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Increased aldehyde-modification of collagen type IV in symptomatic plaques– a possible cause of endothelial dysfunction

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Abstract

Objective

Subendothelial LDL-adhesion and its subsequent oxidation are considered as key events in the development of atherosclerotic lesions. During oxidation of LDL, reactive aldehydes such as malondialdehyde (MDA) are formed, which modify apolipoprotein B100. However, the possibility that these reactive aldehydes could leak out of the LDL-particle and modify surrounding extracellular matrix proteins has been largely unexplored. We have investigated if aldehyde-modification of collagen type IV, one of the major basement membrane components, in plaques is associated with cardiovascular events.

Methods

The amount of MDA-modified collagen type IV and native collagen type IV were determined in homogenates from 155 carotid artery lesions, removed by endarterectomy from patients with or without previous cerebrovascular events.

Results

Plaque MDA-collagen type IV, but not native collagen type IV, correlated with oxidized LDL ($r=0.31$, $P<0.001$) and lipoprotein-associated phospholipase A2 ($r=0.44$, $P<0.001$). MDA-collagen type IV was increased in lesions from symptomatic patients compared to lesions from asymptomatic patients. Autoantibodies against MDA-collagen type IV in plasma correlated with the amount of MDA-collagen type IV in lesions. MDA-modification of collagen type IV decreased endothelial cell attachment. In addition, culture of endothelial cells with MDA-modified collagen type IV increased vascular cell adhesion molecule expression and reduced the anti-coagulant proteins thrombomodulin and endothelial protein C receptor. In the lesions native collagen type IV, but not MDA-collagen type IV, was positively associated with thrombomodulin.

Conclusion

The present observations imply that aldehyde-modification of collagen type IV, associated with LDL oxidation, in atherosclerotic plaques may cause endothelial dysfunction and increase the risk of clinical events.

Introduction

During atherosclerosis LDL-particles diffuse into the vessel wall, where they bind to subendothelial extracellular matrix such as proteoglycans, collagens and fibronectin.^{1, 2} Retention, aggregation and subsequent oxidation of LDL in the vessel wall are considered as key events in the development of atherosclerotic lesions.³ Accordingly, it has been shown that mice expressing proteoglycan-binding-defective LDL develop less atherosclerosis.⁴ During oxidation of LDL, reactive aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) are formed, which modify amino groups in arginine, lysine or histidine residues in apolipoprotein B100, the protein component of LDL.⁵ However, the possibility that these reactive aldehydes could leak out of the LDL-particle and modify surrounding extracellular matrix proteins has been largely unexplored.

Since oxidized LDL is known to induce immune responses associated with atherosclerotic disease,⁶ we hypothesized that modification of matrix proteins, induced by reactive aldehydes, would result in auto-antibodies directed against these modified matrix components. Indeed, by screening human plasma we found both IgM and IgG binding to several MDA-modified extracellular matrix proteins including collagen type I, collagen type III, fibronectin, and the basement membrane protein laminin.^{7, 8} We also showed that MDA can leak out of the LDL-particle and modify extracellular matrix proteins *in vitro*.⁷ In addition, by using in-house produced antibodies recognizing different MDA-modified proteins, we have identified both MDA-modified fibronectin and MDA-modified laminin in atherosclerotic lesions.^{7, 8}

Although previous studies identified antibodies against different modified matrix proteins, the protein levels in atherosclerotic lesions and their relation to plaque stability and

cardiovascular events have not been investigated. Modification of basement membrane components due to nearby lipid oxidation may result in decreased endothelial cell attachment, which will promote cardiovascular events.⁹ Moreover, disturbance of the basement membrane-endothelial cell interaction may lead to endothelial dysfunction. In this paper we measured the amount of MDA-modified collagen IV, the major collagen in basement membranes, in homogenates from 155 carotid artery lesions, removed by endarterectomy from patients with and without previous cerebrovascular symptoms. We found that MDA-modified collagen type IV was significantly associated to oxidized LDL in the lesions and that the amount of MDA-modified collagen type IV was increased in symptomatic lesions. In addition, MDA-modification of collagen type IV decreased endothelial cell attachment, and increased thrombotic and inflammatory properties of endothelial cells, supporting a role for MDA-modification of collagen type IV in endothelial cell dysfunction.

Methods

Patients

Carotid plaques from 155 patients who underwent carotid endarterectomy (87 symptomatic patients who suffered stroke, transient ischemic attack or amaurosis fugax within a month before operation and 68 from asymptomatic patients) were homogenized, as previously described.¹⁰ Informed consent was given by each patient. The study was approved by the local ethical committee.

Sample preparation and histology

Plaques were snap frozen in liquid nitrogen and a one mm fragment from the most stenotic part was taken for histology. The fragments were embedded in O.C.T. compound (Tissue-Tek, Sakura), cryo-sectioned in serial 8- μ m sections, and mounted on coated slides (Superfrost plus). Transversal histological sections were stained with oil red O (for lipids) and with antibodies recognizing CD68 (macrophages), mouse monoclonal anti-human collagen type IV (abcam ab6311), IgY recognizing MDA-collagen type IV or control IgY from the preimmunized hen.

ELISA for measuring native and MDA-modified collagen type IV

The amount of collagen IV and MDA-modified collagen IV in carotid plaque homogenates were detected by ELISA. Twenty μ g/mL plaque proteins in PBS of the respective homogenized plaques were coated on microtiter plates (Nunc MaxiSorp) at 4°C overnight. Unspecific binding were blocked by incubation with Superblock (Thermo Scientific) for 30 minutes at room temperature. The amount of collagen IV was measured by rabbit anti human collagen IV (AbD Serotec) followed by horse radish peroxidase (HRP)-conjugated anti rabbit IgG (Sigma). The amount of MDA-modified collagen IV was measured by chicken anti

MDA-modified collagen IV (described above) followed by HRP-conjugated anti IgY antibody (Sigma). Detection of bound antibodies were developed using TMB substrate kit (Thermo Scientific). All the samples were analyzed at one occasion.

Statistical analysis

Statistical analysis was performed with PASW 18.0 (SPSS) or Graphpad Prism. Native or MDA-collagen type IV in lesions from symptomatic versus non-symptomatic were log-transformed before analysis by independent sample t-test. Correlation analyses were performed using Spearman's rank test. Differences in endothelial cell adhesion and expression were analyzed by unpaired two-tailed t-test. * $p < 0.05$, *** $p < 0.001$

Results

MDA-collagen type IV is present adjacent to endothelial cells and in shoulder regions of lesions

Collagen type IV is one of the major proteins present in the basement membrane underlying the endothelial cells. Since LDL-aggregation in the development of atherosclerotic lesions occurs by binding to subendothelial matrix proteoglycans, we asked whether LDL-oxidation would result in MDA-modifications of proteins present in the closely situated basement membrane. In addition, the identification of auto-antibodies against the MDA-modified basement membrane protein laminin⁸ implied presence of MDA-modified basement membrane components. Thus we stained cross-sections of human carotid lesions with an antibody recognizing MDA-modified collagen type IV produced in hen, a pre-immunization control IgY, anti-collagen type IV, anti-CD68 recognizing macrophages and oil red O staining lipids. MDA-collagen type IV was found adjacent to the endothelial cells, but also in parts of the core of the lesion (Fig. 1A-B). In addition, MDA-modified collagen type IV was present in the fibrous cap and in shoulder regions with macrophage infiltration (Fig. 1C-D), but also in regions devoid of inflammatory cells. In accordance with the notion that MDA-modified matrix proteins are induced by LDL-oxidation, we found MDA-modified collagen type IV in lipid-rich regions. MDA-collagen type IV staining also to a large extent colocalized with native collagen type IV staining.

MDA-collagen type IV is increased in atherosclerotic lesions from symptomatic patients

MDA-modification of collagen type IV could impair the interaction of endothelial cells as well as decrease the stability of the basement membrane and the fibrous cap, and thus influence plaque stability. To determine if MDA-modified collagen type IV is altered in lesions from symptomatic patients, we measured both native and MDA-modified collagen

type IV in homogenates from carotid lesions removed by endarterectomy from 88 symptomatic patients and from 67 asymptomatic patients (Supplementary table 1). The amount of MDA-collagen type IV was increased in homogenates from plaques of symptomatic patients compared to those from asymptomatic patients, whereas the amount of native collagen type IV did not differ (Fig. 2). MDA-collagen type IV did not differ significantly in lesions from diabetic patients compared to lesions from non-diabetic patients (73 (56-97) vs 78 (61-112), n.s.), whereas native collagen type IV was lower in lesions from diabetic patients compared to non-diabetic patients (79 (60-99) vs (85 (70-124), $p < 0.05$).

MDA-collagen type IV was negatively associated with estimated glomerular filtration rate (eGFR) ($r = -0.17$, $p < 0.05$) of the patients. There were no significant associations between native collagen type IV and eGFR.

MDA-collagen type IV is associated with LDL-oxidation and lipoprotein-associated phospholipase A2 in atherosclerotic lesions

Our previous *in vitro* experiments showed that extracellular matrix proteins are MDA-modified via reactive aldehydes formed during LDL-oxidation.⁷ Thus we tested if MDA-modified collagen type IV was associated with LDL-oxidation in atherosclerotic lesions. Indeed we found that the amount of MDA-modified collagen type IV was associated to oxidized LDL measured by ELISA in plaque homogenates and to lipoprotein-associated phospholipase A2 (Lp-PLA2) (Table 1, Supplementary figure 2), which hydrolyzes oxidized phospholipids in oxidized LDL. Moreover, MDA-collagen type IV was associated to lipids measured by oil red O stainings of cross-sections of the carotid lesions. However, there were no associations between the amount of native collagen type IV and oxidized LDL, Lp-PLA2 or area stained for lipids (Table 1, Supplementary figure 2).

MDA-collagen type IV is associated with macrophages and inflammatory chemokines in atherosclerotic lesions

Since retention and subsequent oxidation of LDL in the vessel wall induce an inflammatory response and recruitment of monocytes to the lesion and immunohistochemistry indicated that MDA-collagen IV was localized in inflammatory regions, we tested whether MDA- or native collagen IV was associated with macrophages as well as inflammatory cytokines and chemokines in atherosclerotic lesions. The amount of MDA-collagen type IV was positively associated with macrophages in the lesions, as determined by immunostaining against the macrophage marker CD68 in cross-sections, whereas there was no significant association between native collagen type IV and macrophage content (Table 2, Supplementary figure 3). In addition, MDA-modified collagen type IV, but not native collagen type IV, was significantly associated with the amount of monocyte-chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-1 β (MIP-1 β) in the lesions. Next we analyzed if MDA-collagen type IV was associated with inflammatory cytokines, and found that it was associated with the amount of interleukin (IL)-6 and tumor necrosis factor α (TNF α) in the lesions (Table 2, Supplementary figure 3). Native collagen type IV was associated with the amount of TNF α and with IL-6, but the latter to a lesser degree than MDA-collagen type IV. There was no significant association between native or MDA-collagen type IV and IL-1 β .

Oxidized LDL was associated with both MCP-1 ($r=0.30$, $P<0.001$) and MIP-1 β ($r=0.33$, $P<0.0001$) as well as with TNF α ($r=0.42$, $P<10^{-6}$). In addition a strong and highly significant association was seen between oxidized LDL and the amount of LpPLA2 ($r=0.50$, $P<10^{-9}$). There was no significant difference in oxidized LDL content between lesions from symptomatic and asymptomatic patients.

MDA-modification of collagen type IV results in decreased endothelial cell attachment

Our results of increased levels of MDA-collagen type IV in lesions from symptomatic patients indicate that MDA-modification of collagen type IV influences plaque stability and risk for cardiovascular events. To investigate if MDA-modification of collagen type IV could have a direct effect on endothelial cell behavior promoting cardiovascular events, we performed cell attachment experiments. Fluorescently-labeled HUVECs were allowed to adhere to native and MDA-collagen type IV for 45 minutes and attached cells were then quantified. MDA-modification of collagen type IV was shown to decrease endothelial cell attachment by 34% (Fig. 3A).

MDA-modification of collagen type IV results in decreased anti-coagulant properties and increased VCAM-1 of endothelial cells

Endothelial cell dysfunction is believed to promote cardiovascular events by increased inflammation and decreased anti-thrombotic properties.¹¹ Since the interaction of collagen type IV to endothelial cells is important for endothelial cell function,¹² we cultured HUVECs on either native or MDA-modified collagen type IV and measured anti-coagulant and inflammatory proteins on the cell surface by flow cytometry. Thrombomodulin on the endothelial cell surface catalyzes the conversion of protein C into active protein C by thrombin. This is facilitated by endothelial protein C receptor (EPCR) also present on the endothelial cell surface. Active protein C then will then inactivate cofactor Va and VIIa, which shuts down the coagulation cascade.¹³ Both thrombomodulin and EPCR were decreased on thrombin stimulated endothelial cells situated on MDA-collagen type IV compared to native collagen type IV (Fig. 3B-C) indicating reduced anti-coagulant

properties. Moreover, stimulation of endothelial cells with thrombin and MDA-modified collagen type IV resulted in increased VCAM expression on the cell surface (Fig. 3D).

To test whether native or MDA-modified collagen type IV were related to anticoagulant markers of atherosclerotic lesions, we measured thrombomodulin levels in human plaques. In accordance with *in vitro* data, native collagen type IV showed a strong positive association to thrombomodulin ($r=0.29$, $p<0.001$), whereas there was no significant association between MDA-collagen type IV and thrombomodulin.

Plasma IgM and IgG against MDA-collagen type IV are associated to MDA-collagen IV in lesions

Previously, we have found antibodies directed against several MDA-modified extracellular matrix proteins.^{7,8} To test whether antibodies against MDA-modified collagen type IV were associated to the amount of MDA-modified collagen type IV in the lesions, we measured the amount of IgM and IgG against MDA-modified collagen type IV in plasma from the same patients from whom the carotid lesions were removed. Indeed, both IgM and IgG were positively associated with the amount of MDA-modified collagen type IV, but not with native collagen type IV (Supplementary table 2). IgM against MDA-collagen type IV was negatively associated to the number of days between symptoms and operation ($r=-0.30$, $p=0.006$). Additionally, IgM against MDA-collagen type IV was significantly lower in patients who were operated later than 14 days after symptoms compared to patients operated within 14 days after symptoms (24 ± 43.3 AU vs 40.1 ± 57.4 AU; median \pm interquartile range, $p<0.05$). There was no significant difference in either IgM or IgG against MDA-collagen type IV in lesions from symptomatic patients compared to lesions from asymptomatic patients.

Discussion

During LDL-oxidation the reactive aldehydes MDA and 4-HNE are formed, which modify apolipoprotein B100 in the LDL-particle. We have previously shown that these reactive aldehydes leak out of the LDL-particle and modify surrounding extracellular matrix proteins *in vitro*.⁷ In this study we analyzed the amount of MDA-modified and native collagen type IV in carotid plaque homogenates from symptomatic and asymptomatic patients. We found that the amount of MDA-collagen type IV, but not native collagen type IV, was increased in lesions from symptomatic patients compared to asymptomatic patients. Moreover, the amount of MDA-collagen IV was associated to oxidized LDL, Lp-PLA2, inflammatory cytokines/chemokines and macrophages in lesions. In addition, both IgM and IgG against MDA-collagen type IV in plasma were associated to the amount of MDA-modified collagen type IV in lesions.

Collagen type IV interacts with the other basement membrane proteins perlecan, nidogen, BM-40 and to a lesser extent with laminin forming an intricate network, which is important for the stability of the basement membrane.^{14, 15} In addition, collagen type IV has been shown to be present in the intima of fatty streaks and in advanced lesions.¹⁶ Collagen type IV interaction to endothelial cells is important for maintaining endothelial cell function.¹² This interaction is mediated by integrins on vascular endothelial cells via sequences containing arginine residues,^{17, 18} one of the major amino acids targeted by MDA. Interestingly, Dobler et al found that methylglyoxal, which is increased upon hyperglycemia, results in formation of hydroimidazolone residues in the integrin binding sites of collagen type IV causing endothelial cell detachment.¹⁹ Our data showing localization of MDA-collagen type IV adjacent to endothelial cells in combination with reduced cell attachment implies that MDA-modification of collagen type IV could increase the risk for plaque erosions. In addition, the

decreased expression of anti-coagulant proteins thrombomodulin and EPCR as well as the increase in VCAM-1 of endothelial cells on MDA-collagen type IV indicates endothelial dysfunction, which would further promote cardiovascular events. This was supported by *in vivo* data displaying a positive association of thrombomodulin to native collagen type IV, but not to MDA-modified collagen type IV, in the lesions.

Whether collagen type IV directly interacts with LDL and thereby is exposed to reactive aldehydes, or if this exposure is due to an indirect interaction between collagen type IV and other extracellular matrix proteins/proteoglycans binding LDL, remains to be clarified. It has been shown that collagen type IV interacts both with native and oxidized LDL.^{20, 21} However, the collagen-LDL/oxLDL-interaction is weak and mediated via lysine, arginine and histidine residues. Thus, at least part of the amino acids which are susceptible to modification interact directly with LDL/oxLDL, suggesting that the proximity to oxidizing LDL mainly is mediated via interaction with other matrix components. Interestingly, the heparan sulfate chains of the basement membrane proteoglycan perlecan promote atherosclerosis and play a role in lipid retention.²² Since, collagen type IV has been shown to bind the heparan sulfate chains of perlecan,²³ this interaction could mediate a co-localization of collagen type IV and LDL, resulting in aldehyde modification of collagen type IV. The localization of native collagen type IV demonstrate that collagen type IV is not only present in the basement membrane, but also in the fibrous cap,²⁴ and could thus be modified by oxidized LDL bound to adjacent intimal proteoglycans. Importantly, the highly significant associations of MDA-modified collagen type IV with both oxidized LDL and with Lp-PLA2 support the hypothesis that oxidation of LDL results in aldehyde modification of collagen type IV.

The idea that reactive aldehydes formed during LDL oxidation could leak into the vessel wall and modify nearby extracellular matrix proteins has not been much investigated. However, Takebayashi and coworkers have studied the possibility that increased lipid peroxidation of elastin and collagen could contribute to atherogenesis in hemodialysis patients.^{25, 26} In agreement with those studies we see a negative correlation with MDA-collagen type IV to eGFR, indicating that increased MDA-collagen type IV is associated with decreased kidney function. In addition, Slatter et al have investigated the reactions of MDA with collagen type I. Since MDA has bifunctional aldehydic properties, it has the potential to crosslink collagen molecules and thereby stiffen the collagen fibers.²⁷ Accordingly, MDA has been proposed to be responsible for the increased arterial stiffening seen in diabetic patients.²⁸ However, in our study there was no significant difference between MDA-collagen type IV levels in lesions from diabetic patients compared to non-diabetic patients. In addition to these studies, we have previously shown that LDL-oxidation induces MDA-modification of fibronectin *in vitro*.⁷

A limitation of this study is that the evidence that MDA-modification of collagen type IV induces endothelial dysfunction is provided by *in vitro* studies. Correlation of MDA-modified collagen type IV with measurements of endothelial cell dysfunction of the patients would possibly indicate an *in vivo* relation. However, clinically endothelial cell dysfunction is measured by stimuli that increase endothelium-derived nitric oxide production resulting in endothelium-dependent vasodilation. Thus, this measurement may not reflect increased endothelial cell inflammation and decreased anti-coagulant properties. Furthermore, the positive association between native collagen type IV and thrombomodulin in the lesions supports *in vitro* data showing that endothelial cells on native collagen type IV express increased thrombomodulin. This indicates that collagen type IV needs to be in its native form to maintain anticoagulant properties of endothelial cells.

Conclusion

To our knowledge this is the first study that investigates the association of MDA-modification of collagen with symptomatic lesions and oxidized LDL content. We found that the amount of MDA-collagen type IV is increased in lesions from symptomatic patients and shows a significant association with the amount of oxidized LDL and Lp-PLA2 in atherosclerotic lesions. Our results support the notion that LDL oxidation in atherosclerotic lesions induces MDA-modification of surrounding extracellular matrix proteins. Since collagen type IV is involved in interactions to other extracellular matrix proteins as well as to integrins on endothelial cells, MDA-modification of collagen type IV could contribute to cardiovascular events via impaired endothelial cell attachment and by inducing endothelial cell dysfunction.

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Disclosures: None

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Figure legends

Figure 1

MDA-collagen type IV is present adjacent to endothelial cells and is located in lipid- and inflammatory-rich regions of human plaques.

A-C, Human carotid lesions were stained for lipids (oil red O), macrophages (α -CD68), native collagen type IV (α -Coll IV), IgY against MDA-collagen type IV and IgY control. A, MDA-collagen type IV is present adjacent to endothelial cells in fibrous lesions with thrombosis, but also in parts of the lipid-rich core (arrow). B, High magnification of the boxed area in (A). Thrombus is marked with an *. C, MDA-collagen type IV is present in lipid- and macrophage-rich shoulder regions (*) of the fibrous cap. D, High magnification of the boxed area in (C). Scale bar=2000 μ m (A), scale bar=500 μ m (B, C) and scale bar=250 μ m (D).

2 column color figure

Figure 2

MDA-collagen type IV is increased in lesions from symptomatic patients.

A-B, The amount MDA-collagen type IV (A), but not native collagen type IV (B), is significantly increased in carotid lesions from symptomatic patients compared to asymptomatic patients. Values are presented as mean with 95% confidence interval.

2 column black and white figure

Figure 3

MDA modification of collagen type IV impairs endothelial cell adhesion and induces endothelial dysfunction.

A, Fluorescently-labeled HUVECs were allowed to adhere to native or MDA-collagen type IV for 45 minutes, where after unbound cells were removed by washing and bound cells were measured using an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

B-D, Thrombin stimulated HUVECs were cultured with native or MDA-modified collagen type IV, and the anti-coagulant proteins thrombomodulin (B) and EPCR (C) and the leucocyte attractant VCAM-1 (D) were measured by flow cytometry. Values are presented as individual values and mean \pm SEM are indicated.

2 column black and white figure

Tables

Table 1

Correlation of native and MDA-collagen type IV to lipid, oxidized LDL and Lp-PLA2

	native collagen type IV	MDA collagen type IV
Oil red O	NS	$r=0.18^*$
Oxidized LDL	NS	$r=0.31^{***}$
LpPLA2	NS	$r=0.44^{***}$

* $P<0.05$, *** $P<0.001$, NS not significant

Table 2

Correlation of native and MDA-modified collagen type IV to macrophages and inflammatory chemokines and cytokines

	native collagen type IV	MDA collagen type IV
CD68	NS	$r=0.16^*$
MCP-1	NS	$r=0.26^{**}$
MIP-1 β	NS	$r=0.23^{**}$
IL-6	$r=0.17^*$	$r=0.36^{***}$
TNF α	$r=0.29^{**}$	$r=0.25^{**}$

* $P<0.05$, ** $P<0.01$, *** $P<0.001$, NS not significant

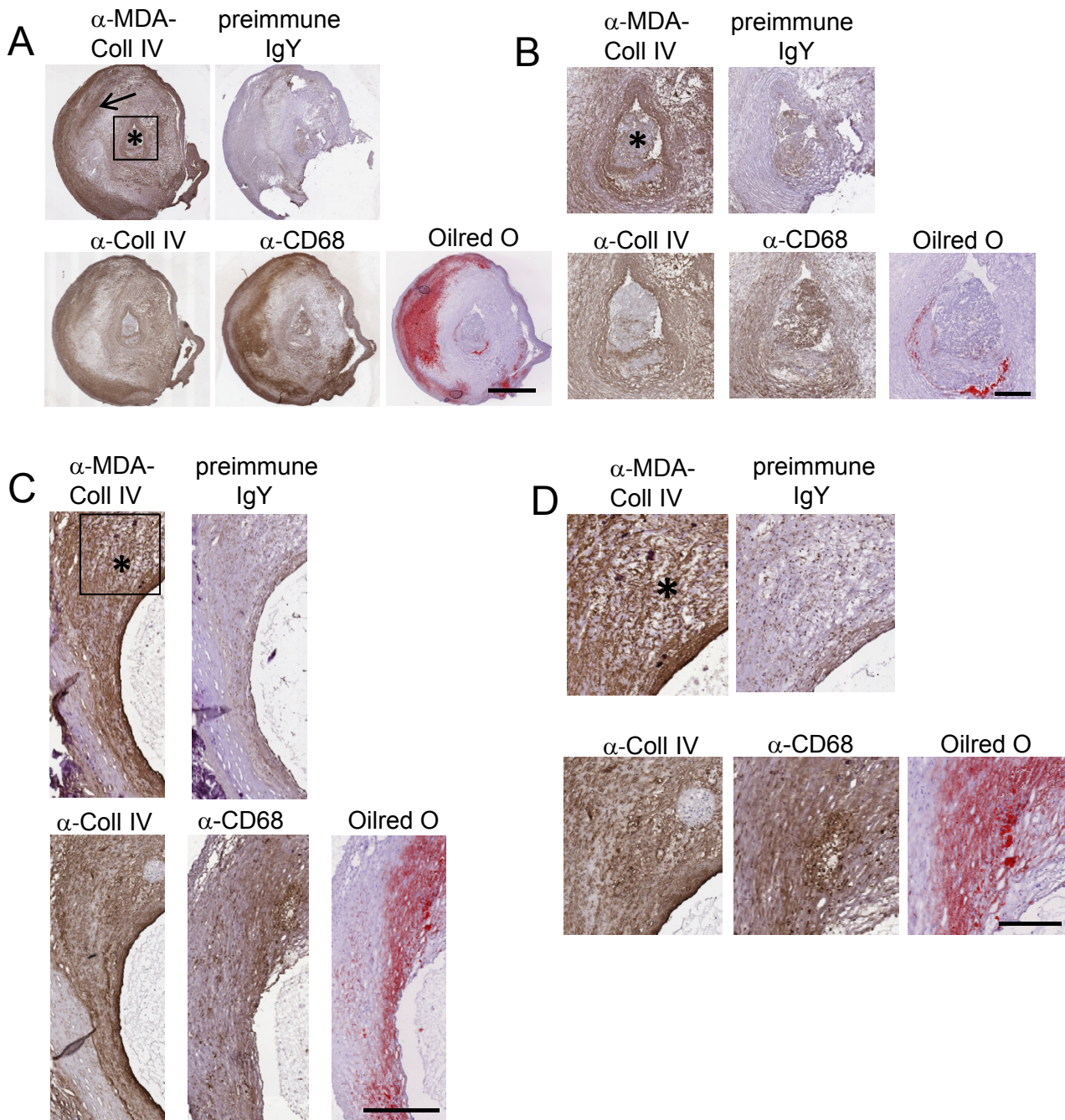


Figure 1

MDA-collagen type IV is present adjacent to endothelial cells and is located in lipid- and inflammatory-rich regions of human plaques.

A-C, Human carotid lesions were stained for lipids (oil red O), macrophages (α -CD68), native collagen type IV (α -Coll IV), IgY against MDA-collagen type IV and IgY control. **A**, MDA-collagen type IV is present adjacent to endothelial cells in fibrous lesions with thrombosis, but also in parts of the lipid-rich core (arrow). **B**, High magnification of the boxed area in (A). Thrombus is marked with an *. **C**, MDA-collagen type IV is present in lipid- and macrophage-rich shoulder regions (*) of the fibrous cap. **D**, High magnification of the boxed area in (C). Scale bar=2000 μ m (A), scale bar=500 μ m (B, C) and scale bar=250 μ m (D).

2 column color figure

Figure 1

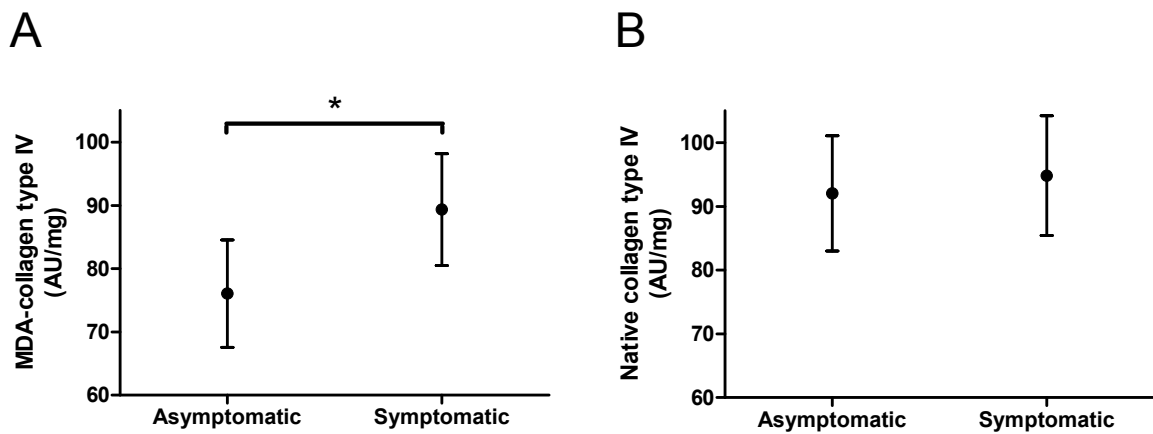


Figure 2

MDA-collagen type IV is increased in lesions from symptomatic patients.

A-B, The amount MDA-collagen type IV (**A**), but not native collagen type IV (**B**), is significantly increased in carotid lesions from symptomatic patients compared to asymptomatic patients. Values are presented as mean with 95% confidence interval.

2 column black and white figure

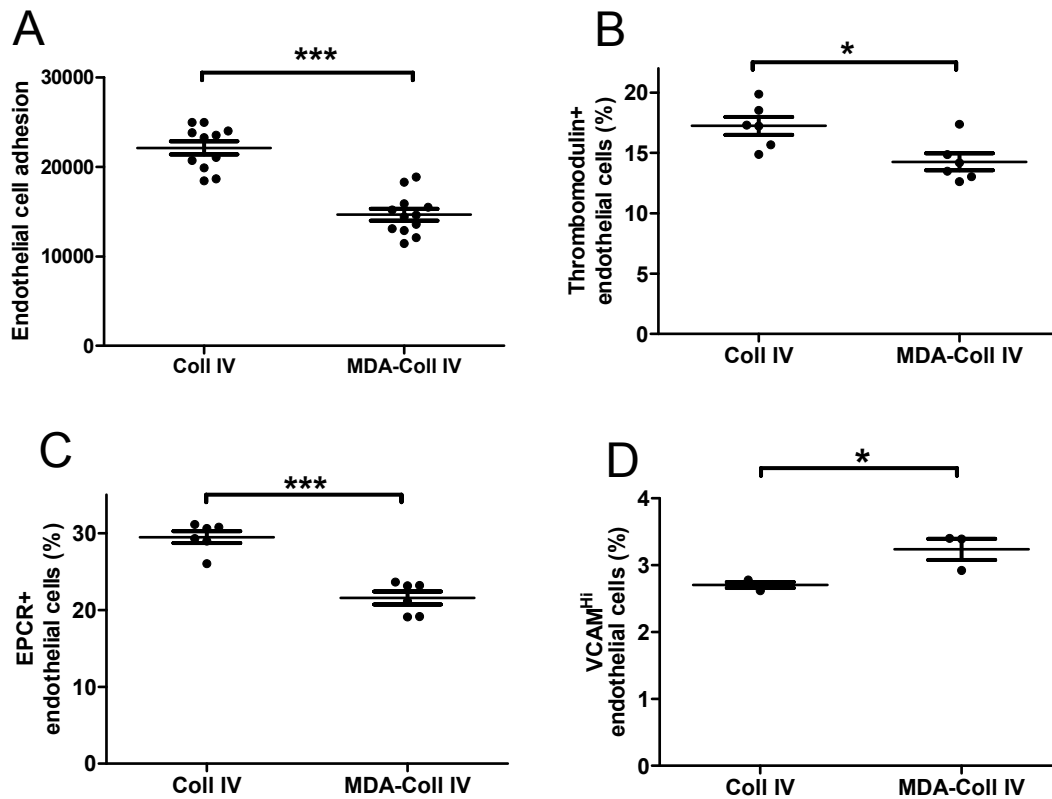


Figure 3

MDA modification of collagen type IV impairs endothelial cell adhesion and induces endothelial dysfunction.

A, Fluorescently-labeled HUVECs were allowed to adhere to native or MDA-collagen type IV for 45 minutes, where after unbound cells were removed by washing and bound cells were measured using an excitation wavelength of 485 nm and an emission wavelength of 520 nm. **B-D**, Thrombin stimulated HUVECs were cultured with native or MDA-modified collagen type IV, and the anti-coagulant proteins thrombomodulin (**B**) and EPCR (**C**) and the leucocyte attractant VCAM-1 (**D**) were measured by flow cytometry. Values are presented as individual values and mean±SEM are indicated.

2 column black and white figure