Atherosclerosis 259 (2017) 60–67

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Free DNA precipitates calcium phosphate apatite crystals in the arterial wall *in vivo*

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ARTICLE INFO

Article history:
Received 19 July 2016
Received in revised form
29 January 2017
Accepted 2 March 2017
Available online 4 March 2017

Keywords:
Free DNA
Calcium score
Smooth muscle cell
Calcification
Aneurysm
Human atherosclerosis

ABSTRACT

Background and aims: The arterial wall calcium score and circulating free DNA levels are now used in clinical practice as biomarkers of cardiovascular risk. Calcium phosphate apatite retention in the arterial wall necessitates precipitation on an anionic platform. Here, we explore the role of tissue-free DNA as such a platform.

Methods: The first step consisted of histological observation of samples from human and rat calcified arteries. Various stains were used to evaluate colocalization of free DNA with calcified tissue (alizarin red, fluorescent Hoechst, DNA immunostaining and TUNEL assay). Sections were treated by EDTA to reveal calcification background. Secondly, a rat model of vascular calcifications induced by intra-aortic infusions of free DNA and elastase + free DNA was developed. Rat aortas underwent a micro-CT for calcium score calculation at 3 weeks. Rat and human calcifications were qualitatively characterized using μFourier Transform Infrared Spectroscopy (μFTIR) and Field Emission-Scanning Electron Microscopy (FE-SEM).

Results: Our histological study shows colocalization of calcified arterial plaques with free DNA. In the intra-aortic infusion model, free DNA was able to penetrate into the arterial wall and induce calcifications whereas no microscopic calcification was seen in control aortas. The calcification score in the elastase + free DNA group was significantly higher than in the control groups. Qualitative evaluation with μFTIR and FE-SEM demonstrated typical calcium phosphate retention in human and rat arterial specimens.

Conclusions: This translational study demonstrates that free DNA could be involved in arterial calcification formation by precipitating calcium phosphate apatite crystals in the vessel wall.

1. Introduction

The arterial wall is a frequent target of the abnormal process of soft tissue calcification, associated with numerous vascular pathologies, including atherosclerosis, aging, renal failure, diabetes and rare genetic diseases [1]. Therefore, the arterial wall calcium score quantified by X-ray scanning is now used in clinical practice as one of the strongest biomarkers of cardiovascular risk [2,3]. Nevertheless, the pathophysiology of initial calcium phosphate precipitation in the vascular system is probably not unique, and remains not completely understood [4]. This process necessitates

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http://dx.doi.org/10.1016/j.atherosclerosis.2017.03.005
0021-9150/

changes in the balance between ionized calcium and phosphate concentrations, under the control of smooth muscle cell (SMC) alkaline phosphatase, which hydrolyzes organic phosphorous [5] as substrate for phosphate release within the arterial tissue. It also necessitates calcium phosphate precipitation on anionic platforms, usually represented by matrix vesicles [6]. Indeed, there are arguments that initial calcium phosphate precipitation [7] could be, at least in part, associated with cell stress and death, via the release of phosphorous from intracellular metabolism and pro-calcifying membrane phospholipid-rich micro-particles [8–10]. On the other hand, the poly-anionic nature of free DNA causes it to strongly interact with cationic calcium phosphate, and DNA is an important source of phosphates. For example, hydroxyapatite columns were initially used to purify DNA [11], and calcium phosphate nanoparticles are now used as vectors for cell DNA transfection [12]. Nevertheless, to the best of our knowledge, the role of arterial wall free DNA in the initiation of calcifications has not yet been explored in the microenvironment of atherosclerotic pathologies.

The purpose of this study was to explore the colocalization of free DNA and calcifying nucleation in initial atheroma in human aortic tissue and in intraluminal buds in the late stages of femoral atherosclerosis, to define the nature of these calcified nuclei, and lastly, to develop an animal model in which arterial calcifications are induced by intraluminal infusion of fragmented autologous DNA.

2. Materials and methods

This study consists of 2 parts: (1) the histological examination of calcifications from human and rat arteries, (2) the characterization of a rat model of vascular calcifications, induced by intra-aortic infusions of free DNA in various situations.

2.1. Histological examination of calcifications from human and rat arteries

2.1.1. Human arteries

Samples of apparently healthy human aorta (n = 6) from the Inserm U1148 Biobank were analyzed. Aortas that displayed early stages of atheroma (fibrous cap atheroma) were analyzed further. Aortas were obtained from deceased organ donors with the authorization of the French Biomedicine Agency (PFS 09-007) and after submission of a legal statement to the French Ministry of Research. Samples were fixed in 5% paraformaldehyde, embedded in paraffin and cut into 6 μm-thick sections for histological analysis. Similarly, samples of stenosed common femoral arteries (n = 6), obtained at endarterectomy from our vascular surgery department (Xavier Bichat Hospital, AP-HP, 75018 Paris), were also fixed, embedded in paraffin and cut for histological analysis with or without prior treatment with ethylenediaminetetraacetic acid (EDTA). This EDTA treatment was used because of its ability to chelate calcium phosphate, thereby revealing background hidden by calcium phosphate precipitation.

2.1.2. Rat aneurysmal aortas

Aortic samples from rats that underwent intraluminal aortic perfusion of pancreatic elastase, along with, or without, repeated injections of *Porphyromonas gingivalis* (PG) as previously described [13], were fixed in 5% paraformaldehyde and embedded in paraffin to obtain 6 μm-thick sections for histological analysis.

2.1.3. Histological examination

Harvested arteries were embedded in paraffin and 6 μm sections were cut. Sections were deparaffinized and rehydrated in toluene and ethanol baths. Several stains were used: hematoxylin-eosin or Masson's trichrome to explore the arterial structure, Alizarin red to detect calcifications, Hoechst (binding of DNA Adenine and Thymine), DNA immunostaining (ab27156, Abcam) and TUNEL assay (fragmented DNA, Roche) before and after decalcification by EDTA incubation, for free DNA visualization.

2.2. Rat model of vascular calcifications

2.2.1. Free DNA preparation

Two Wistar rats were sacrificed under pentobarbital anesthesia (0.3 cc/100 g). Both lungs were harvested and kept at −20 °C. The QIAamp DNA blood maxi (Qiagen) kit was used with some modifications of the protocol. Forty mL of ATL Qiagen lysis buffer and 4 mL of Qiagen proteinase K were added and incubated with lungs overnight at +56 °C to rupture cell membranes. The 46 mL resulting solution was homogenized and distributed into 4 tubes. In each tube, 11.5 mL of AL Qiagen buffer and an equal volume of absolute ethanol were added. Following a 15-min centrifugation at 760*g*, the supernatant was loaded onto 4 Qiagen columns including a silica membrane to fix free DNA.

After two washings of each column, free DNA was then eluted with 1 mL of sterile water. Optical densities were measured at 260 and 280 nm using a NanoDrop spectrophotometer to assess the quality and quantity of the resulting DNA solutions. Aliquots of 300 microL were sonicated for 36 min with intermittent cooling periods at 4 °C each 6 min. The ultrasound waves caused the fragmentation of DNA. Fragment sizes were measured by agarose gel electrophoresis with a DNA scale and were comprised between 100 and 600 bp. Following fragmentation, DNA was mixed with a Hoechst solution.

2.2.2. Surgical procedure: in vivo intra-aortic perfusion of free DNA

Male Wistar rats (7–8 weeks, 300–350 g) were randomized for intra-aortic infusion of various solutions: free DNA alone, elastase infusion followed by free DNA infusion, elastase alone and isotonic saline alone (control group). The procedure and the animal care complied with the principles formulated by the National Society for Medical Research (animal facility agreement: n B75-18-03, experimentation authorization n 75–101), and this study was performed after a favorable decision of the Institutional Review Board for Animal Care, with the declaration (n 5743) to the French Ministry of Research.

The technique was similar to the aneurysm model of intra-aortic perfusion of pancreatic elastase [14]. Briefly, following general anesthesia with pentobarbital and midline laparotomy, the abdominal aorta was exposed over 15 mm. Collaterals were ligated using 9/0 polypropylene sutures. The aorta was clamped below the renal artery and at the level of the bifurcation, this isolating an infra-aortic segment. A distal transversal arteriotomy allowed introduction of a polyethylene microcatheter PE-10 (Clay Adams, Parsippany, NJ) into this segment. The distal aorta was secured to the catheter to avoid leaks and the infrarenal aortic segment was perfused at 0.55 mL/h constant flow for 20 min. After perfusion, the catheter was retrieved and the aortotomy closed using interrupted sutures of polypropylene 10/0. The midline laparotomy was closed in a standard fashion and an analgesic subcutaneous injection of Buprenorphine (0.1 mg/kg) was given.

2.2.3. Perfused solutions

Rats were first perfused with an elastase solution (25%) or with an isotonic solution (NaCl 9‰, control animals) during 5 min. Then, animals were perfused with the fragmented DNA (0.25 μg/μL) or with the same isotonic solution (control animals). This allowed us to constitute four study groups: (1) the control group (solely perfused with isotonic saline solution), (2) a group perfused with elastase alone, (3) the free DNA alone group and (4) the elastase + free DNA group.