active-caspase-9 (1:100), pro-caspase-3 (1:1000), active-caspase-3 (1:500) (R&D Systems; Minneapolis, MN, USA) and β-actin (1:5000) overnight at 4 °C. The membrane was then washed and incubated with horseradish peroxidase conjugated secondary antibodies for 120 min. After washing with TBST for three times, the bound antibodies were detected using enhanced chemiluminescene (ECL) system (ProteinSimple, CA, USA).

2.5. CD11b detection by flow cytometry

Cells at 1×10^5/ml in 50 μl were incubated with PE-CD11b monoclonal antibody or PE-IgG isotype (Becton Dickinson; San Jose, CA, USA) for 30 min at room temperature. Subsequently, the cells were washed three times with phosphate-buffered saline (PBS), spun down and resuspended in PBS. Cells were then analyzed by flow cytometry on a FACS Canto II (Becton Dickinson; San Jose, CA, USA) using the CELL Quest program (Becton Dickinson; San Jose, CA, USA). Results were presented as the percentage of CD11b-positive cells.

2.6. Migration assay

The migration assay was carried out in Transwell chamber (Corning; Corning, NY, USA) with 5 μm porous membrane. Differentiated HL-60 cells (10^5 in 200 μl) were added to the upper chambers and allowed to migrate towards the lower chambers with or without f-MLP (10^{-7} mol/l) for 12 h in the condition of 37 °C and 5% CO_2. Migrated cells collected from the lower chamber were centrifuged at 1500 rpm for 5 min and quantified using a Muse™ smart touch cell analyzer (Merk Millipore; Boston, MA, USA).

2.7. Respiratory burst detection

Differentiated HL-60 cells at a density of 2×10^5 cells/ml were treated with 1 μmol/L DHR123 for 15 min and further incubated in the presence of 1 μmol/l PMA for 30 min at 37 °C, Then the cells were subjected to a FACS Canto II flow cytometry system. The results were expressed as mean fluorescence intensity (MFI).

2.8. Apoptosis determination by flow cytometry

For apoptosis analysis, differentiated HL-60 cells were incubated at 37 °C for 24 h. Then the cells were stained with Annexin V and 7-AAD according to the manufacturer's instructions (Merk Millipore; Boston, MA, USA) and analyzed by a Muse™ smart touch cell analyzer (Merk Millipore; Boston, MA, USA).

2.9. Statistical analysis

Data were expressed as the mean ± SD. The differences between groups were evaluated by one-way ANOVA and the differences in groups were evaluated by paired sample *t*-test by a SPSS16.0 software package (SPSS Inc., Chicago, IL, USA). Significance was defined as a value of $P < 0.05$.

3. Results

3.1. Neutrophil-like cells differentiation of NAP-overexpression in HL-60 cells

To elucidate the role of NAP in neutrophil function, HL-60 cells were transfected with the coding sequence of ALPL by a lentiviral system and vector as a negative control. Then the transfected HL-60 cells were treated with 1.5% DMSO and allowed to differentiate into neutrophil-like cells for 5 days. Over 80% HL-60 cells presented green fluorescence after GFP-NAP infection and puromycin screening (Fig. 1A). In addition, over-expression of NAP in HL-60 cells was confirmed by both Real-time PCR and Western blotting (Fig. 1B, C). The viability of three groups was all above 90%. There was no difference in the viability and proliferation between all three groups (data not show). After 5-day culture with 1.5% DMSO, the bulk of induced cells were smaller and many bumps appeared on the surface of the cells in morphology. The ratio of nuclear and cytoplasmic decreased and nucleolus disappeared. The nuclear chromatin changed from dense to loose and the nuclear morphology appeared to be rod and segmented. (Fig. 2A). Furthermore, the percentage of $CD11b^+$ cell increased from 26.25% to 98.55% (Fig. 2B).