Neutrophil Extracellular Traps and Macrophage Polarization Formation In The Inner And Outer Area of Carotid Plaque Arteries

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Research Article

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Abstract

Introduction: It is noteworthy that vast data exists which links NETs to arterial and venous thrombosis in both animal models and humans. In the current study, the level of extracellular neutrophil networks and macrophage polarization in the area outside and inside the carotid plaque of patients with carotid stenosis were assessed.

Material and Methods: Ten patients were included in this pilot study. Confirmed cases of carotid stenosis were selected to participate in the study using the simple sampling method. Samples of carotid plaques of each patient were divided into two halves with a transverse incision; the terms inner part and outer part were used for the plaque's inner part and the adjacent area, respectively. Carotid plaque was excised, and half of them were sorted in 10% formalin for CD163, CD11c, MPO and histone H3 immunohistochemistry assessment while the other halves were stored at -80 °C for western blotting assay with PDA4 marker. For statistical analysis, we used independent samples T-test or its non-parametric equivalents.

Results: Results of this study showed that the extracellular neutrophil chicks in the inner part of the carotid plaque were significantly increased (P <0.0001), while the number of M1 and M2 macrophages was higher in the inner part compared with the outer part of the carotid plaque (P<0.0001).

Conclusion: NETs and Macrophages have great potential for further investigation to find a better treatment for carotid plaque.

Introduction

Occlusion of the carotid artery is a disease that involves the development of plaque in the carotid arteries. These plaques consist of fibrous tissue, cholesterol, calcium, and cell debris adhering to the artery wall(1). These plaques are known as atherosclerosis. Such plaques allow blood entrance to the brain, causing a disorder. Due to potential brain damage, carotid artery occlusion is considered a serious disease. Findings of the previous studies suggest that NETs are important and significantly influence the development and progression of atherosclerotic plaques(2). In addition, NETs may induce and lead to the formation, stability, and expansion of arterial thrombus. Both neutrophils and macrophages, and less importantly eosinophils and mast cells, produced ETs following the onset of the complications of coronary plaques(3). In turn, ETs formation spans all the steps of the evolution of coronary thrombosis. Various leucocytes types and their ETs are involved in orchestrating the organization and maturation of the thrombus towards stability time-dependently(4). More knowledge regarding ETs’ roles in atherothrombotic disease is crucial since it can provide novel strategies to treat cardiovascular diseases. In the case of totally maximum intima-media thicknesses of the internal carotid artery, heterogeneous plaques were shown to be associated with the risk of lacunar infarction, stroke, cardiovascular disease, and coronary artery disease(5). The morphology of carotid plaques and the internal carotid stenosis degree are mutually dependent factors, both of which show the atherosclerotic disease's severity. The immune cells show a higher inflammatory status in the carotid plaque in comparison with similar cells of
the peripheral blood circulation(6). The expression of both anti-inflammatory and pro-thrombotic/pro-inflammatory mediators are altered in the plaque’s milieu suggesting the significant role of balance between these mediators in the progression of carotid disease(7). Elevated macrophage density in the atherosclerotic plaques of carotid arteries was associated with plaque, lipid content, and elevated plasma levels of low-density lipoprotein cholesterol. Studies revealed that the atherosclerotic plaques’ M1 macrophage content is associated with elevated inflammation or fibrinolysis and the incidence of clinical ischemic stroke(8). The production of NETs was first described as an unrecognized neutrophils’ defense mechanism since it was able to entrap and probably eliminate a wide variety of pathogens(8, 9). Yet, mounting evidence demonstrated that NETs are involved in a variety of pathophysiological conditions. NETs were shown to be present in the atherosclerotic plaques of not only humans but also animal models and are engaged in various mechanisms which lead to atherogenesis. NETs are among the factors that induce oxidative stress and oxidize the particles of high-density lipoprotein, thus decreasing their favorable capacity of cholesterol efflux. Also, NETs induce the dysfunction and apoptosis of endothelial cells and enhance the production of anti-ds-DNA autoantibodies(10, 11). Besides, NETs enhance the pro-thrombotic molecules accumulation, including fibrinogen and von Willebrand factor, thus significantly leading to thrombus formation. It is noteworthy that vast data exists which links NETs to arterial and venous thrombosis in both animal models and humans(12). In the current study, the level of extracellular neutrophil networks and macrophage polarization in the area outside and inside the carotid plaque of patients with carotid stenosis were assessed.

Materials And Methods

Sample collection

The present study was performed with the approval of the Medical Ethics Committee of Mashhad University of Medical Sciences. Confirmed cases of carotid stenosis were selected to participate in the study using the simple sampling method. Samples of carotid plaques of each patient were divided into two halves with a transverse incision; the terms inner part and outer part were used for the plaque’s inner part and the adjacent area, respectively. Patients in this study had no inflammatory or autoimmune diseases and did not takes any immunosuppressive drugs. Ten patients were included in this pilot study. Carotid plaque was excised, and half of them were stored in 10% formalin while the other halves were stored at -80 ° C for molecular studies.

Immunohistochemically and immunofluorescence studies

In the current study, samples of the carotid plaque of each patient were divided into two halves with a transverse incision, and the terms inner part and outer part were used for the plaque’s inner part and the adjacent area, respectively. The immunohistochemistry assay of CD163, CD113, MPO, and Histon H3 marker was done on two groups consisting of both inner and outer parts of the carotid plaque using the Detection Kite.
First, the excised tissues were put in the tissue processor device (Tissue processor, POOYAN model: MIK 2230, serial 223073, made in Iran) before undergoing tissue passage. Then, the tissues were infiltrated using liquid paraffin; then, they were formed into blocks. A microtome was used to cut the blocks; the produced sections were put on glass slides. Then, the slides were stained based on the Perl staining method. Based on Perls Prossian Blue Staining Protocol, a mixture of potassium ferrocyanic 2% and Chloridric acid 2% solutions with a 1:1 ratio was added to the produced slides at 80°C for 5 minutes; then the slides were rinsed using distilled water. Afterward, the slides were added 1% neutral red for 2 minutes and then rinsed using distilled water. When dried, the slides were examined and photographed using a light microscope.

Following preparation, fixation, and molding of the tissue, the slides of the samples were prepared. Deparaffinized slides were added peroxidase inhibitor in the dark for ten minutes to inhibit the endogenous peroxidase. Ten minutes later, the slides were rinsed three times using PBS buffer. Then, the prepared solutions, consisting of primary antibodies for CD163, CD11c, Histone H3, and MPO, were incubated at room temperature for 2-3 hours. Then, the PBS buffer was rinsed three times. Master Polymer (100 microliters) plus HRP were added to any of the samples and then rinsed three times following incubation for 30 minutes at room temperature. Each sample was added to Chromogen solution so that it covered all the slides. Five minutes later, the slides were rinsed using PBS buffer. The prepared slides were investigated using a light microscope.

The samples were incubated with 100 μl of Dil (20 μM; Molecular Probes), a fluorescent dye, for 40 minutes at 37°C. Subsequently, the samples were rinsed with PBS three times, and then, DAPI solution (Sigma) (1 μg/ml) was added to any of the wells. Lastly, fluorescence microscopy was used to visualize the images (Model: BX41; Olympus), and the positive cells were counted in ten high-power random fields (HPF) in each group, and the results were compared.

### Investigation of PAD4 protein expression using Western blotting

The PAD4 level was measured using western blotting; protein lysate (50 μg) was placed in each lane, and electrophoresed in SDS-PAGE (10%), and transferred to the membrane of polyvinylidene difluoride (Merck Millipore). For the immune reaction, a primary antibody was used against PAD4 (Cell Signaling). In brief, bovine serum albumin 1% (Sigma) was used to block the membranes for 1 hour, then the membranes were incubated using the diluted antibody (with 1: 1000 dilution), which contained bovine serum albumin (5% w/v), Tween 20 (0.1%), and 1X Tris-buffered saline, overnight. After rinsing phosphate buffered saline and 0.1% Tween 20 (10 minutes each) for three times, the membranes were incubated using HRP-conjugated secondary antibodies (Cat no:7074; Cell Signaling) at RT for 1 hour. Then, the membranes were rinsed three times with phosphate buffered saline. X-ray films and the ECL system (Roche) were used to visualize the immune-reactive bands. Band densities were calculated using ImageJ software (ver. 1.4). This assay was done in triplicate. The relative PAD4 peptide content was expressed after normalization to the GAPDH housekeeping protein (Abcam).

### Statistical analysis
For statistical analysis, we used independent samples T-test or its non-parametric equivalents, Mann-Whitney U test was used for quantitative variables and Chi-square (or Fisher’s exact test) for qualitative variables. In this study, we have considered \( \alpha = 0.05 \) and \( \beta = 0.2 \).

**Results**

**Confirmation of tumor carotid body in patients**

Physical examination and clinical history are the first steps to begin diagnosing the disease. Carotid artery stenosis can be suspected by listening to the sound of carotid arteries in the neck area with a medical earphone. The presence of sound in this area is one of the characteristics of carotid artery occlusion. Next, the patient’s physical and mental ability (such as strength, memory and speech) should be examined. If carotid stenosis is suspected, the doctor prescribes one of the diagnostic angiographic tests. In Fig. 1, carotid stenosis can be seen in angiographic images by contrast injection.

**Increase of M1 and M2 Macrophages in Outer area incompar to inner area**

The CD163 marker is known as the type 2 macrophage marker. The CD11c marker is known as the type 1 macrophage macrophages. \( P < 0.0001 \). On the other hand, the ratio of M1 to M2 macrophages in the inner region increased from 0.2 to 0.3. In contrast, the ratio of M2 to M1 macrophages decreased from 5 in the inner region to 3.3 in the outer region.

**Increased extracellular network of neutrophils in the inner region of the carotid plaque compared to the outer region of the carotid plaque**

MPO (Myeloperoxidase) MPO is most abundantly expressed in neutrophil granulocytes. The results of immunohistochemistry of carotid plaque tissues showed that the expression of MPO in the inner region of the carotid plaque showed a significant increase compared to the outer region \( (P < 0.001) \). Carotid plaque was significantly increased compared to the outer region \( (P < 0.0001) \). Dapi fluorescence staining showed that chromatin was distributed throughout the tissue and was not found regularly in the cell nucleus. At the tissue level, it shows an increase in the extracellular networks of neutrophils, which has a visible increase in the inner region of the carotid plaque compared to the outer region (Fig. 3).

**Increased expression of PAD4 protein in the inner region of the tumor compared to the outer region**

Peptidyl arginine deiminase-4 (PAD4) is indispensable for generation of neutrophil extracellular traps (NETs). Western blotting showed an increasing in the protein level of PAD4 in inner area in compare with outer area(Fig). as you see increasing of expression MPO in inner area is almost 2 fold increasing incomparable with outer area(Fig. 4A).for Gsk3B arrive to 2 fold a for Nrf2 0.5 fold growing up incomparable with control group.

**Discussion**
Results of this study showed that the extracellular neutrophil chicks in the inner part of the carotid plaque were significantly increased (P < 0.0001), while the number of M1 and M2 macrophages was higher in the inner part compared with the outer part of the carotid plaque (P < 0.0001).

In the early coronary atherosclerosis of humans, fatty streaks are developed with extracellular lipid deposition, which is associated with specific proteoglycans in the outer part of established diffuse intimal thickening. When the lipid content of the fatty streaks increases, macrophages are infiltrated toward these lipid deposits to form the intimal thickening as foam cells(13).

Previous studies show that M1 macrophages are predominant in the rupture-prone shoulder parts, while M2 macrophages are predominant in the adventitia. In the atherosclerotic lesions of humans, M2 macrophages are more commonly found in stable cell-rich areas rather than away from the lipid core(14). Comparing human carotid and femoral atherosclerotic plaques showed an increased number of M1 macrophages in the carotid lesion, while M2 macrophages were predominant in the femoral lesions, which indicated that the accumulation of M1 macrophages might be characteristic for symptomatic lesions(15). In J. LauranStöger et al. study, the histopathological analysis suggested that M1 and M2 macrophages continuously accumulate in the plaques as the lesion severity progresses(16). In the plaque shoulder, as a rupture-prone part of the intima, the pro-thermogenic M1 macrophages are the predominant macrophages, though such a predominance is not observed in the fibrous cap regions. Remarkably, the vascular adventitia's macrophage content strongly conformed to the M2 phenotype. Thus, both M1 and M2 subtypes of macrophages characterize different stages of plaque development in humans, though they localize with distinct lesion morphological features(17, 18).

Investigating the potential reasons for macrophage dispersion in different parts of the carotid plaques Possibly, an effective factor may be wall shear stress, i.e., a frictional force exerted parallel to the vascular wall leading to the altered endothelial cell signaling, endothelial phenotype, gene and protein expression, which leads to a pro-inflammatory phenotype, reduces the nitric oxide availability and disrupts the extracellular matrix, resulting in plaque development. Emerging experimental and clinical data suggests the pathobiology associated with the abnormal wall shear stress leads to the development and progression of atherosclerotic plaques(19). Mat J.Daemen et al.'s study showed that plaque fissures are commonly found in the advanced carotid plaques, which have a grossly normal luminal surface which is associated with fresh hemorrhage in the plaque(20).

It has been shown that neutrophil extracellular traps stimulate the activation of antigen-presenting cells, endothelial cells, and platelets, which leads to a pro-inflammatory immune response. Generally, this finding suggests not only their presence in the plaques and thrombi but also their potentially causative role in promoting the formation of atherosclerotic plaque and arterial thrombosis(21).

Studies suggest the higher usability of the outer layers of the carotid plaques is lower compared with the inner layers; thus, parts of the plaques may separate and enter the peripheral blood circulation and lastly migrate to the other body parts(22). Also, the inner layer of the carotid plaque plays a key role in plaque
development so that the extracellular network accumulation in this area is potentially effective in prompting platelet and lipid accumulation and increasing the plaque size (23).

Knight et al.’s study showed that NET formation might be prevented using chloramine treatment which inhibits PAD4 (playing an important role in the NET formation 32), thereby the size of the atherosclerotic plaque is reduced and carotid artery thrombosis postponed in the mouse model of atherosclerosis (24, 25).

NETs have great potential for further investigation to find a better treatment for venous thromboembolism and atherosclerosis plaques. Therefore, developing drugs to target extracellular networks, including NETs, in addition to macrophage anti-polarization therapies to M1, which form the plaque cores, may weaken the plaque formation primarily and prevent further plaque expansion and carotid artery occlusion (26).

**Abbreviations**

NETs: Neutrophil extracellular traps

ET: Extracellular traps

PAD4: Peptidyl arginine deiminase 4

**Declarations**

**Conflict of interest:** None

**Ethical code:** The proposal (2259) was approved by the Medical Ethics Committee of the University of Mashhad Medical Sciences

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Figures

Figure 1

The angiographic image of the patient with carotid stenosis, carotid stenosis in the carotid bulb region is visible between internal carotid and common carotid.
Figure 2

Immunohistochemistry CD163 and CD11c from inner area toward outer area; A: Increased CD11c expression in the outer area compared to the inner area of the carotid plaque (P<0.0001 ****) B: Increased CD163 expression in the outer area compared to the inner area of the carotid plaque (P<0.0001 ****).
Figure 3

M1(CD11c) to M2(CD163) ratio in outer and inner area of carotid plaque; A: 23% CD11c, 77% CD163 in outer area of carotid plaque B: 17% CD11c and 83% CD163 in inner area of carotid plaque.

Figure 4

Assessment of NETs in outer and inner area of carotid plaque, A; Increased MPO expression in the inner area compared to the outer area of the carotid plaque (P<0.001 ***), B: Increased histone H3 expression in the inner area compared to the outer area of the carotid plaque (P<0.0001****).
Figure 5

Level of PAD4 in inner area and outer area detected by western blotting. Data showed that the PAD4 content was increased in compare to outer layer of carotid plaque.