ORIGINAL RESEARCH ARTICLES

INTRAPLAQUE HEMORRHAGE, A POTENTIAL CONSEQUENCE OF PERIODONTAL BACTERIA GATHERING IN HUMAN CAROTID ATHEROTHROMBOSIS

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Key words
Periodontal disease, periodontitis, keystone periodontal pathogens, carotid atherothrombosis, athrosclerosis, intraplaque hemorrhage

Abstract
Periodontal diseases are multifactorial inflammatory diseases, caused by a bacterial biofilm involving both innate and adaptative immunity, characterized by the destruction of tooth-supporting tissues. In the context of periodontitis, the spread of weak pathogenic bacteria into the bloodstream has been described. These bacteria will preferentially localize to existing clot within the circulation. Atherothrombosis of the carotid arteries is a local pathology and a common cause of cerebral infarction. Intraplaque hemorrhages render the lesion more prone to clinical complications such as stroke. The main objective of this study is to explore the biological relationship between carotid intraplaque hemorrhage and periodontal diseases.
This study included consecutive patients with symptomatic or asymptomatic carotid stenosis, admitted for endarterectomy surgical procedure (n=41). In conditioned media of the carotid samples collected, markers of neutrophil activation (myeloperoxidase or MPO, DNA-MPO complexes) and hemoglobin were quantified. To investigate the presence of DNA from periodontal bacteria in atherosclerotic plaque, PCR analysis using specific primers was performed.
Our preliminary results indicate an association between neutrophil activation and intraplaque hemorrhages, reflected by the release of MPO ($p<0,01$) and MPO-DNA complexes ($p<0,05$). Presence of DNA from periodontitis-associated bacteria was found in 32/41 (78%) atheromatous plaque samples. More specifically, DNA from Pg, Tf, Pi, Aa was found in 46%, 24%, 34% and 68% of the samples, respectively. Hemoglobin levels were higher in conditioned media in carotid samples where the bacteria were found, but this was not statistically significant.
Our data confirm the relationship between intraplaque hemorrhage and neutrophil activation. In addition, the presence of periodontal bacteria DNA in carotid atheromatous plaque, may contribute to this activation. Further analysis is needed to fully explore the raw data and specimens.

Introduction
Atherothrombosis of the carotid arteries is a local pathology associated with the development of systemic atherosclerosis and is a common cause of cerebral infarction, which represents 16.4% of cardiovascular death [1]. The need for surgical resection of atheroma-

tous lesions (endarterectomy) is preemptively indicated by stenosis (above 50 %) and after a cerebral event (stroke). Periodontal diseases are multifactorial inflammatory diseases, caused by a bacterial biofilm [2] involving both innate and adaptative immunity, characterized by the destruction of tooth-supporting tissues (gums, cementum, ligament and alveolar bone). In the context of periodontitis, the spread of weak pathogenic bacteria into the bloodstream has been described [3]. These bacteria will preferentially localize to existing thrombi within the circulatory system [4]. The identification of new prognostic risk factors for atherothrombosis could prevent future vascular events in patients at risk of myocardial or cerebral complications. Periodontal diseases are among the new vascular risk factors that can have an important impact on cardiovascular prevention [5]. The main objective of this translational project is to explore the bioclinical link between chronic periodontitis and carotid atherothrombosis in order to better understand the risk of cardiovascular events.

The morphological parameters of the atherothrombotic plaque (intraplaque hemorrhage, calcification, lipids) has been shown to be associated with the presence of periodontal pathogens [6]. Different bioclinical profiles of patients at risk of cerebral infarction based on periodontal status may be established by comparing the bacterial enrichment associated with chronic periodontitis to the different carotid atherothrombotic lesions.

This translational research is based on a human biobank of tissues (carotid endarterectomy samples), dental plaque samples and blood samples from the Laboratory for Vascular Translational Science at the Bichat Hospital.

Material and Methods

The sample included consecutive patients scheduled for revascularization of carotid stenosis by endarterectomy in the Department of Neurology at the Bichat Hospital. Patients included in the study have benefited from a full mouth periodontal examination by a periodontist. Biological samples (blood and dental plaque) were collected, medical history and treatments were identified. Neurological data included clinical and Doppler imaging. Written Consent was obtained from each patient and the study was approved by the local ethical Commity (CPP 110434).

Conditioned media and post-incubation carotid tissues

Human carotid endarterectomy samples (n=41) were processed within few hours after surgery. They were dissected and incubated 24h at 37°C in a standardized volume (6mL/g of sample wet weight) of RPMI culture medium with antibiotics and an antimycotic.

Quantification of protein in conditioned media

Total protein concentration in conditioned media were quantified using BIO-RAD Protein Assay. Dilution of 50μl of samples (1:40) and standard (BSA 120μg/mL – 7,5μg/mL) in distilled water were performed. BIO-RAD reagent (30%) was added and absorbance was read at 590nm after incubation for 5 min.

Quantification of hemoglobin in conditioned media

Hemoglobin concentration in conditioned media was quantified by the indirect biochemical quantification of heme. Dilution of standard (Hemoglobin Bovine Erythrocyte 5000μg/mL – 19,7μg/mL) in distilled water was performed. Formic acid (70μL) was added to samples (30μL) and standard (30μL) and absorbance was read immediately at 405nm.

Quantification of myeloperoxidase (MPO) in conditioned media

Myeloperoxidase concentration in conditioned media was quantified using “human MPO Elisa kit” (Hycult Biotechnology). Standard was prepared (100 ng/mL – 0ng/mL) by reconstitution with distilled water. Dilution of 100μl of samples (1:10) was performed. Samples and standards were both added in a 96-wells plate coated with a monoclonal antibody against MPO and incubated for 1h at room temperature (RT). After washing (twice with PBS), a biotinylated tracer antibody was added and incubated for 1h at RT. After four washing with PBS, a conjugated solution with streptavidin-peroxidase was added and incubated for 1h at RT. After four washing with PBS, the peroxidase substrate 3, 30, 5, 50-tetramethylbenzidine or TMB was added. The absorbance at 405 nm was measured after 30 min of incubation at RT and addition of STOP solution.

Quantification of MPO-DNA complexes in conditioned media

MPO-DNA complexes level in conditioned media were quantified by combining two different Enzyme Linked Immuno Sorbent Assay (ELISA) tests as previously described [5, 7].

Briefly, samples (100 µl ; dilution 1:10) were added in a 96-well plate coated with a monoclonal antibody against MPO and incubated for 1h at RT. After washing, a peroxidase-labeled anti-DNA antibody was added and incubated for 2h at room temperature. After a second wash, the peroxidase substrate 3, 30, 5, 50-tetramethylbenzidine or TMB was added, and the absorbance at 405 nm was measured after 30 min of incubation at room temperature and addition of STOP solution.

Bacterial DNA extraction from carotid tissues and amplification by PCR

Human carotid samples were cryopulverized and total DNA was extracted from the powder using PrepFiler® BTA Forensic DNA extraction kit (LifeTechologies) following the manufacturer's instructions. The extracted DNA of the samples was amplified using specific sets of primers corresponding to a sequence encoding 16S rRNA of Porphyromonas gingivalis (Pg) (Forward: TACCGAACAACCTACGCACC; Reverse: ACTTGCCTTACAGAGGGGGA), Prevotella intermedia (Pi) (Forward: GCGAACTG GCGGACTTG; Reverse: TTACATCGCACC GTCCCTC), Tannerella forsythia (Tf) (Forward: CAGAACTCCGATTGCGAAGG; Reverse: TCATCCCAACCTTCCTCACAG) and Aggregatibacter actinomycetemcomit-ans (Aa) (Forward: AGGGG GATAACGACGGGAA; Reverse: CTACGCA TTTCACCGCTACAC). Briefly, PCR was carried out in a mixture containing 5 µL of DNA (50 ng), 9 µL of H2O, 4 µL of Master Mix and 2 µL of 0.2 mM PCR primer set.

Amplification (40 to 50 cycles) was performed by real time PCR using a LightCycler® system with SYBR green detection (Roche Applied Biosystems). Products of amplification were then analyzed by electrophoresis with 1% agarose gel stained by ethydium bromide. Genomic DNA samples extracted from bacterial cultures or dental plaque samples were used as positive controls and PCR grade H2O was used as negative control.

Results

There was an association between neutrophil activation and intraplaque hemorrhage ([Hb]>2760µg/g), reflected by the release of MPO (p<0,01 ; Fig A.) and MPO-DNA complexes formation (p<0,05 ; Fig B.).

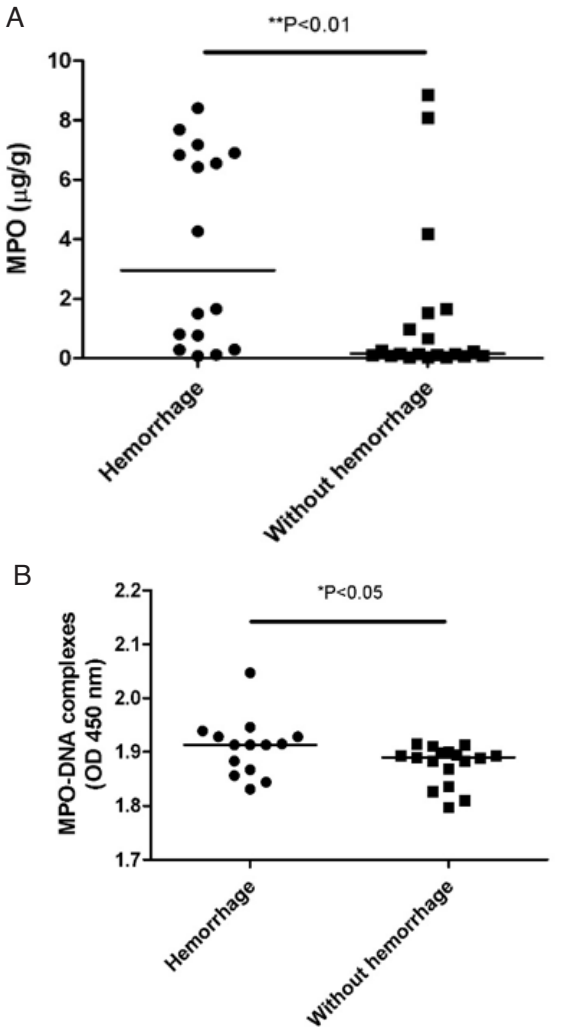

Fig. Increased MPO and MPO-DNA complex levels into the media conditionned by hemorrhagic carotid plaques. A: MPO and B: MPO-DNA levels were determined by ELISA and separated according to their hemorrhagic status.

Presence of DNA from periodontitis-associated bacteria was found in 32/41 (78%) atheromatous plaque samples. More specifically, DNA from Pg, Tf, Pi, Aa was found in 46%, 24%, 34% and 68% of the samples, respectively.

Discussion

The epidemiological link between chronic periodontitis and clinical manifestations of atherothrombosis is now well established [8] but it is necessary to clarify the biological relationship between those conditions. Indeed, we need more information to better understand the mechanisms involved. Most periodontal pathogens are anaerobic bacteria [9]. Their DNA was identified in the atherosclero-

tic plaques of various vascular tissues [10]. However, detection of these microorganisms is difficult to interpret, many other microbiota (intestinal, lung…) may contribute to the development of atherosclerotic plaques. It seems that it is not particularly the type of bacteria that is in question but the innate immune response of the host. Similarly at the sulcus, the role of neutrophils is essential in the development of periodontal disease. Three pathogenic hypotheses [11-14] have been advanced to explain the link between periodontal and cardiovascular diseases: (1) the direct role of bacteria (endotoxins, proteases…) on vascular wall and atherosclerotic plaque; (2) the role of adaptative immune response by elevating circulating and tissular antibody level; and (3) the role of local activation of the innate immune response, mainly with neutrophils. Our hypothesis is that transient bacteremia facilitates coagulum adherence to atherothrombotic plaque of these anaerobic bacteria with low pathogenicity resulting in disproportionate amplification of local tissue innate immune response [15]. This assumption is based on data from the recent biomedical literature and our preliminary results. Indeed, it has been recently demonstrated that the thrombus plays a role as a catalyst of the innate immune response to pathogens in mammal. This role requires the retention of neutrophils and the formation of "Neutrophil Extracellular Traps (NETs)" [4].

In our study we confirm the close relationship between intraplaque hemorrhage and neutrophil activation reflected by the release of MPO ($p<0,01$) and MPO-DNA complexes formation ($p<0,05$). We also confirm the presence of periodontal bacteria DNA in carotid atheromatous plaque (78%) that may contribute to this activation. This preliminary results, in agreement with the literature, have to be completed. Indeed, we need to confirm PCR data with sequencing and we need to quantify more neutrophil activation markers. We also have all neurologic and periodontal clinical informations and biological samples to move forward on the subject.

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