Chlorination and oxidation of the extracellular matrix protein laminin and basement membrane extracts by hypochlorous acid and myeloperoxidase

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ABSTRACT

Basement membranes are specialized extracellular matrices that underlie arterial wall endothelial cells, with laminin being a key structural and biologically-active component. Hypochlorous acid (HOCl), a potent oxidizing and chlorinating agent, is formed in vivo at sites of inflammation via the enzymatic action of myeloperoxidase (MPO), released by activated leukocytes. Considerable data supports a role for MPO-derived oxidants in cardiovascular disease and particularly atherosclerosis. These effects may be mediated via extracellular matrix damage to which MPO binds. Herein we detect and quantify sites of oxidation and chlorination on isolated laminin-111, and laminin in basement membrane extracts (BME), by use of mass spectrometry. Increased modification was detected with increasing oxidant exposure. Mass mapping indicated selectivity in the sites and extent of damage; Met residues were most heavily modified. Fewer modifications were detected with BME, possibly due to the shielding effects. HOCl oxidised 30 (of 56 total) Met and 7 (of 24) Trp residues, and chlorinated 33 (of 99) Tyr residues; 3 Tyr were dichlorinated. An additional 8 Met and 10 Trp oxidations, 14 chlorinations, and 18 dichlorinations were detected with the MPO/H2O2/Cl- system when compared to reagent HOCl. Interestingly, chlorination was detected at Tyr2415 in the integrin-binding region; this may decrease modification was detected with increasing oxidant exposure. Mass mapping indicated selectivity in the sites and extent of damage; Met residues were most heavily modified. Fewer modifications were detected with BME, possibly due to the shielding effects. HOCl oxidised 30 (of 56 total) Met and 7 (of 24) Trp residues, and chlorinated 33 (of 99) Tyr residues; 3 Tyr were dichlorinated. An additional 8 Met and 10 Trp oxidations, 14 chlorinations, and 18 dichlorinations were detected with the MPO/H2O2/Cl- system when compared to reagent HOCl. Interestingly, chlorination was detected at Tyr2415 in the integrin-binding region; this may decrease cellular adhesion. Co-localization of MPO-damaged epitopes and laminin was detected in human atherosclerotic lesions. These data indicate that laminin is extensively modified by MPO-derived oxidants, with structural and functional changes. These modifications, and compromised cell-matrix interactions, may promote endothelial cell dysfunction, weaken the structure of atherosclerotic lesions, and enhance lesion rupture.

1. Introduction

The extracellular matrix (ECM) is a major component of nearly all tissues, with this providing a complex framework and 3-dimensional structure that supports associated cells and defines tissue structure. The ECM has multiple functions including: a) providing mechanical strength and elasticity; b) acting as a scaffold for cell adhesion and growth; c) controlling cell migration and proliferation; d) binding signalling molecules, pro-enzymes, enzyme inhibitors and cytokines; and e) controlling the activity of these materials [1–4]. ECM glycosaminoglycans (GAGs, polymers of acidic or sulfated sugars) can form gels of varying pore size and charge density and thereby regulate molecular traffic. Proteoglycans (proteins with covalently-attached glycosaminoglycan chains) bind secreted signalling molecules (e.g. growth factors, cytokines) and can enhance or inhibit their activity via multiple mechanisms including: (1) immobilization thereby restricting range of action; (2) steric blocking of active sites; (3) providing a reservoir for delayed release; (4) protection from proteolytic degradation thereby prolonging action; (5) via alteration or concentration of the protein for presentation to cell-surface receptors. Alterations may therefore modulate cell
behaviour (e.g. loss of adhesion and enhanced proliferation), and tissue structure, stability and function.

Recent studies have shown that the ECM is highly susceptible to oxidative damage due to its high abundance, its low rate of turnover (which can result in accumulation of damage during ageing and disease) and the relatively low levels of extracellular antioxidant, repairs and catalytic systems [5–7]. Considerable data has been presented that demonstrates that oxidants generated by activated leukocytes (neutrophils, monocytes, tissue macrophages) can induce structural and functional changes to ECM proteins and proteoglycans (proteins with covalently attached GAGs), with damage evident in multiple tissue samples, including within human atherosclerotic lesions [7–11].

Atherosclerosis is characterized by chronic inflammation, large numbers of activated leukocytes, lipid accumulation, endothelial cell dysfunction, smooth muscle cell migration and proliferation, loss of elasticity, calcification and in some cases lesion rupture and thrombus formation (reviewed [12,13]). Convincing data has been presented for the presence of elevated levels of oxidation products at both extra- and intra-cellular sites within plaques (e.g. [14–16]). The majority of this protein damage appears to be present on ECM materials [17].

The presence of elevated numbers of activated neutrophils and monocytes in the inflamed artery wall is known to increase plasma and tissue levels of the heme enzyme myeloperoxidase (MPO) as a result of monocytes in the inflamed artery wall is known to increase plasma and protein damage appears to be present on ECM materials [17].

Intra-cellular sites within plaques (e.g. [14–16]). The majority of this the presence of elevated levels of oxidation products at both extra- and intra-cellular sites within plaques (e.g. [14–16]). The majority of this protein damage appears to be present on ECM materials [17].

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2. Materials and methods

2.1. Reagents and buffers

Murine laminin-111 was purchased from Sigma Aldrich, and murine BME (with reduced growth factors, Cultrex) was purchased from Trevigen. Laminin concentrations were calculated using a protein molecular mass of 722 kDa, based on data from Uniprot entries P19137, P02469, P02468 for the α-1, β-1 and γ-1 subunits respectively. This molecular mass was also used to calculate laminin concentrations in the BME where laminin is the major component. Lysyl endopeptidase (Lys C) was purchased from Wako, and trypsin from Sigma Aldrich. MPO was from Planta Natural Products. All solvents and reagents were MS grade, purchased from Sigma Aldrich, unless otherwise stated. The following antibodies were used: rat anti-mouse laminin (clone AL-2; R&D Systems, MN, USA), mouse anti-laminin γ1 (clone 2E8; Merck Millipore, MA, USA) and a mouse monoclonal against HOCl-modified epitopes (clone 2D10G9) [49]. Biotinylated or HRP-conjugated secondary antibodies used for ELISA and immunoblotting experiments were all from GE (IL, USA). Goat anti-mouse cyanine-2 (Cy-2)-labelled IgG was from Jackson Dianova.

2.2. Oxidation of laminin-111 and BME

Murine laminin-111 or murine BME were suspended in 100 mM sodium phosphate buffer (PBS), pH 7.4, at a concentration of 1 mg mL⁻¹. Treatment with HOCl was carried out by bolus addition of 0, 100 and 500 μM HOCl and incubation for 1 h at 21 °C. Stock concentrations of HOCl were quantified using a molar extinction coefficient ε290 of 17 700 M⁻¹ cm⁻¹ [50]. Enzymatic treatment was carried out at 37 °C by addition of 0.1 μM MPO, 500 μM H₂O₂, and 100 mM NaCl. Controls omitted NaCl, H₂O₂, or MPO, as well as an untreated protein. H₂O₂ was added in 50 μM portions at 10 min intervals to minimize auto-inactivation of MPO by high concentrations of H₂O₂.

2.3. Quantification of HOCl-formation by the MPO system

HOCl production by the MPO/H₂O₂/Cl⁻ system was quantified spectrophotometrically at 290 nm, using the decrease in the mono-chlorodimedone (MCD, 1 M stock) absorbance resulting from MCD oxidation, with a molar extinction coefficient ε290 of 17 700 M⁻¹ cm⁻¹ [51].

2.4. Protein digestion and analysis by mass spectrometry

Laminin-111 (100 μg) was digested in-solution as described
2.5. Quantification of total 3-chlorotyrosine levels by mass spectrometry

of the 3rd isotopic isomer arising from the presence of chlorine isotopes relative to the native peptide to confirm the characteristic enhancement [48]. For chlorinated peptides, the isotopic distribution was inspected. Peptide identifications were validated manually as described previously [48]. Full scans of m/z 400–1400 was recorded with 120,000 resolution, and the top 12 most intense ions were selected for HCD fragmentation using a normalised collision energy (NCE) of 28. Blanks were included between each sample to monitor and prevent carry-over. Data files (.raw, Xcalibur) were analysed using Progenesis QI for proteomics (nonlinear Dynamics, USA) for chromatographic alignment and precursor peak quantification. Proteome Discoverer 2.1 was used for database searches against a murine laminin isofrom database. The following search parameters were used: parent ion tolerance: 4 ppm; fragment ion tolerance: 0.1 Da; trypsin: 2 missed cleavages; fixed modifications: none; variable modifications: chlorination at Tyr (Y) and Trp (W), dichlorination at Y and W, mono-oxidation at Met (M), His (H), Cys (C) and W, di-oxidation at M, C, W, and tri-oxidation at T. The peptide identifications were exported as pepXML files and imported into Progenesis QI for analysis. The resulting data were exported as comma-delimited files (.csv) providing normalised abundance calculated from the extracted ion chromatograms of precursor ions [53]. Peptide identifications were validated manually as described previously [48]. For chlorinated peptides, the isotopic distribution was inspected relative to the native peptide to confirm the characteristic enhancement of the 3rd isotopic isomer arising from the presence of chlorine isotopes [54].

2.6. Quantification of methionine oxidation and methionine sulfoxide formation by amino acid analysis

Laminin-111 (25 μg) was treated with MPO (100 nM), ClO\textsuperscript− (100 mM), and varying concentrations of H\textsubscript{2}O\textsubscript{2} for 2 h at 37 °C. The H\textsubscript{2}O\textsubscript{2} was added in 50 μM aliquots over 10 min intervals. The proteins were then processed and analysed for total 3-chlorotyrosine (3-ClTyr) as described previously [48], using 3-chloroo\textsuperscript{13}C\textsubscript{15}N\textsubscript{2}Tyrosine (100 pmol) as an internal standard [55]. MS experiments which examined TCA precipitation versus spin filters versus for protein purification showed that the use of TCA did not induce artefactual chlorination (T. Nybo, M.J. Davies, A. Rogowska-Wrzesinska, unpublished data).

2.8. Detection of myeloperoxidase-derived damage by ELISA

Laminin-111 (5 μg mL\textsuperscript{−1} in 0.1 M PBS, pH 7.4) was coated onto clear high adhesion 96-well plates over 1 h at 37 °C, or overnight at 4 °C. The wells were then washed twice with 200 μL PBS to remove any non-bound protein. The protein in the wells were then incubated with MPO (20 nM), Cl\textsuperscript− (100 mM) and H\textsubscript{2}O\textsubscript{2} (at the indicated concentrations) for 2 h at 37 °C. The wells were washed twice with 200 μL PBS to remove residual oxidant, and then blocked with 100 μL 0.1% casein (w/v) in PBS for 1 h at 21 °C. The wells were then washed twice with 200 μL PBS before being incubated with 2D10G9 antibody [49] (50 μL; 1:100 dilution) overnight at 4 °C. Unbound antibody was removed (2 × 200 μL PBS containing Tween 20) and then incubated with 50 μL secondary antibody (alkaline phosphatase conjugated anti-mouse IgG; 1:1000 dilution) in 0.1% casein (w/v) in PBS for 1 h at 21 °C. Any unbound secondary antibody was removed by washing (4 × 200 μL TBST) before addition of 50 μL alkaline phosphatase yellow solution. The absorbance of each well at 405 nm was then read every 5 min over a 30 min period using a microplate reader.

2.9. Immunofluorescence analysis of human atherosclerotic lesions

Tissue samples from arteriae femoralis and aortae abdominalis were obtained from 3 subjects (within 12 h post mortem) who died from cerebral haemorrhage. Lesions were classified according to Starry and coworkers [57] with these ranging from microscopically normal to thickened intima (lesion type II-IV). The samples were frozen in a cryostat (Microm HM500 OM; Microm, Walldorf, Germany) using tissue-refreezing medium (Tissue Tec OCT-compound; Miles, Elkhard, Ind., USA). Serial cryosections (5 μm) were collected on glass slides, air-dried (21 °C, 2 h), fixed in acetone (21 °C, 5 min) and stored at −70 °C until examined [11,58]. Before analysis, sections were thawed and fixed again in acetone (21 °C, 5 min). PBS was used to rehydrate sections, followed by blocking with Ultra V block for 10 min (Lab Vision, Fremont, CA, USA). For double immunofluorescence staining, sections were incubated first with monoclonal anti-laminin γ1 (mouse IgG, clone 2E8; 1:100 dilution) followed by goat anti-mouse Cy-2-labelled IgG (1:300) as a secondary antibody [59]. After a blocking step with normal mouse serum (1:25) for 15 min sections were then incubated with Cy-3 labelled monoclonal antibody clone 2D10G9 (1:5) raised against HOCI-modified epitopes [49]. PBS was used for washing steps and Dako antibody diluent for antibody dilutions. All incubation steps were performed at 21 °C in dark moisture chambers. The sections were mounted with Moviol (Calbiochem-Novabiochem, La Jolla, USA), and analysed with a confocal laser-scanning microscope in sequential mode (Leica TCS SP2, Leica Lasertechnik GmbH, Heidelberg, Germany). Analysis for Cy-2 (green staining) used λ\textsubscript{ex} 488 nm, and λ\textsubscript{em} 500–540 nm, and for Cy-3 (red staining) λ\textsubscript{ex} 543 nm, and λ\textsubscript{em} 560–620 nm. Control experiments were performed by omission of the primary antibodies or by replacing them with non-immune mouse or rabbit IgG (Sigma-Aldrich). This study was approved by the ethics committee from the Medical...
University Graz (EK-number: 29–464 ex 16/17). All methods were performed in accordance with the relevant guidelines and regulations.

2.10. Statistics

For the MS data, statistical analysis was performed using Student’s t-test using SAS Enterprise Guide 7.1 (SAS Institute Inc., USA). One-way ANOVA with post-hoc analysis using Dunnett’s multiple comparison test analysis was used for UPLC and ELISA data. Data are representative of at least three independent experiments, with p < 0.05 considered significant.

3. Results

3.1. HOCl induces chlorination and oxidation in murine laminin-111, and laminin chains present in BME

The effects of HOCl (100, and 500 μM) and a MPO/H2O2/Cl− system (with 500 μM H2O2 to generate the equivalent of 500 μM HOCl; see data in [48]) on laminin-111 was studied using both purified murine protein and a BME where laminin is a major component. Sites of modification (chlorination and oxidation) were mapped and quantified by MS using a recently described method ([48]; T. Nybo et al., submitted), in which sequential Lys-C and tryptic digestion are performed without prior reduction and alkylation of the samples, as the latter have been shown to interfere with the detection and quantification of some oxidative modification ([60], T. Nybo et al., submitted). Use of this method resulted in good sequence coverage for the globular domains (LN, L4, LF, and LG) and the triple-helical long arm (I+II) of the untreated pure protein (sequence coverage excluding the LE domains: α1 71.1%, β1 71.3%, γ1 64.0%), but relatively poor coverage of the laminin EGF-like (LE) domains due to the high number of disulfide (cystine) cross-links in these domains (Fig. 1). A small number of peptides specific to the α5, β2 and γ3 chains were also detected in the samples (Table 1), together with a small number of peptides common to both α1 and α5. The presence of these peptides is likely to arise from the presence of low amounts of contaminating α5, β2 and γ3 laminins in the commercial preparation.

Treatment of purified laminin-111 with reagent HOCl (100 and 500 μM) for 1 h at 21 °C and pH 7.4, resulted in the consistent detection of up to 33 Tyr residues (Table 1). Annotated MSMS spectra are reproduced 21 of the Trp and Met oxidation sites, and 10 of the Tyr chlorination sites detected with purified laminin-111. In addition, one novel 3-ClTyr site, two sites with 3,5-Cl2Tyr, two Met oxidation sites, and two Trp oxidation sites were observed. This similarity in the sites of modification suggests that a significant number of the positions in the isolated purified trimeric protein that are accessible to HOCl, remain accessible to the oxidant in the BME. However, the overall number of sites of modification detected with the purified protein was significantly greater than with the BME, suggesting that some regions of the protein are shielded from modification by other components, or that less of the oxidant reacts with the laminin chains in the BME samples.

3.2. The MPO/H2O2/Cl− system induces chlorination and oxidation in murine laminin-111

Analogous experiments to those outlined above were carried out using the MPO/H2O2/Cl− system to determine whether the enzyme system gives rise to an altered distribution or extent of modification. The majority of chlorination and oxidation sites detected on treatment purified laminin with reagent HOCl were reproduced by the MPO/H2O2/Cl− system (Table 1), though the enzyme system also induced modifications at a considerable number of additional sites, including 8 sites of Met oxidation, 10 sites of Trp oxidation, and 14 additional Tyr chlorination sites (Table 1). The number of sites with multiple modifications on a single residue (di-oxygenation, di-chlorination) was significantly greater with the MPO/H2O2/Cl− system than with reagent.
Table 1

List of sites of oxidation (at Met, Trp) and chlorination (Tyr) identified in purified murine laminin-111 (1.18 μM) or laminin present in murine basement membrane extract (ECM; 1 μg protein) treated with 0, 100, and 500 μM HOCI, or a MPO (100 nM)/H₂O₂ (500 μM)/Cl⁻ (100 mM) system in 100 mM PBS, pH 7.4, for 1 h at 21 °C. Data are presented in sequence residue order for each of the laminin chains. The modified residues are indicated together with type of modification and the detected peptide(s) (with start and stop numbering) which contain these residues, with the modified amino acid highlighted. Modifications are coded as follows: Cl = 3-chloroTyr; Cl₂ = 3,5-dichloroTyr; 2 x Cl = two 3-chloroTyr modifications in the peptide at the indicated residues; O₁ = mono-oxygenation; O₂ = di-oxygenation; 2 x O, 3 x O, 4 x O = two, three or four mono-oxygenations at residues within the peptide at the indicated residues. • indicates modification detected with the stated oxidant system.

| Subunit | Residue | Modification | start-stop | Peptide(s) | 100 μM HOCI | 500 μM HOCI | MPO | ECM 100 μM HOCI | ECM 500 μM HOCI |
|---------|---------|--------------|------------|------------|-------------|-------------|-----|----------------|-----------------|
| alpha 1 | 128-7yr | Cl           | 122-132    | GYVQAYYIK  | •           |             |     |                 |                 |
|         |         |              | 133-146;   |            |             |             |     |                 |                 |
| alpha 1 | 142-Trp | Cl           | 139-146    | AANAPRPGMVLER; PGNWALER |             |             |     |                 |                 |
|         |         |              | 133-146    |            |             |             |     |                 |                 |
| alpha 1 | 181-Trp | Cl           | 176-182    | RPGFFYR    | •           |             |     |                 |                 |
|         |         |              | 240-251;   |            |             |             |     |                 |                 |
| alpha 1 | 246-Met | O1          | 238-251    | TlNAo/ltlSEL, HtlnALo/ltlSEL |             |             |     |                 |                 |
| alpha 1 | 574-7yr/575-Tyr | 2 x Cl | 579-586 | LSTYTVAPKPLYGNK |             |             |     |                 |                 |
| alpha 1 | 576-Tyr | O1          | 579-586    | LSTYTVAPKPLYGNK |             |             |     |                 |                 |
|         |         |              | 579-586    |            |             |             |     |                 |                 |
| alpha 1 | 632-Met | Cl           | 596-620    | YTVQGIGYFVSGQLMSHADIK | •           |             |     |                 |                 |
|         |         |              | 630-646    | AEGLSPQPEYPYVR |             |             |     |                 |                 |
| alpha 1 | 638-Tyr | O2          | 630-646    | AEGLSPQPEYPYVR | •           |             |     |                 |                 |
| alpha 1 | 641-Trp | Cl           | 630-646    | AEGLSPQPEYPYVR | •           |             |     |                 |                 |
|         |         |              | 630-646    |            |             |             |     |                 |                 |
| alpha 1 | 1220-Tyr | Cl        | 1277-1277; | QHYAEPYFR  |             |             |     |                 |                 |
|         |         |              | 1277-1277; |            |             |             |     |                 |                 |
| alpha 1 | 1222-Tyr | Cl        | 1277-1277 | QHYAEPYFR, QHYAEPYFR/PK |             |             |     |                 |                 |
|         |         |              | 1277-1277; |            |             |             |     |                 |                 |
| alpha 1 | 1226-Trp | O1          | 1277-1277 | QHYAEPYFR  |             |             |     |                 |                 |
|         |         |              | 1277-1277 |            |             |             |     |                 |                 |
| alpha 1 | 1276-Trp | O2          | 1277-1277 | QHYAEPYFR/PK |             |             |     |                 |                 |
|         |         |              | 1277-1277 |            |             |             |     |                 |                 |
| alpha 1 | 1261-7yr | Cl        | 1244-1268 | LGWSVAYSTGIETSTSN/YPODULIK | •           |             |     |                 |                 |
| alpha 1 | 1261-7yr | O2          | 1252-1268 | STLSGCTSN/YPODULIK | •           |             |     |                 |                 |
| alpha 1 | 1278-7yr | Cl        | 1275-1289 | HVT/MSAAPENGVIR | •           |             |     |                 |                 |
|         |         |              | 1275-1289 |            |             |             |     |                 |                 |
| alpha 1 | 1278-7yr | O2          | 1275-1289 | HVT/MSAAPENGVIR | •           |             |     |                 |                 |
| alpha 1 | 1279-Met | O1          | 1275-1289 | HVT/MSAAPENGVIR |             |             |     |                 |                 |
|         |         |              | 1275-1289 |            |             |             |     |                 |                 |
| alpha 1 | 1296-Met | O1          | 1296-1302 | MXEFINK |             |             |     |                 |                 |
| alpha 1 | 1304-Trp | 2 x O       | 1296-1302 | MXEFINK |             |             |     |                 |                 |
| alpha 1 | 1303-Trp | Cl        | 1303-1310 | YFNSGEK | •           |             |     |                 |                 |
|         |         |              | 1303-1310 |            |             |             |     |                 |                 |
| alpha 1 | 1317-Met; O1 | Cl | 1330-1341 | HYTVLGSMVS,SNLVYLUK; | •           |             |     |                 |                 |
|         |         |              | 1330-1341 |            |             |             |     |                 |                 |
| alpha 1 | 1326-Tyr | Cl           | 1330-1330 | HYTVLGSMVS,SNLVYLUK |             |             |     |                 |                 |
|         |         |              | 1330-1330 |            |             |             |     |                 |                 |
| alpha 1 | 1326-Tyr | C2          | 1330-1330 | HYTVLGSMVS,SNLVYLUK |             |             |     |                 |                 |
| alpha 1 | 1333-Tyr | Cl           | 1335-1335 | AYFGSFIQOSR | •           |             |     |                 |                 |
|         |         |              | 1335-1335 |            |             |             |     |                 |                 |
| alpha 1 | 1333-Tyr | C2          | 1335-1335 | AYFGSFIQOSR | •           |             |     |                 |                 |
| alpha 1 | 1824-Trp | O1          | 1823-1854 | G垂NVDMASTHTAAQDTITQLLEHREDELLWAR; | •           |             |     |                 |                 |
|         |         |              | 1823-1854 |            |             |             |     |                 |                 |
| alpha 1 | 1828-Trp | O2          | 1821-1854 | G垂NVDMASTHTAAQDTITQLLEHREDELLWAR |             |             |     |                 |                 |
|         |         |              | 1821-1854 |            |             |             |     |                 |                 |
| alpha 1 | 1832-Trp | O1          | 1821-1854 | G垂NVDMASTHTAAQDTITQLLEHREDELLWAR |             |             |     |                 |                 |
| alpha 1 | 1856-Met; 2 x O | 1856-1869 | ISVGYDVLQSVQMSK, NHYDVLQSVQMSK, NHYDVLQSVQMSK | •           |             |     |                 |                 |
| alpha 1 | 1867-Met | 2 x O | 1885-1890 | IRTGYDVLYMQKSV, SHYDVLQSVQMSK, SHYDVLQSVQMSK | •           |             |     |                 |                 |
| alpha 1 | 1926-Met | O1          | 1937-1993 | IQT,TEAEHNLAAHAK | •           |             |     |                 |                 |
| alpha 1 | 2070-Met | O1          | 2086-2077 | SHTTFLLAGR | •           |             |     |                 |                 |
|         |         |              | 2086-2099; |            |             |             |     |                 |                 |
| alpha 1 | 2086-Met; 2 x O | 2086-2094 | DAEVQGVALLDLQ, DAEVQGVALLDLQ, MHDVEMQGVALDLQ | •           |             |     |                 |                 |
| alpha 1 | 2138-Tyr | Cl           | 2137-2155 | AYFGQTSNFIYNTLLNVK | •           |             |     |                 |                 |

(continued on next page)
Table 1 (continued)

| apha 1 | 2138-Tyr | C2 | 2137-2155 | FYQPQSTYNTLYNLVR | * |
|--------|----------|----|-----------|-------------------|---|
| apha 1 | 2147-Tyr | C1 | 2137-2155 | FYQPQSTYNTLYNLVR | * | * |
| apha 1 | 2147-Tyr | C2 | 2137-2155 | FYQPQSTYNTLYNLVR | * |
| apha 1 | 2165-Tyr | C1 | 2156-2180 | TQEPDPDLLGSSGSSEDQALEVMR | * |
| apha 1 | 2179-Met | O1 | 2156-2180 | TQEPDPDLLGSSGSSEDQALEVMR | * |
|        |          |    |           | 2184-2196; | * |
| apha 1 | 2188-Tyr | O1 |            | 2184-2208; 2184-2216 | * |
|        |          |    |           | VALVILGSSGTTLFPFSINNIA; VALVILGSSGTTLFPFSINNIA | * |
| apha 1 | 2209-Tyr | O1 | 2209-2216 | WSHVYTR | * |
| apha 1 | 2213-Tyr | C1 | 2209-2216 | WSHVYTR | * |
| apha 1 | 2220-Met | O1 | 2217-2226 | FGNNAGSLYVWL | * | * | * |
|        |          |    |           | 2289-2298; 2299-2301 | * | * |
| apha 1 | 2293-Tyr | O1 | 2289-2301 | SGILVNYERI; SGILVNYEREGRK | * | * |
| apha 1 | 2295-Tyr | C1 | 2289-2301 | SGILVNYERI | * | * |
| apha 1 | 2374-Met | O1 | 2373-2388 | VAVDILGSSPLTLYVTR | * | * | * | * |
| apha 1 | 2385-Tyr | C2 | 2406-2420 | GQILAVIDEVQTSOK | * | * |
| apha 1 | 2415-Tyr | C2 | 2406-2420 | GQILAVIDEVQTSOK | * |
| apha 1 | 2443-Tyr | C1 | 2440-2451 | DLVYVGGSLHPSK | * |
| apha 1 | 2443-Tyr | C2 | 2440-2451 | DLVYVGGSLHPSK | * |
| apha 1 | 2504-Tyr | C1 | 2502-2510 | GGVYVEMPPK | * |
| apha 1 | 2504-Tyr | C2 | 2502-2510 | GGVYVEMPPK | * |
| apha 1 | 2555-Met | O1 | 2538-2560; | DAEAGGDAHVPTFSIRK; DAEAGGDAHVPTFSIRK | * | * |
|        |          |    | 2538-2574 | DAEAGGDAHVPTFSIRK; DAEAGGDAHVPTFSIRK | * |
| apha 1 | 2585-Tyr | C1 | 2576-2597 | ALLHAPFSGSDGHEHHSLVR | * | * | * | * |
| apha 1 | 2585-Tyr | C2 | 2576-2597 | ALLHAPFSGSDGHEHHSLVR | * | * |
| apha 1 | 2614-Met | O1 | 2601-2615; 2602-2615 | RHTLQVQSPESNPVEK; RHTLQVQSPESNPVEK | * | * | * | * |
| apha 1 | 2631-Tyr | C1 | 2624-2939 | TDISILVYGGLEDK | * | * | * |
| apha 1 | 2631-Tyr | C2 | 2624-2939 | TDISILVYGGLEDK | * |
| apha 1 | 2785-Met | O1 | 2782-2790 | LHHFMFDGSK | * |
| apha 1 | 2864-Tyr | C1 | 2845-2855 | LYYGLPSHWR | * |
| apha 1 | 2864-Tyr | C2 | 2845-2855 | LYYGLPSHWR | * |
| apha 1 | 2973-Tyr | C1 | 2969-2976 | ITATQPR | * |
| apha 1 | 2973-Tyr | C2 | 2969-2976 | ITATQPR | * |
| apha 1/5 | 265-Tyr | C1 | 264-269 | RYYSIK; YYYSIK | * |
| apha 1/5 | 265-Tyr | C2 | 263-269 | RYYSIK | * |
| apha 1/5 | 266-Tyr | C1 | 264-269 | YYYSIK; YYYSIK | * |
| apha 1/5 | 266-Tyr | C2 | 263-269 | YYYSIK | * |
| apha 5 | 914-Met/915 Met | O1 | 909-920 | GYAHMAAQPR | * |
| apha 5 | 2954-Met | O1 | 2550-2558 | TWEMPVQR | * |
| apha 5 | 2655-Met | O1 | 2636-2657 | AVAAEAALTVHVTGQESDLGDAQ | * |
| apha 5 | 3190-Met | O1 | 3189-3198 | PMSQETQOR | * |
| beta 1 | 148-Met | O1 | 342-352 | TRPAMXIER | * | * |
| beta 1 | 182-Met | O1 | 373-383 | SQGSTGPMX | * |
| beta 1 | 220-Tyr | C1 | 216-223 | IEEDPSPR | * | * | * | * |
| beta 1 | 259-Tyr | C1 | 258-268 | YYAVDVMVR | * |
| beta 1 | 263-Tyr | C1 | 258-268 | YYAVDVMVR | * |
| beta 1 | 265-Met | O1 | 258-268 | YYAVDVMVR | * |
| beta 1 | 575-Tyr | C1 | 574-590 | QYQDR; QYQDR; DSLGQVPSWFGQVR | * | * |

(continued on next page)
| Beta 1  | 575-Tyr | C1  | 574-590 | QYDRIPSWTCGPPRVR |
|--------|---------|-----|---------|------------------|
| Beta 1  | 583-Tyr | C1  | 580-590 | IPWSWTPGPPRVR, QYDRIPSWTPGPPRVR |
| Beta 1  | 583-Tyr | C2  | 580-590 | IPWSWTPGPPRVR, QYDRIPSWTPGPPRVR |
| Beta 1  | 731-Tyr | C1  | 712-736 | SLFDPVGSQGLDVTNYSAWFTQFQ |
| Beta 1  | 731-Tyr | C2  | 712-736 | SLFDPVGSQGLDVTNYSAWFTQFQ |
| Beta 1  | 1216-Tyr | C1  | 1209-1217; 1209-1225 | ISGVGIPFR, ISGVGIPFRTVDSVEK |
| Beta 1  | 1216-Tyr | C2  | 1209-1225 | ISGVGIPFR, ISGVGIPFRTVDSVEK |
| Beta 1  | 1264-Met | C1  | 1264-1270 | MAQVEVK |
| Beta 1  | 1324-Tyr | C1  | 1324-1333 | YFMMSAEK |
| Beta 1  | 1324-Tyr | C2  | 1324-1333 | YFMMSAEK |
| Beta 1  | 1327-Met | C1  | 1324-1333 | YFMMSAEK |
| Beta 1  | 1327-Met | C2  | 1324-1333 | YFMMSAEK |
| Beta 1  | 1360-Met | C1  | 1354-1363; 1356-1368; 1354-1363; | VEDVLLER, VEDVLLERESPFK, DRVEDVLLER, DRVEDVLLERESPFK |
| Beta 1  | 1671-Tyr | C1  | 1662-1674 | AAQNSGEAFYEK |
| Beta 1  | 1671-Tyr | C2  | 1662-1674 | AAQNSGEAFYEK |
| Beta 1  | 1698-Tyr | C1  | 1689-1699 | TLDGEELKFYK |
| Gamma 1 | 145-Tyr | C1  | 140-149 | AFQTVK |
| Gamma 1 | 230-Tyr | C1  | 227-248 | PSAVINTKPSLQENATATCR |
| Gamma 1 | 273-Tyr | C1  | 270-283 | SYYA/SEPFAVGR |
| Gamma 1 | 272-Tyr, 273-Tyr | C1 | 270-283 | SYYA/SEPFAVGR |
| Gamma 1 | 538-Tyr | C1  | 530-555 | DSGEASLEWSDQRDIHAVDSDYYFR |
| Gamma 1 | 552-Tyr | C1  | 530-555 | DSGEASLEWSDQRDIHAVDSDYYFR |
| Gamma 1 | 556-Tyr | C1  | 556-562 | YHAPXK |
| Gamma 1 | 613-Tyr | C1  | 602-620 | VSVPLAGQNSFVTSETVK |
| Gamma 1 | 613-Tyr | C2  | 602-620 | VSVPLAGQNSFVTSETVK |
| Gamma 1 | 633-Tyr | C1  | 625-645 | LHEADYPWIRPSPEFQK |
| Gamma 1 | 633-Tyr | C2  | 625-645 | LHEADYPWIRPSPEFQK |
| Gamma 1 | 658-Tyr | C1  | 654-661 | IRGT/5SER |
| Gamma 1 | 1063-Met | C1  | 1047-1074; 1047-1072 | IQEUESLNIQGTDQDSTDQFDRIK; IQEUESLNIQGTDQDSTDQFDRIK |
| Gamma 1 | 1098-Met | C1  | 1092-1100 | DVQDN/LMR |
| Gamma 1 | 1266-Tyr | C1  | 1262-1286 | AVQISAVQIQVTQVOSLEALENANK |
| Gamma 1 | 1266-Tyr | C2  | 1262-1286 | AVQISAVQIQVTQVOSLEALENANK |
| Gamma 1 | 1462-Met | C1  | 1448-1474; 1448-1464 | TGGEVTDLDNEVNGMALKELEAQELN; TGGEVTDLDNEVNGMAL |
| Gamma 1 | 1485-Met | C1  | 1476-1502 | KQQDAQQQ/MMMG/MAGASQAQELNAR; KQQDAQQQ/MMMG/MAGASQAQELNAR |
| Gamma 1 | 1485-Met | C2  | 1476-1502 | KQQDAQQQ/MMMG/MAGASQAQELNAR; KQQDAQQQ/MMMG/MAGASQAQELNAR |
| Gamma 1 | 1488-Met | C1  | 1476-1502; 1476-1503; 1476-1503; 1476-1503; 1476-1503 | KQQDAQQQ/MMMG/MAGASQAQELNAR; KQQDAQQQ/MMMG/MAGASQAQELNAR |
| Gamma 1 | 1488-Met | C2  | 1476-1502; | KQQDAQQQ/MMMG/MAGASQAQELNAR; KQQDAQQQ/MMMG/MAGASQAQELNAR |
| Gamma 1 | 1517-Met | C1  | 1556-1575; 1566-1575; 1566-1575; 1566-1575; | GEAA/MYDRNL; GEAA/MYDRNLEAKI; GEAA/MYDRNL; GEAA/MYDRNL |
| Gamma 1 | 1517-Met | C2  | 1585-1582; 1585-1582; 1585-1582; 1585-1582; | GEAA/MYDRNL; GEAA/MYDRNLEAKI; GEAA/MYDRNL |
| Gamma 1 | 1573-Tyr | C1  | 1565-1575 | KQEAAIHM2NR, KQEAAIHM2NR, KQEAAIHM2NR, KQEAAIHM2NR |
| Gamma 1 | 1573-Tyr | C2  | 1565-1575 | KQEAAIHM2NR |
HOCI, as were the number of modifications detected on the (contaminating) α5, β2 and γ3 chains (Table 1). Experiments with the MPO/H₂O₂/Cl⁻ system and BME were not carried out as MPO is known to bind to multiple BME components [61,62], with this potentially confounding the data analysis.

3.3. Laminin is highly susceptible to damage induced by the MPO/H₂O₂/Cl⁻ system

The extent of modification at each of the identified sites of modification on laminin was evaluated by the relative site occupancy (RSO; the % conversion of a specific site to a particular product) [63]. Fig. 2 shows the site assignment and RSO of the modifications induced by 500 μM HOCI and the MPO/H₂O₂/Cl⁻ system (with 500 μM H₂O₂, and hence ~ 500 μM HOCI [48]) on purified laminin (PL-HOCI and PL-MPO respectively), and by 500 μM HOCI on laminin in the BME (BME-HOCI). Peptide quantification data are presented in Supplementary Tables 1–3 and Supplementary Figs. 2–7. As indicated in Fig. 2A, the MPO-system induced a markedly greater extent of modification than reagent HOCI, particularly on the α1-chain. This difference was less dramatic with the β1 and γ1 chains, suggesting that MPO may associate with, and hence direct oxidation to particular regions on this chain.

Although fewer sites were identified on the laminin chains present in the BME than with isolated laminin (Table 1), the RSO values detected for the BME were generally comparable with those detected with the HOCI-treated purified protein. These data are consistent with the hypothesis that the composition, or the interactions between laminin and other components in the BM, affect the regions of the protein that are exposed to the oxidant.

3.4. Quantification of isobaric peptide forms

A number of peptides were detected that contained more than a single site of modification with this resulting in the detection of isobaric (identical mass) species. In some cases, these species could be separated by chromatography on the basis of the altered hydrophobicity of the peptide. Thus, Tyr₆₃₈ and Tyr₆₄₈ were both detected as sites of modification in the peptide, 6₃₀₉₅₉₆₆₈₇₈₉₉₆₈₉₉₆₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉₉}_{503}
CITyr detected, relative to H₂O₂ initially supplied (and hence HOCl formed), indicates that this product accounts for ~0.22% of the HOCl generated, indicating that this is a low abundance modification, as reported previously [23,66].

3.8. Detection and quantification of MPO/H₂O₂/Cl⁻-induced modifications on laminin using ELISA

Previous studies have employed a monoclonal antibody, 2D10G9, to detect HOCl-induced damage in biological samples (e.g.
This antibody was therefore used to examine the formation of MPO/H₂O₂/Cl⁻-induced epitopes on both isolated laminin-111 using an ELISA assay, and also the presence of these epitopes in human atherosclerotic lesions (see below) by immunofluorescence.

Treatment of isolated laminin-111 with the MPO/H₂O₂/Cl⁻ system, with varying amounts of H₂O₂, provided evidence for HOCl-generated epitopes on laminin, detected by the 2D10G9 antibody, with greater recognition observed with higher oxidant doses with statistically-significant increases in recognition detected with the complete enzyme system containing ≥10 μM H₂O₂ (Fig. 8). Little recognition was observed for control (untreated) protein samples, or when individual components of the enzyme system were omitted (data not shown). The increase in recognition was not linear, as expected, due to the presence of other reactive targets [23] on the protein.
3.9. Structural changes induced on laminin by exposure to reagent HOCl and a MPO enzyme system

The above data indicate that HOCl and the MPO/H$_2$O$_2$/Cl$^-$ system can induce significant changes on laminin-111 at the residue level. Whether these modifications result in structural changes was examined using SDS-PAGE with silver staining (under both non-reducing and reducing conditions) or immunoblotting using an anti-murine laminin antibody (clone AL-2).

SDS-PAGE carried out under non-reducing conditions on samples treated with both HOCl (Fig. 9A) and the MPO/H$_2$O$_2$/Cl$^-$ system (Fig. 9C), resulted in the detection, by silver staining, of large amounts of material which migrated only a short distance into the gels from the loading wells, consistent with the presence of high molecular mass, intact trimeric laminin-111 protein (Fig. 9A). This material was not detected under reducing conditions (Fig. 9B,D). Increasing oxidant exposure resulted in modest smearing of this band to lower masses, with this being most obvious for the HOCl system (Fig. 9A). Under non-reducing conditions, weaker bands were detected at ~ 460 kDa (assigned to low levels of dissociated α1 chains; red arrows) and ~ 200 kDa (blue arrows; assigned to β1 and γ1 chains which have similar masses). These bands do not correspond to the masses indicated by the molecular mass standards (Fig. 9A,C), as the markers are reduced protein samples, which migrate at a different rate to the non-reduced proteins. On the reducing gels (Fig. 9B,D), these lower mass bands migrate at approximately the expected masses. With increasing oxidant exposure the intensity of the staining of the bands assigned to the α1, β1 and γ1 chains decreased. These changes were more marked for the samples run under non-reducing conditions, presumably due to the lower concentrations of the protein present in a dissociated form. The bands assigned to the parent α1, β1 and γ1 chains showed increased smearing with higher oxidant exposure, with this being ascribed to the presence of multiple proteins conformations, and/or modest changes in molecular mass, arising from oxidation. A weak band was also detected at low molecular mass on the reducing gels (Fig. 9B,D), with the staining intensity of this band increasing with increasing oxidant exposure for both the HOCl and enzyme systems. The origin of this material is unclear, though it may be a protein fragment induced by oxidation that is only released and detected under reducing conditions, possibly as a result of disulfide bond cleavage.

Immunoblotting of samples separated under reducing conditions with the rat anti-murine laminin antibody (clone AL-2), provided evidence, for the HOCl system (Fig. 9E), for reduced recognition of the α1 chain on increasing HOCl exposure. The β1 and γ1 chains did not give rise to significant signals, possibly as a result of the preferential recognition of α1 chain epitopes by this antibody. Corresponding blots were not carried out on samples separated under non-reducing conditions due to the low intensity of the bands from the dissociated chains, and the poor migration of the trimeric protein. Immunoblotting experiments with the 2D10G9 antibody did not provide usable data, consistent with previous experience with this antibody and gels (Chuang et al., unpublished data).

3.10. In vivo evidence for laminin modification by the MPO/H$_2$O$_2$/Cl$^-$ system

As laminins are major proteins of the BM that underlies the endothelial cells of the artery wall, and the evidence for HOCl-mediated damage during the development of atherosclerosis [15,26,35,36], we sought evidence for the presence of laminin, and HOCl-mediated damage to laminin, in human atherosclerotic lesions. Examination of frozen sections of type II-IV human lesions demonstrated the presence of high levels of laminin detected by a monoclonal antibody that recognises laminin γ1 in the BM underlying the endothelial cells (Fig. 10A–C, left panel) and also in the BM of smaller vasa vasorum in deeper regions of the tissue (Fig. 10C, left panel). When the same tissue sections were stained for HOCl-generated epitopes, strong fluorescence was detected in similar regions (Fig. 10A–C, middle panel). In addition to staining near the surface of the aortae, consistent with the BM, areas of the vasa vasorum also showed pronounced positivity for HOCl-modified epitopes (Fig. 10A–C, middle panel). Merging of images from Fig. 10A–C (left and middle panel) reveal intense staining for laminin and HOCl-modified epitopes in close proximity with partial colocalization within the BM underneath the endothelial layers of the aortae (Fig. 10A–C, right panel) as well as the endothelial layers of vasa vasorum (Fig. 10C, right panel). Control experiments in which the primary antibodies were omitted, or experiments using non-immune mouse or rabbit IgG, showed no artefactual staining (data not shown).
Fig. 5. Fragment ion spectra of the peptide $^{2081}_{\text{DMEMQANLLLD}}^{2092}$ from murine laminin-111 for: (A) the native peptide, (B), peptide with mono-oxygenation (sulfoxide formation) at Met$^{2082}$, and (C) peptide with mono-oxygenation (sulfoxide formation) at Met$^{2084}$. Data from murine laminin-111 (1.18 μM) treated with 0, 100, and 500 μM HOCI. Annotated MS/MS spectra and fragment ion tables for all identified peptides are available in Supplementary Fig. 4.

Fig. 6. Concentration dependence of modifications at Met residues in the peptides: (A) $^{2081}_{\text{DMEMQANLLLD}}^{2092}$ and (B) $^{1476}_{\text{KQDDADQDDMGMASQQEAELNAR}}^{1502}$. In (A), the Met residues at position 2084 and 2082 appear to be modified sequentially and in this order. In (B), modification occurs at each of the 4 Met residues with little apparent site specificity at any of the Met residues. With increasing HOCI exposure all 4 Met residues become oxidised. Data from murine laminin-111 (1.18 μM) treated with 0, 100, and 500 μM HOCI as indicated.
Evidence has also been presented for ECMzyme would target matrix materials. Considerable data support thisinflammation, such as peroxynitrous acid, and for the presence ofdamage by other oxidants associated with both normal metabolism andvascular diseases, and predictive of adverse outcomes (e.g. [28,30,70]).plasma/serum levels of MPO can be both diagnostic of major cardioand sudden cardiac death. It has been shown that blood orstroke, and modified laminin-111 in human atherosclerotic lesions. The data obtained indicate that some sites on laminin-111 are more susceptible to modification than others, as judged by both the sitespecificity and extent of modification as determined by RSO. Treatment with reagent HOCl resulted in the detection, by MS mass mapping, of oxidation at 30 Met and 7 Trp residues, and chlorination of 33 Tyr residues (with 3 Tyr also detected as 3,5-Cl2Tyr), out of the 56 Met, 24 Trp and 99 Tyr residues present in the native protein. Treatment of the isolated laminin-111 with the MPO/H2O2/Cl- system, supplied with 500 μM H2O2 to give ~500 μM HOCl, showed some similarities, and also some significant differences, with the regard to the both the sites and extents of modification. This may be due to an association or binding of MPO to particular sequences within the trimeric laminin structure. A recent study, using an identical MS approach, quantified modifications induced by reagent HOCl, versus MPO/H2O2/Cl- on human plasma fibronectin, showed an overall decrease in the number of sites, and extent of modification, [48]. In contrast, for laminin, a significant increase in the number of sites, and a greater extent of modification were observed with MPO/H2O2/Cl- compared to HOCl. Thus, an additional 8 Met and 10 Trp oxidation sites, and 14 3-ClTyr, and 18 3,3′,5,5′-tetramethylbenzidine [48]). This increase in number and extent of modifications is surprising, as MPO protein might be expected to act as an alternative target for the generated oxidant, and therefore diminish the oxidant concentration available for reaction with the laminin. It is unclear, at present, why there are these differences. It ispossible that interactions between MPO and the ECM proteins, results ina higher concentration or efficiency of oxidant formation in the case of association with laminin, and/or that association of MPO with fibronectin inhibits MPO activity and thereby decreases the extent of fibronectin modification (cf. the inhibition, via complex formation of plasma ceruloplasmin on MPO activity [73,74]).Both the MS and amino acid analyses indicate that Met residues are...
a major target for HOCl with this protein; this is in accord with the high rate constant for reaction of HOCl with this side-chain ($k = 3 \times 10^7$ to $1 \times 10^8$ M$^{-1}$ s$^{-1}$) [23, 75]. Cys and cystine are also highly reactive with HOCl [23, 75, 76], with $k$ for Cys (typically) higher than for Met [75]. However, there is a very low abundance of Cys (but a moderate number of cystine) residues in laminin, and other ECM proteins in general, suggesting that these do not consume a significant amount of the HOCl provided. This does not however preclude reaction at the few Cys residues being functionally important. The rate constants, $k$, for reaction of HOCl with disulfides vary significantly with their environment (e.g. presence of neighboring groups) and conformation [76], suggesting that the cystine groups present in the laminin EGF-like (EL) domains of laminin may be significant targets. Reaction at such sites is however difficult to detect and quantify due to the cross-linked nature of these structures, and the destruction of these modifications during the standard “reduce and alkylate” protocols of most MS mass mapping approaches. New approaches are therefore needed to test this suggestion.

The quantitative data obtained from the amino acid analyses indicate that ~ 15% of the HOCl generated by the enzyme system, is consumed by Met residues. 3-ClTyr formation accounts for an additional ~ 0.2%, with an additional (not quantified) small amount used to convert 3-ClTyr to 3,5-diClTyr. A similar analysis was not carried out for Trp. These data, together with that from the MS mass mapping, indicate that the products detected account for only a modest amount of the HOCl added or generated. A significant proportion of the “missing” HOCl is likely to have reacted with Lys, His (and possibly Arg) residues, as these are important kinetic targets [23, 24], and highly abundant in laminin-111 (cf. 316 Lys, 161 His residues; data from Uniprot entries P19137, P02469, P02468). Reaction at these residues gives unstable chloramines (RNHCl species, and dichloramines, RNCl$_2$, at higher oxidant concentrations) that decay over minutes-hours. Decomposition of these species yields multiple materials, including regeneration of the parent amino acid, radicals [77, 78], carbonyl compounds [77, 79–81],

Fig. 9. Structural changes induced on laminin-111 and individual laminin chains induced by reagent HOCl (panels A, B) or a MPO/H$_2$O$_2$/Cl$^-$ system (panels C, D) detected by SDS-PAGE run under non-reducing (panels A, C) or reducing conditions (panels B, D) with detection of protein bands by silver staining. Laminin-111 (2.5 μg in 50 μL) was treated with reagent HOCl at the indicated molar excesses (panels A, B) or for panels (C, D) with a MPO (100 nM)/H$_2$O$_2$ (at the indicated molar excesses, added in multiple aliquots)/Cl$^-$ (100 mM) system for 2 h at 37 °C in 100 mM PBS, pH 7.4, before analysis. In panels A–D, the lane labelled “C” is control, non-oxidised protein. In panels C, D, the lane labelled “MPO-” is the complete reaction system without MPO (i.e. laminin incubated with H$_2$O$_2$ alone). The extreme left lane (and extreme right lane for panels A, B) on each gel contains molecular mass markers for reduced proteins of the indicated masses. Red arrows indicate the bands assigned to the α1 chains, and the blue arrows indicate bands assigned to the β1 and γ1 chains that have very similar masses. Panel E: Reaction system as panel B (i.e. treatment of laminin-111 with reagent HOCl at the indicated molar excesses, and SDS-PAGE separation run under reducing conditions) except the resulting gel was blotted and probed for recognition of laminin chains using a rat anti-murine laminin monoclonal antibody (clone AL-2) and subsequent chemiluminescence detection (for details see Materials and methods). The major band detected is from the α1 chain. Representative gels and blots are presented, typical of 3 independent experiments.
carboxylic acids [82], and nitriles [82]. This complexity makes quantification complex [77–79].

Mapping of the detected modifications onto a complete 3D structure cannot be accomplished at present, as only a small number of partial structures are available. A recently structure (PDB entry: 5MC9) [83] contains the coordinates of the xLG1–3 domains, as well as ~50 residues of the coiled coil (α1β1γ1). These sequences contain 20 Tyr, of which 11 were identified as chlorination targets, 14 Met, of which 7 were detected as sulfoxides, and 4 Trp, with 3 of these detected as oxidised species (Fig. 11A, Supplementary Fig. 8). While most of these modified residues appear to be surface exposed, Tyr 2138, Tyr2147, Trp2293, Tyr2295, and Met2554, which were detected as modified species with both HOCl and MPO/H2O2/Cl-, appear to be buried within the structure; this is also true for Met2179, which was modified solely by the enzyme system. These data indicate either that (neutral) HOCl can penetrate and react within the interior of a protein, or that the crystal structure represents a “snap shot” of a system with considerable flexibility. The detection of modifications to (surface-exposed) Tyr2415 is of particular interest as it is situated close to Glu1605 (indicated in brown, in Fig. 11, panel A), which is essential for interactions with integrins [83–85]. Modification of this residue, and possibly others nearby, may therefore affect integrin binding to laminins, and hence cell binding to the ECM [4,85].

Similar analyses (Fig. 11, panels B-E) have been carried out using the structures of the short arms of the β1 and γ1 chains (PDB entries: 4AQS and 4AQT [86]), which contain both the N-terminal (LN) and part of the first EL-like domains. As little sequence coverage (and hence no modifications) was obtained for the LE domains due to the presence of disulfide bonds (cf. Fig. 1), the renderings of these structures are displayed both with the LE domains included, and with these greyed out (Fig. 11B-E). The β1 structure contains 12 Tyr, 5 Met, and 4 Trp residues, with 3-ClTyr formation detected at 3 Tyr (Tyr-220, Tyr-259, Tyr 263) and sulfoxide formation at 3 Met (Met-148, Met-182, Met-265). The γ1 structures contains 13 Tyr, 2 Met, and 4 Trp, with 4 of the Tyr residues detected as 3-ClTyr (Tyr-145, Tyr-230, Tyr-272, Tyr-273). Interestingly, the sites that were most readily modified are, in general, not those that are most surface-exposed. The majority of the modifications detected are present in the highly conserved portions of both structures that appear to be involved in binding to other chains [86]. Laminin polymerisation has been proposed to occur, at least in vitro, via a two-step process initiated by association of the β and γ chains through the LN-terminal domains, followed by association with the α chains [87], so these data suggest that the ECM assembly may be affected by modifications in the LN domains.

The LE domains contain a large number of Tyr and Trp residues, which may be potential modification targets overlooked by the current method. The LE domains are highly preserved structural elements present in a wide array of extracellular proteins [88,89], and are known to mediate in the case of laminin γ1, binding to other BM materials such as nidogen [90]. Modification to the LE domains may therefore have functional consequences and stresses the need for efficient methods to analyse disulfide-rich peptides.
The potential functional importance of these modifications is indicated by enhanced recognition of modified laminin-111 by the 2D10G9 antibody that recognises HOCl-mediated damage (Fig. 9), and the detection of material recognised by this antibody in human type II-IV atherosclerotic lesions (Fig. 10). The close co-localization of epitopes recognised by 2D10G9 with those recognised by a laminin antibody in human lesion material provides strong support for the presence of HOCl-modified laminin and other extracellular matrix materials in this major human disease. Whether this is a contributing factor to the disease or merely a consequence of ongoing oxidation within the artery wall remains to be established, however it is well established that endothelial cell dysfunction and denudation are key early events in the development of atherosclerosis, and it is possible that modifications to the integrin binding site, as detected here, may be a key factor in the known loss of endothelial cell adherence.
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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.redox.2018.10.022.

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