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Original Article

Histone acetylation and methylation significantly change with severity of atherosclerosis in human carotid plaques

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ABSTRACT

Background: The aim of the study was to analyze histone acetylation, methylation, and the expression of their corresponding transferases in atherosclerotic plaques of patients with carotid artery stenosis.
Methods: Atherosclerotic tissue from our biobank ($n=80$) was divided into various segments covering all plaque stages and classified according to the American Heart Association. The plaques were assigned to early (types I–III) or advanced (types V–VII) stage group of atherosclerosis. Ten healthy carotid arteries from transplant donors served as controls. The expression of histone acetyltransferases (GNAT group: *GCN5L*, P300/CBP group: *P300*, MYST group: *MYST1* and *MYST2*) and histone methyltransferases (H3K4: *MLL2/4*, *SET7/9*, and *hSET1A*; H3K9: *SUV39H1*, *SUV39H2*, *ESET/SETDB1*, and *EHMT1*; H3K27: *EZH2* and *G9a*) was analyzed by SYBR-green-based real-time polymerase chain reaction. Histone acetylation/methylation in the cells within atherosclerotic plaques was determined by immunohistochemistry.
Results: Increased histone acetylation was observed on H3K9 and H3K27 in smooth muscle cells (SMCs) in advanced atherosclerotic lesions compared to healthy vessels ($P=.002$ and .034). H3K9 acetylation in SMCs and macrophages was associated with plaque severity of atherosclerosis ($P=.048$ and $<.001$). Expression of GCN5L and MYST1 also correlated with the severity of atherosclerosis ($P<.001$). Methylation of H3K9 and H3K27 was significantly reduced in atherosclerotic plaques in SMCs and inflammatory cells ($P<.001$ and .026). Methylation on H3K4 was significantly associated with the severity of atherosclerosis. Expression of methyltransferase MLL2/4 was increased in advanced stages of atherosclerosis ($P<.001$).
Conclusions: Histone acetylation and methylation seem to play a decisive role in atherosclerosis, showing significant differences between healthy vessels and vessels at different stages of atherosclerosis.

1. Introduction

Epigenetics describe heritable posttranslational modifications of DNA or histones, while the DNA sequence itself remains unchanged [1]. These processes are influenced by various physiological alterations (e.g., development, aging) and environmental factors (diet, drugs, smoking, etc.). Furthermore, epigenetic state can be altered by various human diseases [2]. In the nucleus, DNA is wrapped around octamers composed of dimeric histones H2A, H2B, H3, and H4, forming chromatin. Different modifications of their N-terminal tails, such as acetylation, methylation, phosphorylation, ubiquitination, and SUMOylation, regulate the accessibility of DNA for transcription factors [3]. Histone acetylation and methylation are mechanisms of particular importance in the modulation of chromatin structure. Transfer of acetyl groups to amino groups of lysines by histone acetyltransferases (HATs), as well as the effect of stearic hindrance, facilitates the binding of transcription factors to DNA and consequently increases gene expression. HAT families consist of the GNAT/MYST and P300/CPB groups, basal transcription factors, and nuclear receptor cofactors [4,5]. Transfer of acetyl groups to histone lysine residues seems to follow a global pattern, but acetylation on specific lysine residues is also possible [4]. Histone deacetylases (HDACs) can remove the acetyl groups and have a reverse effect on transcription [5]. In this context, epigenetic modification is mostly a dynamic process. In contrast to acetylation, methylation of histones is a more complex procedure that does not always lead to gene activation [6]. Whether genes are silenced or activated depends on the histone methylation site and whether monomethylation, dimethylation, or trimethylation takes place [7]. As the epigenome is influenced by many interacting external factors, understanding its modification is thought to be one of the keys to comprehend diseases of multifactorial

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genesis. A lot of work has been done in the field of cancer research in this regard [4,8]. Interestingly, however, almost nothing is known about the influence of epigenetics on cardiovascular diseases. Currently, there is increasing evidence that atherosclerotic processes might also be affected by epigenetic mechanisms. Growth, differentiation, migration, contractility, inflammation, and deposition of extracellular matrix by smooth muscle cells (SMCs) have been shown to be regulated by HAT and HDAC activity [9]. Increased inflammation due to endothelial injury has already been associated with DNA hypermethylation [10], resulting in the recruitment of inflammatory cells, which are known to be regulated by lysine acetylation [11]. The adhesion of monocytes to endothelial cells via VCAM-1 leads to progression of atherosclerosis and is regulated e.g. by the activity of HATs [12]. Another important factor for the function of the vascular endothelium is nitric oxide synthase (eNOS), which is controlled by both histone acetylation and methylation [13].

Given the evidence that epigenetic mechanisms might be closely related to the development and/or progression of atherosclerosis, this study focuses on the two most important histone modifications: acetylation and methylation. Our intention was to determine whether any differences appear concerning these histone modifications in early and advanced atherosclerotic plaques compared with healthy human carotid arteries. In addition, we attempted to assign specific histone alterations to individual cell types within the carotid atherosclerotic plaques by means of immunohistochemistry. Moreover, expression of selected corresponding methyltransferase and acetyltransferases was analyzed in order to reveal epigenetic factors involved in the pathogenesis of atherosclerosis.

2. Materials and methods

2.1. Study population

Carotid tissue samples from 80 patients who underwent carotid endarterectomy were selected from our biobank in the Department of Vascular and Endovascular Surgery (Klinikum rechts der Isar der Technischen Universität Muenchen, Germany). The patient demographic data are summarized in Table 1. Carotid lesions were dissected into three to five pieces dependent upon the size of the plaque tissue, in order to cover the different plaque stages, characterized according to the American Heart Association (AHA) classification and divided into early (types I–III) or advanced (types V–VII) atherosclerosis stage groups [14–16]. Atherosclerosis-free carotid vessels ($n=10$) of individuals obtained from the Department of Trauma Surgery served as controls (Table 1). The study was performed according to the guidelines of the World Medical Association Declaration of Helsinki. The local ethics committee of our university hospital approved the study and written informed consent was given by all patients.

Table 1
Patient characteristics

	Carotid patients ($n=80$)	Healthy controls ($n=10$)
Age (years)	70.5±8.9	59.8±5.2
Gender (male)	61.3%	60%
Neurological symptom	41.3%	0%
Hypertension	82.5%	*N.N.*
Hyperlipidemia	48.8%	*N.N.*
Smoking	36.3%	*N.N.*
Diabetes mellitus	35.0%	*N.N.*
Chronic kidney disease	5.0%	*N.N.*
Coronary disease	23.8%	*N.N.*
Aspirin/clopidogrel	93.8%	*N.N.*
Beta-blocker	48.8%	*N.N.*
ACE inhibitors	35.0%	*N.N.*
Statins	73.8%	*N.N.*

N.N. — not known.

2.2. Histology and plaque characterization

Carotid plaques were segmented in blocks of 3–4 mm, fixed in formalin, and embedded in paraffin (FFPE). As described above, segmentation was performed to cover different stages of atherosclerosis between type I and type VII. Hematoxylin–eosin and Elastica van Gieson staining were performed in order to assess the type of atherosclerotic lesions. The evaluation of plaque morphology and assessment of the intensity of staining for each antibody and all tissue samples was performed by two independent investigators blinded for the study outcome. In addition, the results were discussed with an experienced pathologist. Histological classification of carotid atherosclerotic lesions was performed as described by Stary and colleagues and approved by AHA [14–16].

2.3. Immunohistochemistry

All slides were coated with poly-L-lysine solution (0.1% w/v in water; Sigma-Aldrich, St. Louis, USA) in order to improve specimen adhesion during the staining procedure. Immunohistochemistry was performed on all tissue samples in order to differentiate endothelial cells (antihuman-CD31, mouse monoclonal, clone JC70A, dilution 1:100; Dako, Glostrup, Denmark), lymphocytes (antihuman-CD45 common leukocyte antigen, mouse monoclonal, clones 2B11 and PD7I26, dilution 1:200; Dako), macrophages/monocytes (anti-CD68, mouse monoclonal, clone KP1, dilution 1:2000; Dako), and SMCs (antismooth muscle actin, mouse monoclonal, clone HHF35, dilution 1:200; Dako). Subsequently, the following antibodies were used in order to detect the specific acetylation sites on histones: antihistone H3 (acetylK27) antibody ([EP8654] (ab45173), rabbit monoclonal, dilution 1:4000; Abcam, Cambridge, UK) and antihistone H3 (acetylK9) antibody ([Y28] (ab32129), rabbit monoclonal, dilution 1:2000, Abcam). For detection of methylation sites, the following antibodies were applied: dimethylhistone H3 (Lys4) [(C64G9), rabbit monoclonal, dilution 1:3000; Cell Signaling Technology Inc., Danvers, USA], dimethylhistone H3 (Lys9) (polyclonal, source: rabbit, dilution 1:200; Cell Signaling Technology Inc.), and dimethylhistone H3 (Lys27) [(D18C8)XP, rabbit monoclonal, dilution 1:1000; Cell Signaling Technology Inc.]. For visualization, the APAAP method was used for antismooth muscle actin and the LSAB method for the rest of the antibodies (REAL Detection System APAAP Mouse and Peroxidase/DAB+ Rabbit/Mouse; Dako).

2.4. Semiquantitative analysis of histone acetylation and methylation in the individual cells within carotid atherosclerotic plaques

In all cases, immunohistochemical analyses of histone acetylation and methylation were performed on slides consecutive to the stained SMCs (SM-actin), lymphocytes (CD45), endothelial cells (neovascularisation) (CD31), and macrophages/macrophage-derived foam cells (CD68) (as an example of consecutive staining, see supplementary Fig. 1). In order to evaluate the expression level in a semiquantitative manner, the staining intensity was analyzed in the individual cells over the entire carotid plaque located on the slide by two independent investigators. First, the maximal staining intensity was assessed for each individual cell type and the corresponding histone acetylation (H3K9 and H3K27) and methylation (H3K4, H3K9, and H3K27). Then, semiquantitative scoring between 0 (no staining) and 1 (maximal observed positive staining for the individual antibody used) was estimated. The graduation was performed in five steps assigning maximal staining intensity 100% (or 1.0) as +++++, 80% (0.8) as ++++, 60% (0.6) as +++, 40% (0.4) as ++, 20% (0.2) as +, and 0% (0) − as a negative staining. These analyses were performed for all slides and median and rage were calculated accordingly.

2.5. Quantitative real-time PCR

RNA was extracted from FFPE samples using the High Pure RNA Paraffin Kit and following the manufacturer's instructions (Roche,