Abstract

The role of well-characterized post-translational protein modifications, such as phosphorylation or methylation, in regulating cellular metabolism is widely described. However, in this context, protein citrullination is one of the less studied modifications. Citrullination, also known as deimination, involves the conversion of peptidylarginine to peptidylcitrulline. The reaction has significant molecular consequences for modified proteins *via* altering their charge, which in turn impacts their interactions with other proteins or modifies the active sites, thereby inhibiting enzyme activity. Citrullination is catalyzed by enzymes from the peptidylarginine deiminase (PAD) family. To date five PAD isoforms are known, of which PAD2 and PAD4 have been most extensively studied. These isoforms are also the only ones capable of histone citrullination, suggesting their involvement in the epigenetic regulation of gene expression. However, the physiological roles of this modification remain poorly understood. Existing data largely focus on the influence of citrullination and increased PAD activity on autoimmune diseases. Recently there is also gathering evidence suggesting PADs involvement in the progression of cancer.

In the presented study, human endothelial cells were used as the primary research model. Including the immortalized human microvascular endothelial cell line (HMEC-1) and primary endothelial cells isolated from the human umbilical vein (HUVEC). In the body, endothelial cells not only form a barrier between circulating blood and tissues but also perform many critical regulatory functions. These include regulating blood flow, blood pressure, coagulation, and fibrinolysis. One of the key functions of endothelial cells is the regulation of angiogenesis, the formation of new blood vessels from pre-existing ones. Angiogenesis is vital for not only for physiological processes, such as wound healing, but also plays a role in pathological conditions. In cancer, pathological angiogenesis is particularly significant as it supplies oxygen and nutrients to tumor, facilitating its growth and, in later stages, enabling metastasis. In endothelial cells, angiogenesis is regulated by numerous mechanisms, including epigenetics. Inhibiting pathological angiogenesis is currently an attractive strategy for cancer therapy, leading to ongoing research into novel mechanisms that regulate this process. While the role of well-studied protein modifications in modulating endothelial cell functions has been widely described, no conclusive information is currently available on the role of citrullination in this context.

This dissertation investigates the impact of inhibiting PAD activity, and thus citrullination of proteins, including histones, on selected functions and parameters of the epigenome of endothelial cells. Two methods were employed to inhibit PAD activity: (i) pharmacological approach using commercially available inhibitors, and (ii) a genetic silencing approach involving transcriptional silencing of the PAD4 gene. In the pharmacological model, three commercial PAD inhibitors were used: BB-Cl-amidine (BBCLA), Cl-amidine (CLA), and F-amidine (FA). PAD4 expression in HMEC-1 cells was silenced using shRNA delivered via lentiviral transduction.

The cytotoxicity of the selected inhibitors was analyzed using the resazurin reduction assay. Cytotoxic effects were observed only for BBCLA at concentrations >1.75 μM in both endothelial cell

types. The effect of the inhibitors on H3 citrullination levels in endothelial cells was analyzed using Western blotting. BBCLA exhibited the highest inhibitory potential against H3 citrullination in both cell types, reducing this modification to approximately 20% of control levels (*p<0.0001). None of the PAD inhibitors significantly altered the progression of the HMEC-1 cell cycle, as analyzed by flow cytometry. However, significant changes were observed in the expression profile of selected cyclins, which was analyzed using qPCR.

The effects of pharmacological inhibition of citrullination on the angiogenic potential of endothelial cells were assessed using functional assays and by analyzing secretory and transcriptional profiles. Each inhibitor reduced both the migratory capacity and pseudocapillary structure formation potential of endothelial cells. PAD activity inhibition in HMEC-1 cells with BBCLA or CLA significantly decreased NO• secretion (*p<0.001 or *p<0.05, respectively). Additionally, BBCLA significantly inhibited total MMP activity (*p<0.001) in HMEC-1. Furthermore, each inhibitor increased mRNA expression and secretion of PEDF, a potent angiogenesis inhibitor. PAD activity inhibition also decreased VEGFA secretion, and in the case of BBCLA and CLA, a reduction in the expression of the gene encoding this factor was observed. Pharmacological inhibition of PAD activity in HMEC-1 cells also resulted in a cytoprotective effect against H_2O_2, significantly (*p<0.0001) reducing DNA damage caused by this factor. Pre-incubation of HMEC-1 cells with the inhibitors also reduced ROS production under oxidative stress induced by t-BOOH (*p<0.001). At the transcriptional level, a significant increase in Nrf2 expression was noted, a key transcription factor regulating genes related to antioxidant potential, which may explain the effects described above. Thus, PAD involvement in regulating selected redox homeostasis parameters was demonstrated. To elucidate the molecular basis of the inhibitors' antiangiogenic effects and PAD inhibition in endothelial cells, the levels of selected elements of the PI3K/Akt signaling pathway were analyzed. Each inhibitor significantly reduced the level of active Akt kinase (pH-Akt S473) to approximately 40% of control levels (*p<0.0001 or *p<0.05), suggesting PAD involvement in regulating the PI3K/Akt pathway, which plays a critical role in shaping the angiogenic potential of endothelial cells as well as their other functions. These findings indicate that PAD activity is crucial for regulating endothelial cell functions, including their angiogenic potential.

HMEC-1 PAD4 KD cells were generated using RNA interference mechanisms, specifically shRNA. In this model, selected effects observed in the pharmacological model were verified. A similar statistically significant decrease in active Akt kinase levels (pH-Akt S473) to approximately 40% of control (*p<0.05) was found, confirming PAD involvement, particularly PAD4, in regulating the PI3K/Akt pathway in HMEC-1 cells. A relationship between PAD4 activity and the expression of PEDF and PAI-1 was also confirmed, with significantly increased mRNA levels observed for these factors (*p<0.05 or *p<0.001). No changes in VEGFA expression were observed, suggesting that the changes induced by BBCLA and CLA may result from non-specific effects or inhibition of other PAD isoforms. Functionally, HMEC-1 PAD4 KD cells exhibited a significantly reduced ability to form pseudocapillary structures, similar to the pharmacological model. These findings confirm the conclusions from the

pharmacological model and highlight PAD involvement in regulating endothelial cell functions, particularly their angiogenic potential.

Selected epigenome parameters were analyzed for potential indirect interactions between PAD activity/citrullination and other histone PTMs. In HMEC-1 cells, a potential indirect interaction between PADs and HDACs was demonstrated. Pharmacological inhibition of PADs significantly increased HDAC3 and HDAC5 expression, while PAD4 silencing showed this effect only for HDAC3. Notably, these isoforms are described in the literature as negative regulators of angiogenesis. Using the HDAC inhibitor SAHA significantly (*$p<0.001$) reduced H3 citrullination levels, confirming the validity of further studies on potential direct or indirect interactions between PADs and HDACs. The literature provides more information on potential interactions between citrullination and histone methylation. This study analyzed these interactions at the level of SET7/9 methyltransferase activity. Specific silencing of SET7/9 expression in HMEC-1 cells significantly (*$p<0.05$) reduced the expression and levels of PAD2 and PAD4 isoforms, as well as histone citrullination. A similar decrease in H3 citrullination was observed in cells with silenced LSD1 or G9a expression. This suggests a potential indirect interaction between SET7/9, LSD1, and G9a activity and the status of protein citrullination, including histones. Pharmacological inhibition of PADs did not significantly affect SET7/9, LSD1, or G9a levels. However, in HMEC-1 PAD4 KD cells, increased mRNA expression for SET7/9 and LSD1 was observed. PAD inhibitor treatment did not result in significant changes in H3K9me3 and H3K27me3 levels, which, considering the literature, is surprising. PAD inhibition did not significantly affect chromatin structure, as analyzed by HP1 isoform levels, while a significant increase (*$p<0.05$) in CAF1A was observed only in cells treated with BBCLA or CLA.

In summary, the presented results confirm PAD involvement in shaping endothelial cell functions, particularly their angiogenic potential. PAD activity, specifically PAD4, is crucial for regulating the PI3K/Akt pathway. Potential indirect interactions between citrullination and other histone PTMs in HMEC-1 cells were also confirmed, particularly at the PAD–HDAC and SET7/9–PAD levels. This dissertation also underscores the importance of further research into protein citrullination, especially concerning the regulatory function of this modification beyond inflammation and immune response context.

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