Comparative analyses of PAD expression and activity in myeloid cell lines

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Abstract

Protein arginine deiminase (PAD) enzymes and the arginine to citrulline reaction that they catalyze have increased in prominence in recent years, as dysregulation is associated with multiple inflammatory and autoimmune diseases. Under normal circumstances, PAD4 is required for the generation of neutrophil extracellular traps (NETs) by neutrophils. PAD4 citrullinates histone H3, initiating chromatin decondensation and subsequent extrusion of nuclear DNA to form a net-like structure. These NETs are important in innate immune responses to infection, as loss of components of this process can lead to the inability to eradicate usually harmless pathogens. We have developed tools to better understand the complete picture of PAD2- and PAD4-catalyzed citrullination and its effects in various systems. HL-60 and THP-1 cells differentially expressed PAD2 and PAD4, depending on differentiation and activation stimuli, as shown by ELISA. The activity of these PAD enzymes was also highly dependent on the differentiation and activation status of these cells. The PAD4 target histone H3 was found to be citrullinated only upon activation of differentiated cells, although PAD4 was expressed in differentiated cells that had not been activated. These data help to elucidate appropriate model systems for researchers looking to investigate PAD activation and downstream targets in immune cells.

Background

Citrullination is the process of deimination of charged arginine residues to neutral citrulline residues within proteins (Figure 1). The first antibodies against citrullinated proteins were identified in rheumatoid arthritis (RA) patients in 1998.[1] This eventually led to a diagnostic test for anti-citrullinated protein antibodies (ACPA) in patients suspected to have RA. Citrullination is catalyzed by PAD enzymes in a calcium-dependent manner. PAD activity is largely restricted to specific tissues and is regulated transcriptionally, translationally, and by the availability of calcium.[2] Both PAD2 and PAD4 are expressed in immune cells, and overexpression and increased activity have been described in several autoimmune diseases as well as cancer.[3-5]

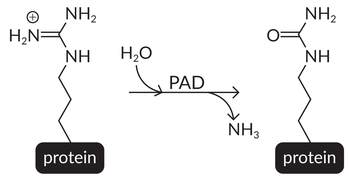


Figure 1 – Arginine to citrulline reaction catalyzed by PADs.

Under normal conditions, PAD4, which is expressed by differentiated neutrophils, has been shown to be important in host defense. The loss of PAD4 prevents neutrophils from forming NETs, web-like extruded complexes consisting of DNA and antimicrobial agents, which are critical for neutrophil-mediated killing of some pathogens.[6] During NET formation, PAD4 citrullinates histones, which reduces their binding affinity for DNA and promotes the decondensation of chromatin. Subsequent extrusion of double-stranded DNA (dsDNA) studded with histones, antimicrobial enzymes, and defensin peptides allows the capture, killing, and phagocytosis of bacteria and other pathogens.[7-9] NET clearance is an equally important process, as excessive circulating NET fragments could be toxic and could stimulate autoantibody production. This process is thought to be involved in the pathogenesis of systemic lupus erythematosus, which is characterized by accumulation of autoantibodies to dsDNA and histones.[10] These studies are quickly building up to a critical importance for PAD activity and NET formation and clearance in the pathogenesis of multiple inflammatory and autoimmune disorders.[3-4,11-12]

The transient nature of NETs can make them challenging to study. Cayman Chemical has worked to develop a series of tools to enable researchers to study the processes involved in PAD activation and NETosis. Selective inhibition of PADs may prove to be a therapeutic target for some autoimmune disorders,[13] and development of simple methods for evaluating expression and activity in cells and *in vitro* will be useful in the pursuit of effective drugs. Here, we outline how these tools perform in a myelomonocytic cell line, HL-60, which can be differentiated into a neutrophil-like cell.

Results

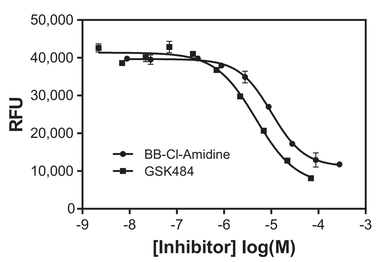


Figure 2 – Inhibition of human recombinant PAD4 by BB-Cl-Amidine and GSK484.
Inhibition of human recombinant PAD4 was tested using Cayman Chemical's PAD4 Inhibitor Screening Assay Kit (Ammonia) (Item No. 700560). BB-Cl-Amidine (Item No. 17079) and GSK484 (Item No. 17488) inhibited PAD4 activity with pIC_{50} values of 4.99±0.03 and 5.206±0.07, respectively.

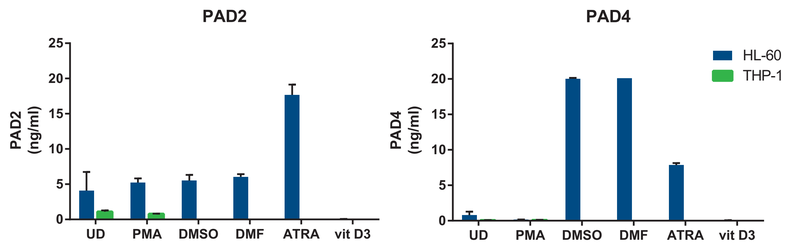


Figure 3 – HL-60 cells can be induced to upregulate expression of PAD enzymes by specific differentiation stimuli.
HL-60 cells (blue) were left undifferentiated (UD) or differentiated for 72 hours at 37°C with the following stimuli: 25 nM PMA (Item No. 10008014), 1.25% DMSO, 100 mM DMF, 0.1 µM all-*trans* retinoic acid (ATRA, Item No. 11017), or 100 nM vitamin D_3 (vit D3, Item No. 11792). THP-1 cells (green) were left undifferentiated or differentiated with 25 nM PMA. Cells were lysed by three freeze-thaw cycles in PBS. PAD2 and PAD4 were quantified in clarified lysates by ELISA using Cayman Chemical's PAD2 (human) ELISA Kit (Item No. 501450) and PAD4 (human) ELISA Kit (Item No. 501460). PAD2 expression was greatest in HL-60 cells after differentiation with retinoic acid, while PAD4 was induced in HL-60 cells by DMSO and DMF treatment, which are known to be strong inducers of neutrophilic differentiation.

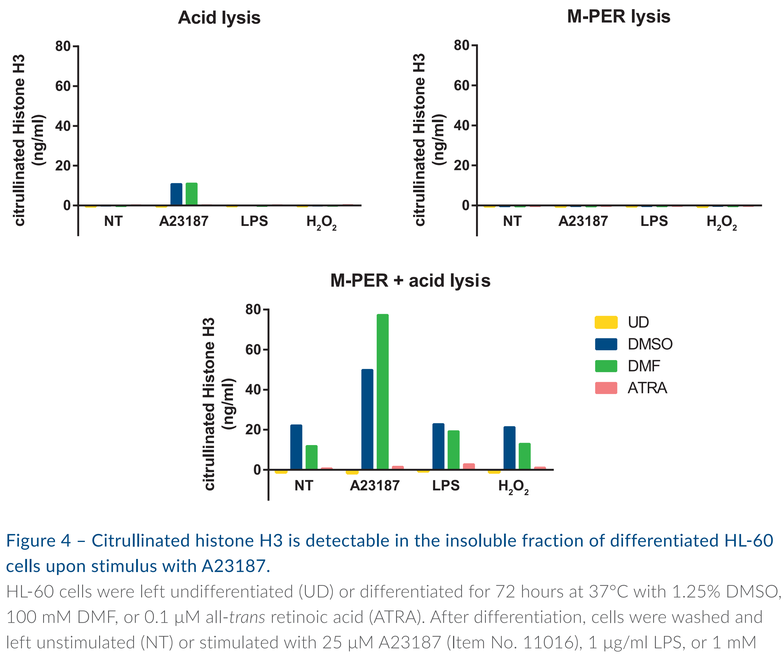


Figure 4 – Citrullinated histone H3 is detectable in the insoluble fraction of differentiated HL-60 cells upon stimulus with A23187.
HL-60 cells were left undifferentiated (UD) or differentiated for 72 hours at 37°C with 1.25% DMSO, 100 mM DMF, or 0.1 µM all-*trans* retinoic acid (ATRA). After differentiation, cells were washed and left unstimulated (NT) or stimulated with 25 µM A23187 (Item No. 11016), 1 µg/ml LPS, or 1 mM H_2O_2 in RPMI containing BSA and $CaCl_2$ for 2 hours. Cells were lysed with either 0.4 M H_2SO_4 or M-PER lysis buffer containing protease inhibitors followed by extraction of the insoluble fraction using 0.4 M H_2SO_4. Citrullinated histone H3 was quantified by ELISA in all lysates using Cayman Chemical's Citrullinated Histone H3 ELISA Kit (Item No. 501440). Notably, histone H3 citrullination was found only in the detergent-insoluble, acid-extracted fraction. A23187 induced citrullination of histone H3 in differentiated HL-60 cells under conditions favorable for PAD4 expression.

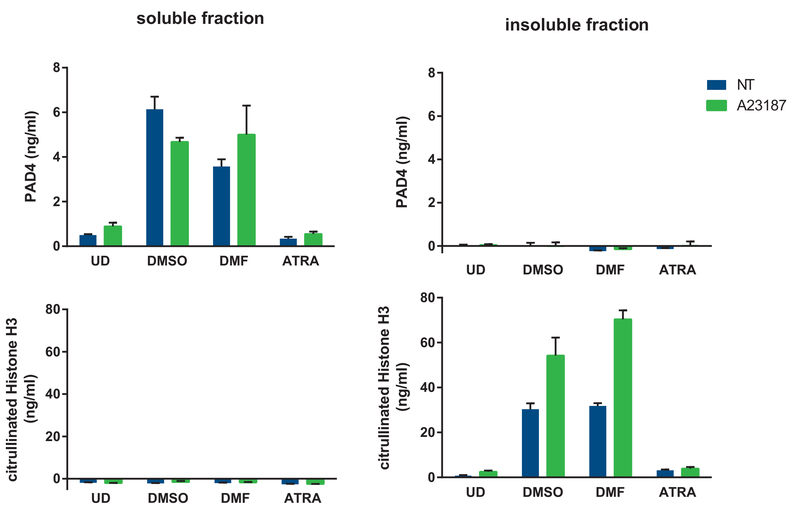


Figure 5 – Citrullinated histone H3 and PAD4 are detectable in different fractions of differentiated HL-60 cells after stimulus with A23187.
HL-60 cells were left undifferentiated (UD) or differentiated for 72 hours at 37°C with 1.25% DMSO, 100 mM DMF, or 0.1 µM all-*trans* retinoic acid (ATRA). After differentiation, cells were washed and left unstimulated (NT, blue) or stimulated with 25 µM A23187 (green) in RPMI containing BSA and $CaCl_2$ for 2 hours. Cells were lysed with M-PER lysis buffer containing protease inhibitors (soluble fraction) followed by extraction of the insoluble fraction using 0.4 M H_2SO_4. Citrullinated histone H3 and PAD4 were quantified by ELISA in all lysates. PAD4 expression, only found in the soluble fraction, was unaffected by A23187 treatment, while the citrullination of histone H3, a product of PAD4 activation, was elevated by treatment with calcium ionophore.

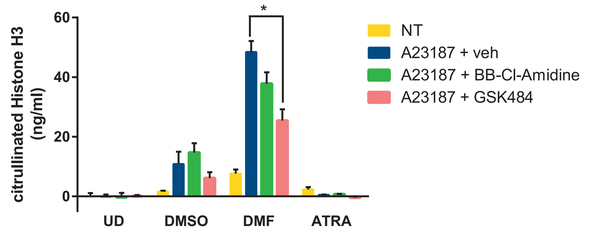


Figure 6 – PAD inhibitors block some citrullination of histone H3 in differentiated HL-60 cells.
HL-60 cells were left undifferentiated (UD) or differentiated for 72 hours at 37°C with 1.25% DMSO, 100 mM DMF, or 0.1 µM all-*trans* retinoic acid (ATRA). After differentiation, cells were washed and left unstimulated (NT, yellow) or stimulated with 25 µM A23187 in the presence of vehicle (blue), 10 µM BB-Cl-Amidine (green), or 10 µM GSK484 (pink) in RPMI containing BSA and $CaCl_2$ for 2 hours. Cells were lysed with M-PER lysis buffer containing protease inhibitors followed by extraction of the insoluble fraction using 0.4 M H_2SO_4. Citrullinated histone H3 was quantified by ELISA in all lysates. GSK484 showed significant decrease in citrullination of histone H3 in DMF-differentiated, A23187-stimulated HL-60 cells (t-test, p = 0.03).

Conclusions

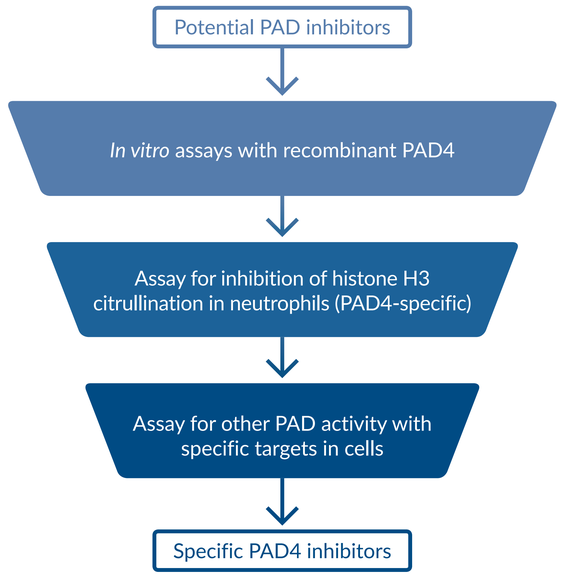
1. HL-60 cells do not express significant quantities of neutrophil elastase in response to differentiation and activation stimuli, as determined by activity assay (data not shown; Item No. 600610).

2. HL-60 cells express significant levels of PAD2 and PAD4 upon specific differentiation signals.

3. Stimulation of PAD4-expressing (but not PAD2-expressing) HL-60 cells with calcium ionophore induces the citrullination of histone H3, indicating that the enzyme is active and citrullinating its typical targets.

   A. It should be noted that the apparent presence of all of the PAD4 in the soluble fraction does not imply differential compartmentalization of PAD4 and histone H3.
   B. Method: Acid extraction of the insoluble fraction yields the greatest citrullinated histone signal.

4. Established PAD inhibitors can decrease citrullination of histone H3 in PAD4-expressing cells.
   A. Confirmation of PAD specificity of citrullination
   B. "*in vivo*" screening for PAD4 inhibitors



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