Extracellular Traps in Lipid-Rich Lesions of Carotid Atherosclerotic Plaques: Implications for Lipoprotein Retention and Lesion Progression

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ABSTRACT

Purpose: To investigate the presence and location of extracellular traps (ETs) in atherosclerotic plaques and to determine whether they are spatially associated with inflammatory cells and the lipid core.

Materials and Methods: Human carotid atherosclerotic plaques were collected from seven patients after surgical endarterectomy. Sequential tissue sections were stained with hematoxylin-eosin or subjected to immunohistochemistry to detect ETs, neutrophils and macrophages or apolipoprotein B (ApoB). To demonstrate the specificity of the antibody used to detect ETs, the adjacent tissue section was pretreated with deoxyribonuclease-1 (DNase-1) before immunostaining for ETs.

Results: All seven carotid plaques demonstrated advanced atherosclerotic lesions. Extensive ET and ApoB immunostaining was detected predominantly within the acellular lipid core. Along the edges of the lipid core, confocal microscopy revealed areas suggestive of active release of ETs from MPO-positive cells. Pretreatment of tissue sections with DNase-1 abolished ET signal in the extracellular matrix, but not the signal within the cells along the margins of the core.

Conclusions: The localization of ETs to the lipid core suggests a possible binding site for lipoproteins, which may further promote lesion progression and inflammation.

ABBREVIATIONS

ApoB = apolipoprotein B, DAPI = 4′,6-diamidino-2-phenylindole, DNase-1 = deoxyribonuclease-1, ET = extracellular trap, MPO = myeloperoxidase

The response-to-retention hypothesis of atherosclerosis states that atherogenic lipoproteins are retained within the arterial wall triggering an inflammatory response that initiates the development of early atherosclerotic lesions (1–4). With increasing inflammatory response and infiltration of immune cells in atherosclerotic lesions, the possibility of extracellular traps (ETs) deposited into the extracellular matrix by neutrophils and macrophages was explored. ETs were first described in 2004 to have an antimicrobial purpose, released by neutrophils in response to infection (5). Since their description, ETs have been shown to be produced by various immune cells and have been detected in various conditions, including autoimmune diseases, thrombosis, sepsis, and ischemic-reperfusion injury (6–10).

The detection of ETs within atherosclerotic lesions may have two important consequences: (*i*) ETs may promote inflammation (eg, via the activation of the toll-like receptors of the innate immune system), and (*ii*) the release of the highly negatively charged DNA, the backbone of ETs, may provide binding sites for lipoproteins (11,12). Lipoproteins containing apolipoprotein B (ApoB) have been shown to have the ability to cross the intact endothelium of the intimal layer via transcytosis (3,13,14). There is evidence that these lipoproteins may be retained in the arterial wall by the negatively charged proteoglycans preventing their transcytosis into the vessels of the adventitia (1,13,15).

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From the SIR 2013 Annual Meeting.

None of the authors have identified a conflict of interest.

J Vasc Interv Radiol 2014; 25:631–634

http://dx.doi.org/10.1016/j.jvir.2013.12.567

Because inflammation is an important component of atherosclerosis, even in its early stages, we hypothesized that ETs released from infiltrating inflammatory cells may also play a role in the retention of lipoproteins and contribute to the progression of atherosclerotic lesions. Carotid artery atherosclerotic plaques were evaluated for the presence of ETs, and detection of ETs was correlated with myeloperoxidase (MPO)-positive cells, such as neutrophils and macrophages, and detection of ApoB using fluorescent immunohistochemistry and confocal microscopy. In addition, the ability of deoxyribonuclease-1 (DNase-1) treatment of the carotid artery sections to reduce immunostaining for ETs as a marker of specificity of the ET antibody was tested.

MATERIALS AND METHODS

Following institutional review board approval, seven human carotid atherosclerotic plaques were collected from patients undergoing surgical endarterectomy. On surgical removal of the tissues, the samples were placed in 10% formalin solution for fixation. After fixation overnight, the tissues were decalcified and embedded in paraffin. Sections 7 μm thick were mounted on positively charged slides, and paraffin was removed from sections as previously published (16). Deparaffinized slides containing sections of carotid artery were rinsed in phosphate-buffered saline and stained with hematoxylin-eosin to visualize tissue morphology.

For immunohistochemistry, Tyramide Signal Amplification Plus Cyanine 3 system (Perkin Elmer, Boston, Massachusetts) was used following the manufacturer's instructions. The following primary antibodies were used: mouse monoclonal anti-histone H2A/H2B/DNA complex antibody to detect ETs (5,17–19), rabbit polyclonal anti-MPO (Abcam, Cambridge, Massachusetts), and rabbit polyclonal anti-ApoB (Santa Cruz Biotechnology, Dallas, Texas). In each assay, 1 μg/mL dilution of the primary and secondary antibodies was used. Sections for immunofluorescence were mounted using mounting medium (Vectashield; Vector Laboratories, Burlingame, California) containing 4′,6-diamidino-2-phenylindole (DAPI). A Carl Zeiss 100TV microscope (Carl Zeiss Microscopy, Thornwood, New York) with a multiband filter block, NanoZoomer 2.0-HT slide scanner (Hamamatsu Corporation, Bridgewater, New Jersey), and Olympus multiphoton confocal microscope (FV1000-MPE; Olympus America, Center Valley, PA) were used to visualize the tissue sections. For negative controls, MPO, Apo-B, and ET antibodies were omitted and substituted with 1 μg/mL dilution of the appropriate control IgG (anti–rabbit IgG and anti–mouse IgG; Vector Laboratories). To demonstrate the specificity of the mouse monoclonal anti-histone H2A/H2B/DNA complex antibody to detect ETs, a subset of tissue sections was initially treated with 10 units of DNase-1 enzyme (New England Biolabs, Ipswich, Massachusetts) for 30 minutes at 37°C. Additionally, to minimize permeabilization, these sections were not treated with Triton X-100 (Sigma-Aldrich, St. Louis, MO). Subsequent steps for immunostaining for ETs were as described earlier. The rationale for this experiment was to show that nuclease treatment would lead to a loss of ET signal as the ET antibody recognizes an epitope comprising DNA and histones.

RESULTS

Immunostaining for ETs demonstrated marked signal detection in the regions of atherosclerotic lipid core in each of the seven specimens examined (**Fig, a–i**). The signal predominated in regions that were acellular as shown by the lack of nuclei staining in both DAPI-stained and hematoxylin-eosin–stained sections (**Fig, d, e**). Minimal immunofluorescent signal from ETs was detected along the border of the lipid-filled lesions, which were highly cellular as shown by nuclear specific stains (**Fig, d, e**). The fibrous regions of the atherosclerotic plaque and the media layer of the vessel wall also showed minimal immunostaining for ETs.

To demonstrate the specificity of the antibody for detecting ETs, sections were initially incubated with the DNase-1 enzyme to degrade DNA predominantly in the extracellular matrix. These sections were not treated with Triton X-100 to minimize permeabilization of the cells and nuclei. After a period of incubation with the DNase-1 enzyme, immunofluorescent staining for ETs demonstrated loss of signal in the lipid-filled lesions indicating that the epitope of the antibody recognized a specific combination of DNA and histones (**Fig, f**). The only remaining immunofluorescent signal detected using the ET antibody in these sections was, as expected, within the cell nuclei.

Because ETs are known to be predominantly produced by neutrophils, immunostaining for MPO was performed to detect neutrophils and macrophages (7), which are known to be present within plaques, to see if the areas bordering the lipid lesions contained cells that may potentially be the source of ETs. The cellular margins surrounding the lipid cores demonstrated a rind of MPO-positive cells suggesting that these cells may be the source of the ETs causing the lipid core to expand peripherally (**Fig, i**). Confocal microscopy further showed cells along these margins that are MPO-positive either actively releasing ETs into the matrix or engulfing ETs from the matrix (**Fig, h**). Given the intense negative charge of the DNA backbone, we next explored the possibility that this negatively charged DNA may potentially serve as the binding sites for ApoB. Immunofluorescent staining of ApoB demonstrated a marked level of detection of ApoB within the lipid core of the atherosclerotic lesions suggesting an association with ETs in the same area (**Fig, g**).