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Functional Consequences of Inhibition of Histone H3 Citrullination in Neutrophils

Milan Číž*

Department of Experimental Biology, Faculty of Science, Masaryk University, Czech Republic.

Abstract

Introduction: Neutrophils play a dual role in oncology, where they can either support anti-tumor immunity or, in their tumor-associated form, promote cancer progression through immunosuppression and angiogenesis. Their abundance and phenotype in the tumor microenvironment are increasingly studied as prognostic biomarkers and potential targets for cancer immunotherapy. The effects of Calcium Ionophore (CaI)-induced citrullination of histone H3 on the physiological manifestations of human neutrophils (oxidative burst, Myeloperoxidase (MPO) activity, cytokine production and release) were evaluated.

Methods: Neutrophils were isolated from peripheral blood of healthy volunteers and pre-treated with Cl-amidine (pan-PAD inhibitor) or TDFA (specific PAD4 inhibitor). CaI was added to the samples for activation of PAD. Production of reactive oxygen species in neutrophils was assessed using luminol-dependent chemiluminescence. Supernatants were used for measuring cytokine release from the cells (ELISA) and determination of activity of released MPO (colorimetric assay). Citrullination of histone H3 (western blotting) and production of cytokines (ELISA) was detected in cell lysates.

Results: The results from the analysis of oxidative burst showed different inhibitory effects possibly due to the impact of Cl-amidine on the inhibition of other PAD isoforms and its potential effect on NADPH oxidase subunits. Activity of MPO released from neutrophils was slightly inhibited by Cl-amidine due to the potential impact of histone H3 citrullination on release of MPO during NETosis. The changes in IL-8 production and release could be caused by altered gene expression of neutrophils after PAD activation.

Conclusion: Citrullination of histone H3 has already been shown to play a role during formation of NETs. However, the participation of histone H3 citrullination in other neutrophil defense mechanisms is not fully understood. Studying histone H3 citrullination thus represent a promising target in the treatment of inflammatory conditions.

Keywords: Neutrophil; Citrullination; Histone H3; Oxidative Burst; Myeloperoxidase; Cytokines.

Introduction

Neutrophils are cells of non-specific immune response. They are the first white blood cell population to arrive to the site of inflammation, where they destroy foreign particles by various defense mechanisms and provide additional signaling molecules that recruit macrophages to the inflammatory site. Neutrophil defense mechanisms include phagocytosis, the formation of reactive oxygen species (ROS), degranulation, and last but not least, the ability to form neutrophil extracellular traps (NETs). For recent reviews see [1-4].

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Correspondance: *Milan Číž, Department of Experimental Biology, Faculty of Science, Masaryk University, Czech Republic.*

Email: milan.ciz@sci.muni.cz

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NETs play a pro-tumorigenic role in cancer biology by shaping the tumor microenvironment [5], promoting immune evasion, and facilitating metastatic spread [6]. NETs can shield tumor cells from T-cell and NK-cell attack, support primary tumor growth, and enhance cancer cell migration and invasion [7]. They also capture circulating tumor cells, promote their extravasation, and can even awaken dormant cancer cells by remodeling the extracellular matrix, thereby contributing to metastatic relapse [8]. Elevated NET levels are associated with poor overall and disease-free survival, underscoring their importance as both biomarkers and potential therapeutic targets [9].

Modification by citrullination of histone H3 is thought to be involved in the in vitro formation of NETs [10]. Peptidyl arginine deiminases (PADs) convert arginine residues to the non-standard amino acid citrulline in a variety of protein substrates [11]. PAD4 isoform is expressed in granulocytes and is essential for the formation of NETs via PAD4-mediated histone citrullination [12]. Normally, PAD4 is situated in the cytoplasm of the cell, but it moves to the nucleus during cell activation where its substrate histone H3 is located [13]. PAD4 is the only PAD isoform which has a signal for translocation to the nucleus [12]. Citrullination of histones is thought to promote NET formation by inducing chromatin decompensation and facilitating the expulsion of chromosomal DNA. Numerous stimuli have been reported to lead to PAD4 activation and NET formation [13]. However, how this signaling process proceeds and how PAD4 becomes activated in cells is largely unknown. A comprehensive survey of the receptors and signaling pathways regulating PAD4 activation is important for our understanding of innate immunity. The identification of signaling intermediates in PAD4 activation may also lead to the generation of pharmaceuticals to target NET-related pathogenesis.

While citrullination of histone H3 has already been shown to play a significant role during formation of NETs [10], the participation of histone H3 citrullination in other neutrophil defense mechanisms is not fully understood. Therefore, we provide some evidence suggesting changes in PAD activity in neutrophils may lead to the altered activity of myeloperoxidase (MPO), ROS formation and production of pro-inflammatory cytokines, such as interleukin (IL)-8.

During the citrullination of histones, a positive charge is lost. This results in weakening of histone-DNA interactions which leads to the chromatin decompensation and allows the attachment of transcription factors to DNA. Thus, citrullination is capable of affecting gene expression and is one of the epigenetic modifications [14]. The inflammation, as a typical innate immune response, is very complex and insists on the precise control of many functional mechanisms acting at different levels, including also the regulation of gene expression by epigenetic modifications [15]. Dysregulation of histone citrullination was observed during several diseases associated with inflammation, e.g., rheumatoid arthritis [16,17], and multiple sclerosis [18,19]. Studying histone H3 citrullination thus represent a promising target in the treatment of immune system related disorders.

We hypothesize that enhanced activity of PAD and production of citrullinated proteins, such as histone H3, in neutrophils leads to chromatin decompensation, which results in altered gene expression profile. Thus, citrullination of histone H3 protein is manifested by phenotype plasticity and functional variability in neutrophils.

Materials and methods

Human neutrophil isolation: The described work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans, the volunteers gave informed consent. Blood was taken from the cubital vein of healthy volunteer to the syringe with anti-coagulant (sodium citrate). Blood was mixed with 3% dextran and sedimented for 45 min. The upper fraction was layered on the Histopaque 1077 and centrifuged (390 g, 30 min, without acceleration and brake). The pellet was quickly but gently hemolysed with sterile water and washed with PBS. Isolated neutrophils were re-suspended in RPMI medium, counted (CASY, Innovati) and immediately used for the experiments.

Sample preparation: The neutrophils were diluted in RPMI and seeded to the 2 ml Eppendorf tubes, each tube containing $4x10^6$ cells. For the various citrullination stimuli testing, cells were treated with Calcium-Ionophore A23187 (CaI), phorbol myristate acetate (PMA), opsonized zymosan particles (OZP), N-formyl methionyl-leucyl-phenylalanine (FMLP), or HBBS (negative control) for 3 h at 37°C. For the testing with PAD inhibitors, cells were treated with Cl-amidine (pan-PAD inhibitor, Cayman Chemical, USA), Thru-Asp-F-amidine (TDFA specific PAD4 inhibitor), or HBBS (control) for 30 min at 37°C. CAI (final concentration 7.5 µM) or HBBS (negative control) was added to the samples and incubated for another 30 min at 37°C. The final volume of each sample was 2 ml. Samples were centrifuged and the medium was collected. Samples were kept on ice. The cells were washed with cold PBS and lysed in 200 µl IP buffer. Lysates were sonicated by Sonopuls (Bandelin) for 5 s, centrifuged (16000 g, 5 min, 4°C) and the supernatant was collected. Mediums and lysates were used for further analysis (see below). All analyses with exception of chemiluminescence were run with this sample preparation set-up.

Western blot analysis (Histone H3 detection): The protein concentration was measured in lysates by BCA assay. The samples were diluted to the same concentration and mixed with Lemli buffer. Proteins were resolved in a 15% SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked 1 h with 1% gelatin, incubated overnight at 4°C with Anti-Histone H3 (citrulline R2 + R8 + R17) antibody (1:500; ab5103, Abcam) or Anti-Histone H3 antibody (1:1000; ab10799, Abcam), washed and incubated 1 h with peroxidase conjugated anti-rabbit antibody (1:5000) or anti-mouse antibody (1:10000), respectively. Blots were analyzed via enhanced chemiluminescence (Thermo Fisher Scientific), densitometric analysis was performed in ImageJ program.

ELISA (Cytokine measurement): The concentration of produced and released IL-6, IL-8, IL-10, and TNF-α was detected in cell lysates and mediums using commercial ELISA kits (Thermo Fisher Scientific, USA).

TMB assay (MPO detection): The MPO activity was detected by colorimetric TMB (tetramethylbenzidine) assay. Briefly, 105 µl of reaction mixture (2 mM TMB, 0.5 mM H_2O_2 in 300 mM sodium acetate, pH=4.4) was added to the 15 µl of sample medium. The absorbance was measured at 655 nm by Infinite M200 (Tecan) microplate reader after 20 min of incubation at room temperature.

Chemiluminescence measurement (ROS detection): The chemiluminescence was measured using the microtiter plate luminometer Orion II (Berthold Detection systems GmbH, Germany). The principle of the method was previously described [20]. The neutrophils were seeded to the 96-well luminometric plate in concentration $1x10^5$ cells per well and treated with Cl-amidine, TDFA, or HBBS (control) for 30 min at 37°C. Luminol was added to the cells in final concentration 1 mM, followed CaI in final concentration 7.5 µM; the final volume of the sample was 300 µM. All samples were performed in duplicates. Immediately after CaI addition, the plate was placed into the luminometer and measurement was started. Chemiluminescence was measured repeatedly during 30 min, the samples were incubated in the apparatus at 37°C.

All reagents were purchased from Merck (Germany), unless stated otherwise.

Statistical analysis

Results are reported as mean + standard error of the mean (SEM). Results were normalized with reference to the CaI-activated control cells in each run-on account of the variability due to individual donors. Data were statistically analyzed using one sample t test in GraphPad Prism version 8.01 for Windows (GraphPad Software, USA).

Results

We observed histone H3 citrullination in neutrophils activated with various types of stimuli. Our results demonstrate that CaI, a compound increasing the intracellular calcium concentration, is the most potent inductor of histone H3 citrullination when compared to common neutrophil activators PMA, OZP, and FMLP (Figure 1).

Further, we tested the effects of Cl-amidine (pan PAD inhibitor) and TDFA (PAD4 inhibitor) on histone H3 citrullination induced by CaI. The inhibition of citrullination of histone H3 was achieved using both types of inhibitors, Cl-amidine or TDFA (Figure 2). It is obvious that both PAD4 and other cytoplasmic PAD isoforms contributed to histone H3 citrullination.

The effects of histone H3 citrullination on physiological manifestations of neutrophils - oxidative burst, activity of MPO and production and release of IL-8 - have been demonstrated.

The oxidative burst was slightly inhibited in neutrophils pre-treated with Cl-amidine in a dose dependent manner, being statistically significant only in the concentration of 50 µM. On the other hand, no inhibitory effect of TDFA on the production of ROS was observed (Figure 3). Similarly, Cl-amidine in both used concentrations slightly decreased the activity of MPO released from activated neutrophils while TDFA had no effect (Figure 4).

Finally, the amount of cytokine production and release from neutrophils was detected. No changes were observed for TNF-α, IL-6 and IL-10 (data not shown). The only significant changes were observed in IL-8 production and release. Interestingly, only Cl-amidine decreased the concentration of IL-8 in cell lysates while both Cl-amidine and TDFA decreased the concentration of IL-8 in neutrophil supernatants (Figure 5).

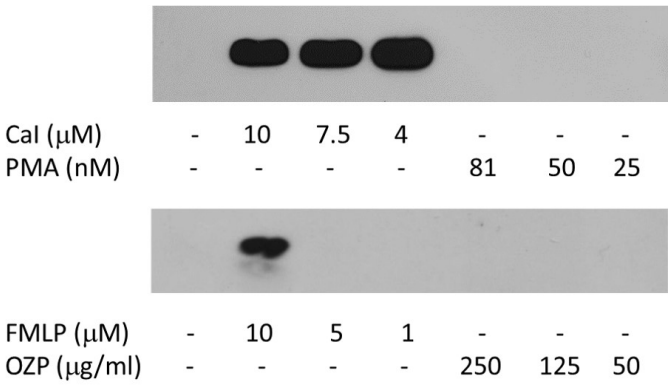

Figure 1: Histone H3 citrullination in human neutrophils. Neutrophils isolated from healthy donors were treated by three concentrations of CaI, PMA, FMLP, and OZP for 3 h. Representative Western blot pictures show citrullination of histone H3. Protein samples were prepared from lysates of $2x10^6$ cells/ml.

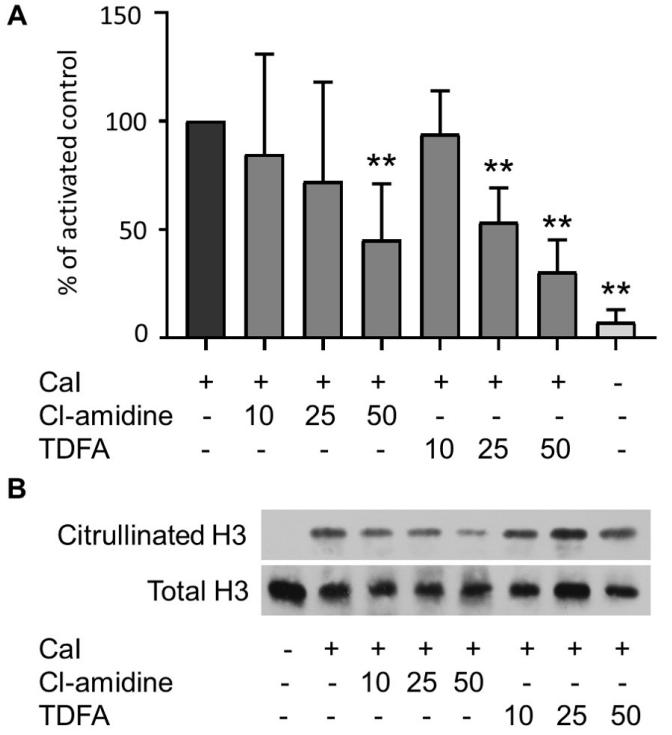

Figure 2: (A) Histone H3 citrullination in neutrophils. The result of optical density of citrullinated histone H3 was evaluated with total histone H3. The results from 6 independent experiments were converted to a % of positive control (neutrophils with CaI) and expressed as the mean + SEM. Statistically significant differences between samples with inhibitor or negative control and positive control are shown (**p<0.01, *p<0.05). Panel **(B)** Representative Western blot pictures show citrullination of histone H3.

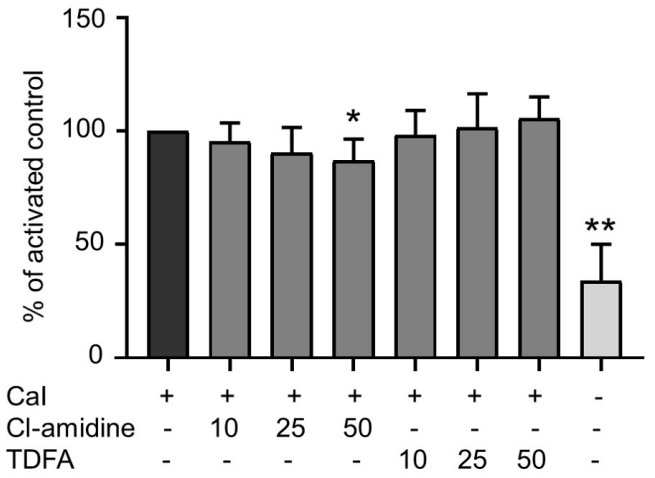

Figure 3: Oxidative burst of neutrophils. The results from 4 - 8 independent experiments were converted to a % of positive control and are expressed as the mean + SEM. Statistically significant differences between samples or negative control and positive control are shown (**p < 0.01, *p < 0.05).

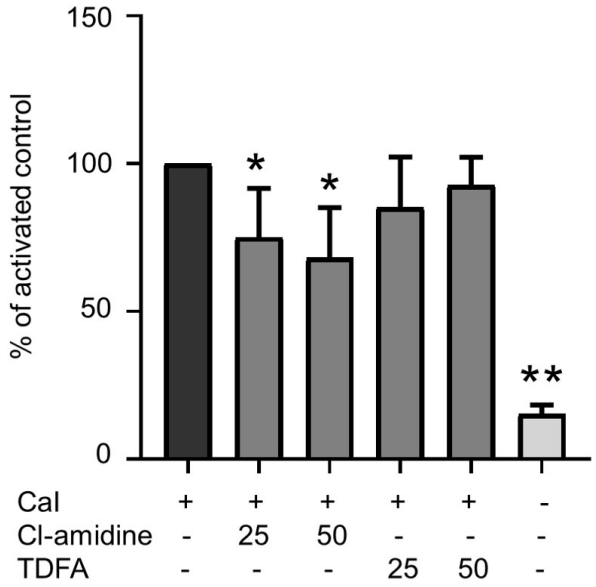

Figure 4: Activity of myeloperoxidase released from neutrophils. The data of colorimetric measurement were converted to a % of positive control (neutrophils activated with Cal). The results from 5 independent experiments are expressed as the mean + SEM. Statistically significant differences between samples or negative control and positive control are shown (**p<0.01, *p<0.05).

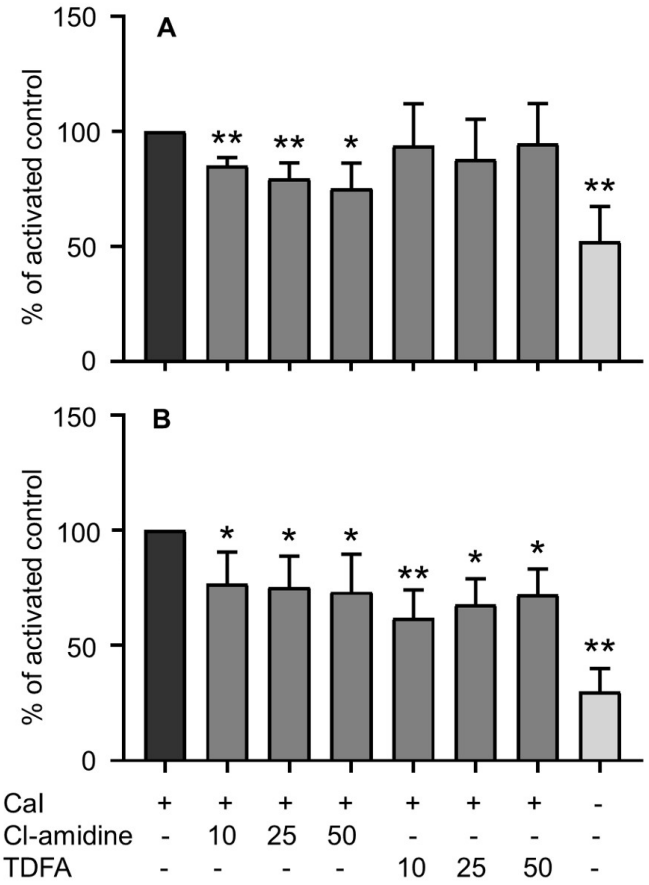

Figure 5: Activity of myeloperoxidase released from neutrophils. The data of colorimetric measurement were converted to a % of positive control (neutrophils activated with Cal). The results from 5 independent experiments are expressed as the mean + SEM. Statistically significant differences between samples or negative control and positive control are shown (**p<0.01, *p<0.05).

Discussion

Nowadays, several approaches how to study the effect of PADs on the citrullination of histones in various cells exist. A key regulator of PADs is calcium [21]. Its cytoplasmic concentration ranges from 10^{-8} to 10^{-6} M under physiological conditions. In vitro, citrullination is observed in the presence of high calcium concentration, which can be reached in cells using calcium ionophores (A23187) [22]. Another approach is to use inhibitors with different PAD selectivity [23]. According to the literature, two inhibitors have been selected to determine whether inhibition of histone H3 citrullination in neutrophil granulocytes would affect their functional manifestations. Widely used and well characterized irreversible PAD inhibitor is Cl-amidine [24]. It is derived from benzoyl arginine and contains reactive positively charged halo acetamidine moiety that mimics the PAD substrate - the guanidine moiety. Cl-amidine covalently binds to the active site of PADs, blocking the binding of original substrate. In order to increase the effect and specificity of the inhibitor, several compounds have been derived from Cl-amidine, e.g., tripeptide TDFA containing fluoroacetamide. TDFA exhibits higher PAD4 selectivity over other PAD isoforms [23].

Despite the general opinion that PAD4 is the major isoform responsible for histone H3 citrullination [25], we observed that Cl-amidine (pan-PAD inhibitor) is more effective than TDFA (selective PAD4 inhibitor). From these results it is clear that PAD isoforms other than PAD4 were responsible for the observed effects. One such candidate isoform is PAD2, another important neutrophil citrullination enzyme. It was shown previously that PAD2 deficiency decreased NET formation [26]. Similarly, it has been recently demonstrated that PAD2 inhibition could reduce NETosis and decrease inflammatory cytokine production in a mouse model

of lethal lipopolysaccharide (LPS)-induced endotoxic shock [27]. In contrary, Darrah [28], reported that PADs expressed in human neutrophils have unique substrate specificities, independent of their subcellular distribution, histone H3 being citrullinated only by PAD4.

It was also found that Cl-amidine inhibited nitric oxide (NO) generation in a time- and dose-dependent manner in maturing DCs activated by LPS. This suppression of NO generation was independent of changes in NO synthase (NOS) enzyme activity levels but was instead dependent on changes in inducible NO synthase (iNOS) transcription and expression levels [29]. This phenomenon could contribute to a decreased chemiluminescence response in Cl-amidine treated cells in our experiments since NO contributes to the overall CL response of phagocytes.

We observed that CaI is the most potent inductor of histone H3 citrullination when compared to common neutrophil activators. This finding was supported by the data of de Bont et al. [30]. They observed that the processes leading to NET formation after various types of stimuli proceed via different pathways, which do not need to be associated with citrullinated histones presence. Due to their opinion, it can be caused by the activity of proteases in some cases when the citrullinated histones are readily cleaved. Similarly, Zhou et al. [31], observed that agents triggering a sufficiently large influx of extracellular calcium ions induced a marked citrullination of multiple proteins in human neutrophils while other neutrophil activating stimuli, such as formyl-peptides or phorbol esters, did not induce any detectable increase in protein citrullination, suggesting that receptor-induced calcium mobilization is insufficient to trigger hypercitrullination. Moreover, stronger neutrophil activators lead to the over-activation of NADPH oxidase and increase production of ROS. ROS were proved to inhibit the activity of PAD2 and PAD4 [32].

In 2018, Zhou et al. reported direct effects of PAD4 on NADPH oxidase, the crucial enzyme of neutrophil oxidative burst [33]. They observed that PAD4 was associated with the cytosolic subunits $p47^{phox}$ and $p67^{phox}$ of NADPH oxidase. Activation of PAD4 led to rapid citrullination of $p47^{phox}$ and $p67^{phox}$, as well as their dissociation from PAD4. This dissociation prevented the assembly of an active NADPH oxidase complex and an oxidative burst in neutrophils.

Citrullination-induced NETs are characterized by a mixing of nuclear chromatin with cytoplasmic proteins including MPO in the extracellular space [34]. In line with this we found Cl-amidine was able to decrease the activity of MPO in neutrophil supernatants. On the other hand, MPO itself might be important for NETs formation, at least under certain circumstances [35].

We also observed a partial inhibition of IL-8 production and release from neutrophils after treatment with PAD inhibitors. These results are in accordance with findings of Raup-Konsavage et al. [36], who reported lowered expression of pro-inflammatory cytokines including IL-8 in PAD4-deficient mice.

Conclusion

Using two inhibitors, Cl-amidine and TDFA, the impact of histone H3 citrullination on physiological manifestations of neutrophils has been demonstrated. The results from the analysis of oxidative burst showed different inhibitory effects possibly due to the impact of Cl-amidine on the inhibition of other than PAD4 isoforms and its potential effect on NADPH oxidase subunits. Activity of MPO released from neutrophils was slightly inhibited only by Cl-amidine due to the potential impact of histone H3 citrullination on release of MPO during NETosis. Comparison of inhibitors shows more effective inhibition of PAD caused by Cl-amidine, possibly due to the concomitant inhibition of other PAD isoforms. The changes in IL-8 production and release could be caused by altered gene expression after PAD activation.

Declarations

Conflicts of interest: The author declares that there are no conflicts of interest.

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