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Research Paper

Chlorination and oxidation of human plasma fibronectin by myeloperoxidase-derived oxidants, and its consequences for smooth muscle cell function

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ABSTRACT

Fibronectin (FN) occurs as both a soluble form, in plasma and at sites of tissue injury, and a cellular form in tissue extracellular matrices (ECM). FN is critical to wound repair, ECM structure and assembly, cell adhesion and proliferation. FN is reported to play a critical role in the development, progression and stability of cardiovascular atherosclerotic lesions, with high FN levels associated with a thick fibrotic cap, stable disease and a low risk of rupture. Evidence has been presented for FN modification by inflammatory oxidants, and particularly myeloperoxidase (MPO)-derived species including hypochlorous acid (HOCl). The targets and consequences of FN modification are poorly understood. Here we show, using a newly-developed MS protocol, that HOCl and an enzymatic MPO system, generate site-specific dose-dependent Tyr chlorination and dichlorination (up to 16 of 100 residues modified), and oxidation of Trp (7 of 39 residues), Met (3 of 26) and His (1 of 55) within selected FN domains, and particularly the heparin- and cell-binding regions. These alterations increase FN binding to heparin-containing columns. Studies using primary human coronary artery smooth muscle cells (HCASMC) show that exposure to HOCl-modified FN, results in decreased adherence, increased proliferation and altered expression of genes involved in ECM synthesis and remodelling. These findings indicate that the presence of modified fibronectin may play a major role in the formation, development and stabilisation of fibrous caps in atherosclerotic lesions and may play a key role in the switching of quiescent contractile smooth muscle cells to a migratory, synthetic and proliferative phenotype.

1. Introduction

Fibronectin (FN) is a large plasma and extracellular matrix glycoprotein, composed of two nearly identical subunits (~ 230–270 kDa) linked by two disulfide-bonds located near the carboxyl termini [1,2]. The protein exists in two forms: a soluble form predominantly found in plasma, but also in abundance at sites of tissue injury, and a cellular form present in tissue extracellular matrix (ECM). The plasma form plays a key role in the early physiological responses to tissue injury as it binds to fibrin fibres via multiple domains and hence is a key component of blood clots [3]. The resulting fibrin-FN network supports migration and adhesion of fibroblast and endothelial cells which over time replace this provisional matrix structure with cell-derived FN, collagen, laminins and other ECM components [4,5]. Cell-derived FN, which is synthesized by endothelial, smooth muscle and fibroblast cells, amongst others, is structurally-related to the plasma form, but also contains extra domains (extra domains A and B) which are alternatively spliced type III modules that are not present in the plasma form [6].

FN is present in the ECM of the arterial wall under normal physiological conditions [7], but a significant increase in concentration, and different isoforms, have been reported in atherosclerotic lesions [8]; these changes appear to occur during lesion development [9]. Patients with coronary artery disease also have elevated levels of plasma FN [10]. These changes may be associated with remodelling of the vascular

Abbreviations: 3-ClTyr, 3-chlorotyrosine; 3,5-Cl_2Tyr, 3,5-dichlorotyrosine; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; FN, human plasma fibronectin; HCASMC, human coronary artery smooth muscle cells; HOCl, the physiological mixture of hypochlorous acid and its anion $^{-}$OCl; MCD, monochlorodimedone; MPO, myeloperoxidase; MSA, methanesulfonic acid; RSO, relative site occupancy; SDC, sodium deoxycholate; SMC, smooth muscle cell; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TMB, 3,3′,5,5′-tetramethylbenzidine

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wall during the development of atherosclerosis, with considerable evidence for increased ECM deposition in the fibrous caps of atherosclerotic plaques [11]. The formation of a thick fibrous cap is widely regarded as being beneficial with regard to plaque stability [11]. Rupture of the lesion, exposure of the highly thrombogenic substrata, and subsequent thrombus formation, is a major cause of heart attacks and strokes [11]. Rupture prone plaques typically have a thin fibrous cap, increased numbers of activated inflammatory cells capable of producing oxidants, high levels of lipids, and low numbers of smooth muscle cells [12,13]. Rupture occurs most commonly in the shoulder regions where macrophages and other leukocytes accumulate [14,15]. Studies on atherosclerosis-prone apo $E^{-/-}$ mice that do not express plasma-derived FN, have shown a reduction in number and size of atherosclerotic lesions, however mice deficient in plasma-derived FN lacked vascular smooth muscle cell infiltration and failed to develop a fibrous cap [16]. These data are consistent with the hypothesis that FN plays a critical role in generating and stabilizing the fibrous cap of atherosclerotic lesions, and plays a major role in determining lesion stability and propensity to rupture. The colocalization of inflammatory cells with FN in plaque shoulder regions, and FN degradation during the progression of atherosclerosis, supports the hypothesis that plaques are damaged and destabilized by inflammatory cell oxidants, with this occurring, at least in part, via ECM damage [17,18].

Oxidants are intentionally generated during many physiologic and pathological processes [19]. Activated leukocytes generate superoxide radicals ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) via NADPH oxidase (NOX) enzymes, and neutrophils, monocytes and some tissue macrophages release the heme enzyme myeloperoxidase (MPO) from intracellular storage granules [20]. In the presence of chloride ions (Cl^-), MPO utilizes H_2O_2 to generate the potent oxidant hypochlorous acid (HOCl) [20,21]. HOCl reacts rapidly with many biological targets, with kinetic data indicating that proteins are major targets, due to their abundance and high reactivity [21,22]. Although HOCl plays an important role in killing invading pathogens, excessive or misplaced generation can result in host tissue damage, with this being associated with multiple human pathologies involving acute or chronic inflammation, including atherosclerosis [21,23–25].

There is limited data on HOCl-induced modifications to isolated FN, or FN in the arterial wall, though it is known that FN can be modified by HOCl both *in vitro,* and in basement membrane preparations from other tissues [26–28]. Modified FN colocalizes with leukocyte-derived MPO in human atherosclerotic lesions [29], but the nature of the modifications induced on FN by MPO-derived oxidants are unknown. Sulfur-containing amino acids (Cys, Met and cystine) are major targets for HOCl [22,30,31], however FN has low levels of Cys, though a large number of Met and disulfide (cystine) bonds; these are therefore likely to be major targets, if they are accessible. HOCl can also modify His, Trp, Lys and Tyr residues [22] though the chloramines (RNHCl species) formed on His and Lys have limited stability and hence cannot be easily quantified *in vivo* [32,33]. Reaction of HOCl and chloramines with Tyr generates the well-established biomarker 3-chlorotyrosine (3Cl-Tyr) [34–36]. This stable product is characteristic of MPO-mediated damage, as it is the only enzyme known to induce significant levels of chlorination [35]. Elevated 3Cl-Tyr levels have been detected on low- and high-density lipoproteins extracted from atherosclerotic lesions, and also on plasma proteins from people with cardiovascular disease [36–40].

The studies reported here aimed to determine whether human plasma FN is susceptible to damage induced by HOCl and a MPO-catalysed system, to identify the nature and sites of damage using a recently developed proteomics approach [41], and to examine whether oxidant-modified FN has functional effects on human coronary artery smooth muscle cells, a key cell type within the artery wall.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma Aldrich except for: human plasma fibronectin (FN) (Corning or Sigma-Aldrich), human myeloperoxidase (Planta Natural Products), lysyl endopeptidase (Lys-C) (Wako), and 3-chloro-[$^{13}C_6$] tyrosine (Cambridge Isotope Laboratories). All solvents were HPLC or LCMS grade. RNA was extracted from cell cultures using an RNeasy kit (Qiagen, Valencia, CA) according to the manufacturers protocol, with cDNA synthesis and quantitative real-time PCR carried out using SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen) and SYBR® GreenER™ qPCR SuperMix Universal (Invitrogen), respectively. Human interleukin-6 (IL-6) was determined using an ELISA kit (Biolegend; San Diego, USA) as described by the manufacturer. Human coronary artery smooth muscle cells (donor 1596), SMC growth medium and SMC basal medium were from Cell Applications (San Diego, USA).

2.2. Quantification of HOCl formation using 3,3′,5,5′-tetramethylbenzidine (TMB) or monochlorodimedone (MCD)

TMB was used to quantify HOCl production by the MPO-H_2O_2-Cl^- system as outlined previously [42]. The developing reagent consisted of 20 mM TMB in dimethylformamide, and 2 mM NaI in sodium acetate buffer (0.44 M, pH 5.4) prepared immediately prior to use. The MPO-H_2O_2-Cl^- (20 nM MPO, 0–200 μM H_2O_2, 200 mM Cl^-) or reagent HOCl (0–200 μM) was incubated with taurine (10 mM) at 37 °C for 2 h. Then, 50 μL of TMB reagent was added to each well and incubated for 5 min at 21 °C. The absorbance at 645 nm was then measured on a Spectra Max® i3x microplate reader. The concentration of HOCl formed and trapped by taurine, was calculated using a standard curve generated using reagent HOCl (0–200 μM). HOCl production was also quantified by determining the loss of parent MCD spectrophotometrically, using a molar extinction coefficient ε_{290} 17,700 M^{-1} cm^{-1} [43].

2.3. Oxidation of human plasma fibronectin (FN)

Purified FN was prepared in 100 mM sodium phosphate buffer, pH 7.4, at a concentration of 1 mg mL^{-1} (2.27 μM). HOCl was added at 0, 100 or 500 μM and incubated for 1 h at 21 °C. HOCl stocks were quantified spectrophotometrically at 292 nm using a molar extinction coefficient (ε_{292}) of 350 M^{-1} cm^{-1}. For MPO-mediated oxidation, aliquots of FN were incubated (unless otherwise indicated) with 0.1 μM MPO, 100 mM NaCl and 500 μM H_2O_2, with the H_2O_2 added as 10 × 50 μM aliquots at 10 min intervals at 37 °C on a thermo-shaker, and incubated for a further 10 min after the final addition. Controls with no MPO, no H_2O_2, no NaCl, and untreated FN were included.

2.4. Protein digestion for mass spectrometry

Modified and non-modified proteins were digested in-solution using an optimised protocol without the use of reduction and alkylation, as these reduce the yield of chlorinated products (T. Nybo, M.J. Davies, A. Rogowska-Wrzesinska, unpublished data). Briefly, residual reactants and salts were removed using 10 kDa spin-filters (Amicon Ultra-0.5 Ultracel-10K, Merck Millipore, Ireland), with buffer exchange into 50 mM triethyl ammonium bicarbonate buffer supplemented with 4 M urea and 1% sodium deoxycholate (SDC) to induce denaturation. Proteins were incubated for > 3 h, followed by a two-step digestion using Lys-C for 2 h in 4 M urea, followed by trypsin for 18 h in 1 M urea. The temperature was kept at 30 °C to minimize protein carbamylation. SDC was removed using acidification and ethyl acetate phase transfer as described previously [44].