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MMP-7 mediates cleavage of N-cadherin and promotes smooth muscle cell apoptosis

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Aims Vascular smooth muscle cell (VSMC) apoptosis can lead to thinning of the fibrous cap and plaque instability. We previously showed that cell–cell contacts mediated by N-cadherin reduce VSMC apoptosis. This study aimed to determine whether matrix-degrading metalloproteinase (MMP)-dependent N-cadherin cleavage causes VSMC apoptosis.

Methods and results Induction of human VSMC apoptosis using different approaches, including 200 ng/mL Fas ligand (Fas-L) and culture in suspension, caused N-cadherin cleavage and resulted in the appearance of a C-terminal fragment of N-cadherin (∼35 kDa). Appearance of this fragment during apoptosis was inhibited by 47% with the broad-spectrum MMP inhibitor BB-94. We observed retarded cleavage of N-cadherin after treatment with Fas-L in aortic mouse VSMCs lacking MMP-7. Furthermore, VSMC apoptosis, measured by quantification of cleaved caspase-3, was 43% lower in MMP-7 knockout mouse VSMCs compared with wild-type VSMCs following treatment with Fas-L. Addition of recombinant active MMP-7 increased the amount of N-cadherin fragment by 82% and augmented apoptosis by 53%. The involvement of MMP-7 was corroborated using human cells, where a MMP-7 selective inhibitor reduced the amount of fragment formed by 51%. Importantly, we observed that treatment with Fas-L increased levels of active MMP-7 by 80%. Finally, we observed significantly increased cleavage of N-cadherin, MMP-7 activity, and apoptosis in human atherosclerotic plaques compared with control arteries, and a significant reduction in apoptosis in atherosclerotic plaques from MMP-7 knockout mice.

Conclusion This study demonstrates that MMP-7 is involved in the cleavage of N-cadherin and modulates VSMC apoptosis, and may therefore contribute to plaque development and rupture.

Keywords Vascular smooth muscle • Apoptosis • Atherosclerosis • Matrix-degrading metalloproteinase-7 • N-cadherin

1. Introduction

Rupture of atherosclerotic plaques is the major cause of myocardial infarction and stroke.[1] Vascular smooth muscle cells (VSMCs) form a protective fibrous cap over atherosclerotic plaques. VSMC apoptosis in the fibrous cap[2] and subsequent weakening of the cap[3] is thought to be an important regulator of plaque stability. In normal blood vessels VSMC apoptosis is rare, however increased apoptosis is observed in unstable human atherosclerotic plaques.[4] Recently, mouse models of atherosclerosis have provided direct evidence that VSMC apoptosis causes plaque instability.[5–7] Consequently, reducing VSMC apoptosis is an attractive strategy for attenuating plaque instability. A greater understanding of the mechanisms underlying apoptosis of VSMCs in the fibrous cap may aid the development of new therapies for reducing plaque instability.

We have previously demonstrated that N-cadherin, a predominant cell–cell junction protein in VSMCs,[8] provides a vital pro-survival signal to VSMCs via PI3-kinase-dependent activation of Akt.[9] Over-expression of N-cadherin reduced apoptosis of VSMCs in suspension culture, while conversely, over-expression of dominant negative

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N-cadherin increased apoptosis of VSMCs. Additionally, we recently showed that a soluble form of N-cadherin, composed of the extracellular domain, acts as a mimetic reducing VSMC apoptosis, as well as features of atherosclerotic plaque instability *in vivo*.[10]

Studies in other cell types have suggested that cadherin cleavage by matrix-degrading metalloproteinases (MMPs) or a disintegrin and metalloproteases (ADAMs) occurs during apoptosis.[11–14] Although we have previously observed that MMPs, specifically MMP-9 and MMP-12, cleave N-cadherin during VSMC proliferation,[8,15] it was unknown whether MMP-dependent cleavage of N-cadherin modulates VSMC apoptosis. We therefore aimed to determine whether proteolytic cleavage of N-cadherin by MMPs leads to VSMC apoptosis and is therefore likely to contribute to plaque development and rupture.

2. Methods

2.1 Husbandry

Knockout mice were kindly provided by Roger Lijnen (University of Leuven, Belgium) and then bred within the University of Bristol animal unit. C57BL/6J mice were obtained from Charles River and used as wild-type controls. Apolipoprotein E knockout mice ($ApoE^{-/-}$) were crossed with MMP-7 knockout mice to generate $ApoE^{-/-}$/$MMP\text{-}7^{-/-}$ as well as strain-matched controls ($ApoE^{-/-}$/$MMP\text{-}7^{+/+}$). These mice were placed on high-fat diet for 8 weeks and brachiocephalic arteries removed as previously described.[16] Housing, care, and all procedures were performed in accordance with the guidelines and regulations of the University of Bristol and the United Kingdom Home Office. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2 Cell culture

The investigation conforms to the principles outlined in the Declaration of Helsinki. Human saphenous vein segments were obtained from consenting coronary artery bypass graft patients (Local ethics board REC#05/Q2007/77) and VSMCs grown from medial explants as described previously.[8] Mouse VSMCs were grown from explants of mouse aorta removed from either MMP knockout or wild-type mice or C57BL/6J controls, as described previously.[15] Each experiment was performed with VSMCs at passage 2–10 from at least three different individuals.

Apoptosis was induced either by culturing VSMCs (5×10^4 cells/well in 24 well plates) in serum free media supplemented with 300 ng/mL human recombinant soluble Fas ligand (Fas-L), 50 ng/mL tumour necrosis factor-α (TNFα), 100 μM H_2O_2 or 50 μM BCl_2 antagonist (HA-14, Calbiochem), or by culturing VSMCs in suspension culture as described previously.[17]

MMP activity was inhibited by 1 μM BB-94 (Batimastat, a widely used and specific MMP inhibitor which inhibits many MMPs including MMPs 1, 2, 3, 7, 9, 12, 13 with K_i values of 0.15–2.3 nM,[18] was a kind gift of Dr Clive Long, Pfizer), 2 μM MMP-2 inhibitor[19] (K_i values of 1.7 μM for MMP-2, 444244, Calbiochem), 100 nM MMP-12 inhibitor[20,21] (IC_{50} 14 nM for MMP-12 and >270 nM for MMPs 2, 3, 8, 9, and 13, PF-356231, Biomol), or 10 nM MMP-7 inhibitor (IC_{50} 10 nM for MMP-7, 444264, Calbiochem). γ-Secretase, plasmin, and caspase activity were inhibited by 10 μM γ-secretase inhibitor (565771, Calbiochem), 10 μM NALME[22,23] (Sigma), and 20 μM pan caspase inhibitor (Z-VAD-FMK) inhibitor (550377, Calbiochem), respectively. DMSO was added to act as a vehicle control in all experiments. VSMCs were also treated with 60 nM active recombinant human MMP-7 (444270 Calbiochem) and 2 μg/mL cycloheximide (to prevent protein synthesis and thereby inhibit replenishment of full-length N-cadherin).

2.3 Atherosclerotic plaques

Human carotid endarterectomy samples were obtained (local ethical approval #E3111) as described previously.[24] Human right coronary artery plaques were removed from hearts collected for valve retrieval (local ethical approval REC#08/H0107/48). Surplus segments of internal mammary artery (IMA) were obtained from consenting patients undergoing coronary artery bypass grafting (local ethical approval REC#04/Q2007/6). Frozen samples were extracted in SDS lysis buffer or MMP-7 assay buffer (1 mM monothioglycerol, 50 mM Tris–HCl, pH 7.4) and analysed by western blotting and MMP-7 activity assay, respectively, or frozen sections were cut for *in situ* zymography.

2.4 Cleavage of recombinant N-cadherin by MMP-7

As described previously,[15] active MMP-7 (2 nM) was incubated with 2 nM purified soluble N-cadherin-Fc (SNC-Fc; consisting only of the extracellular domain), for 30 min at 37°C and then subjected to western blotting.

2.5 Western blotting

Western blotting for N-cadherin, phosphorylated Akt (pAkt), total Akt, and β-tubulin was performed with equal protein concentrations, as described previously.[8,9] The anti-N-cadherin antibody (610920, BD Transduction Laboratories) recognizes amino acids 802–819 in the C-terminus of N-cadherin. To detect SNC-Fc, western blotting for the Fc domain of mouse IgG was performed as described previously.[15] Cleaved caspase-3 was detected using rabbit anti-cleaved caspase-3 antibody diluted 1:1000 (Cell Signalling No. 9661). Densitometric scanning was performed to quantify the optical density of detected bands (O.D. $\times$ mm^2) and normalized to β-tubulin values.

2.6 Measurement of apoptosis

Apoptosis was assessed *in vitro* by measurement of cleaved caspase-3 levels using the Caspase-Glo luminescent assay (Promega) as described in the manufacturer's instructions. Immunocytochemistry for cleaved caspase-3 was performed as described previously.[10] Apoptosis was also measured in paraffin-wax embedded sections using *in situ* end labelling (ISEL) as described previously.[25] The number of ISEL positive plaque cells was counted and expressed as a percentage of the total number of plaque cells.

2.7 MMP-7 activity assay

Ten times concentrated conditioned media and tissue lysates were assessed for MMP-7 activity using a fluorimetric assay. Samples and standards were incubated with 0.14 mg/mL DQ-gelatin fluorescent substrate (Invitrogen) with and without 10 nM MMP-7 inhibitor (444264, Calbiochem) and fluorescence read using a Fluostar Optima fluorimeter from 0 to 90 min until peak fluorescence was achieved. MMP-7 activity was calculated as that inhibited by the MMP-7 inhibitor.

2.8 *In situ* zymography

MMP activity was detected as described previously.[26] Sections were incubated with 10 nM MMP-7 inhibitor to identify MMP-7 activity. In some cases, immunohistochemistry for cleaved caspase-3 was performed as described previously[10] after *in situ* zymography. MMP activity was quantified using image analysis and expressed as fluorescent arbitrary units (FAU).

2.9 Statistics

Experiments were carried out at least three times with VSMCs from different sources. Data were analysed by Student's *t*-test or ANOVA and Student Newman–Keuls post-test and a significant difference accepted when $P < 0.05$.

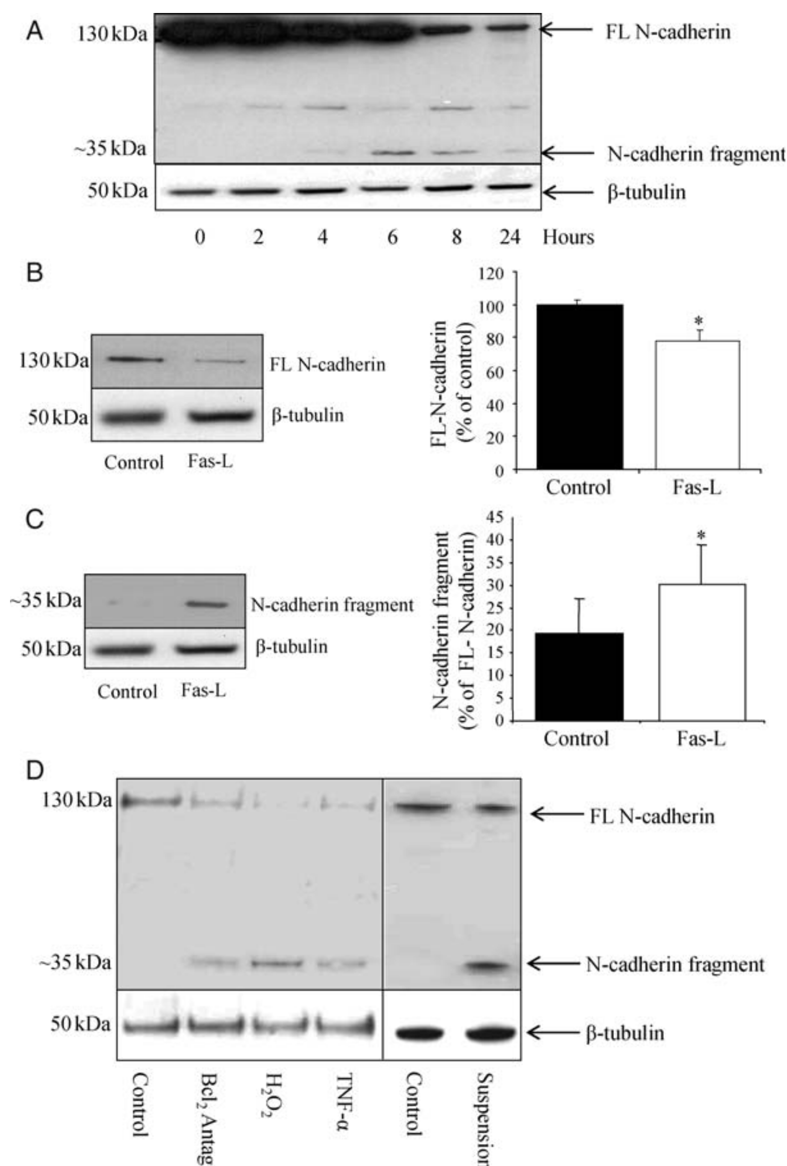

Figure 1 N-cadherin cleavage occurs during VSMC apoptosis. Representative western blot for full-length (FL, 130 kDa) and ~35 kDa C-terminal fragment of N-cadherin protein in human VSMCs after induction of apoptosis with Fas-L at various time-points (*A*), full-length N-cadherin at 4 h (*B*), and ~35 kDa C-terminal fragment of N-cadherin after 4 h (*C*). Bar charts show densitometric analysis of western blots (*B* and *C*) and * indicates a significant difference ($P < 0.05$) from control, $n = 5$. (*D*) Representative western blot for full-length (130 kDa) and ~35 kDa C-terminal fragment of N-cadherin protein in human VSMCs after induction of apoptosis with 50 μM Bcl_2 antagonist (Bcl_2 antag) for 18 h, 100 μM H_2O_2, or 50 ng/mL TNFα for 22 h, and suspension culture for 24 h. β-Tubulin is shown as loading control.

3. Results

3.1 N-cadherin is cleaved during VSMC apoptosis

Treatment with Fas-L resulted in a time-dependent cleavage of full-length N-cadherin in human VSMCs (*Figure 1A*), which reached significance after 4 h of treatment (*Figure 1B*). The appearance of ~35 kDa fragment of N-cadherin was also observed after 4 h (*Figure 1A* and *C*). Other inducers of apoptosis (Bcl_2 antagonist, TNFα, H_2O_2, or suspension culture) also caused cleavage of full-length N-cadherin and the appearance of the ~35 kDa N-cadherin fragment (*Figure 1D*), confirming that this was not a unique effect of Fas-L-induced apoptosis. Using western blotting, we were unable to detect release of the extracellular domain of N-cadherin into the conditioned media (data not shown).

3.2 MMPs contribute to N-cadherin cleavage

Addition of the MMP inhibitor BB-94 (*Figure 2A*) significantly reduced the amount of N-cadherin fragment produced in response to Fas-L and increased full-length N-cadherin correspondingly, indicating that MMPs are involved in N-cadherin cleavage. In contrast, N-cadherin cleavage was not retarded by treatment of human VSMCs with a plasmin inhibitor (NALME) with or without BB-94 (see

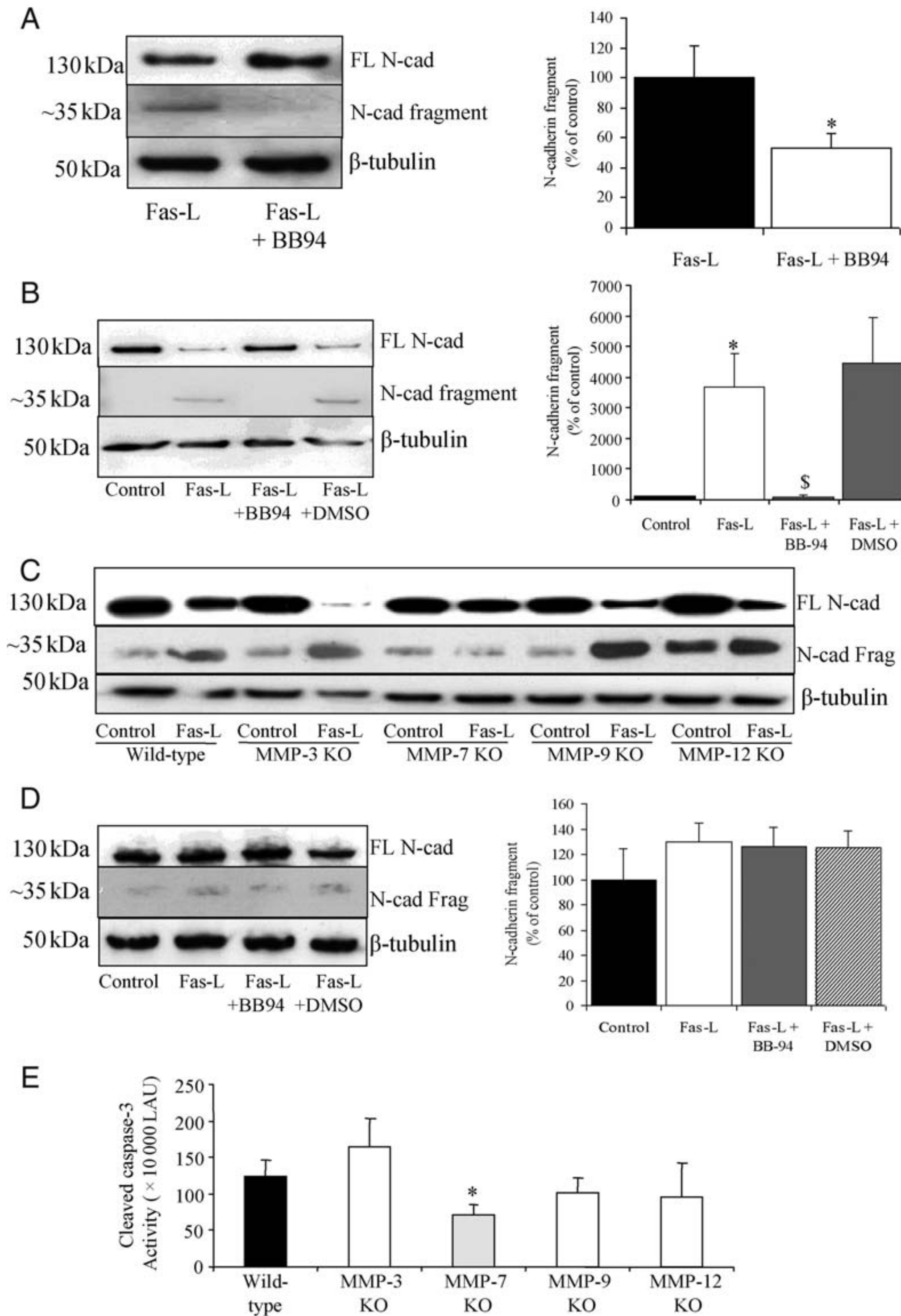

Figure 2 N-cadherin cleavage during apoptosis is mediated by MMPs. (*A*) Representative western blots ($n = 6$) for N-cadherin protein in human VSMCs 4 h after the addition of Fas-L and 1 μM BB-94 or DMSO (vehicle control). (*B*) Representative western blots ($n = 3$) N-cadherin protein in mouse wild-type VSMCs 4 h after the addition of Fas-L with or without 1 μM BB-94 or DMSO vehicle control. (*C*) Representative western blots ($n = 3$) for N-cadherin protein in mouse wild-type or MMP-3, -7, -9, or -12 knockout VSMCs 4 h after the addition of Fas-L. (*D*) Representative western blots ($n = 3$) for N-cadherin protein in mouse MMP-7 knockout VSMCs 4 h after the addition of Fas-L with or without 1 μM BB-94 or DMSO vehicle control. Bar charts (*A*, *B*, and *C*) show densitometric analysis of western blots for ~35 kDa C-terminal fragment of N-cadherin, data are shown as percent of control, and * and $ indicate a significant difference ($P < 0.05$) from control and Fas-L + DMSO, respectively. β-Tubulin is shown as loading control. (*E*) Cleaved caspase-3 activity expressed as LAU in mouse wild-type or MMP-3, -7, -9, or -12 knockout (KO) VSMCs 8 h after the addition of Fas-L. * indicates a significant difference ($P < 0.05$) from wild-type control, $n = 6$.

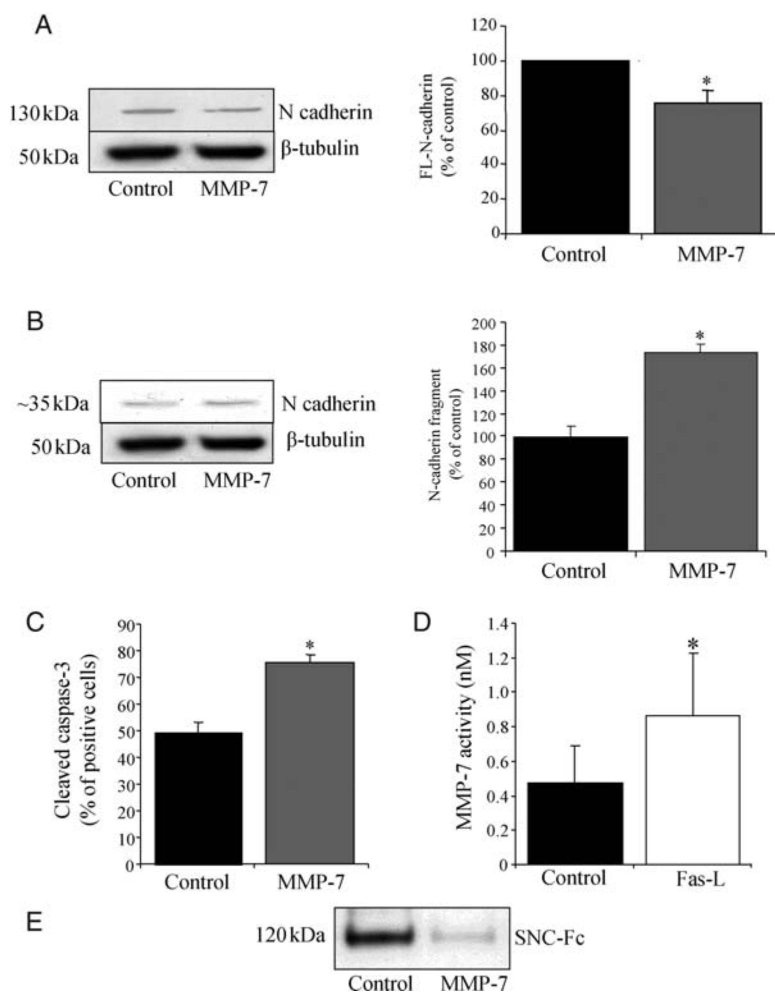

Figure 3 MMP-7 promotes N-cadherin cleavage and apoptosis. Representative Western blots for full-length N-cadherin (*A*), and ~35 kDa C-terminal fragment of N-cadherin (*B*) at 4 h after addition of 60 nM recombinant MMP-7. β-Tubulin is shown as loading control. Bar charts show densitometric analysis of western blots (*A* and *B*), data are shown as % of control and * indicates a significant difference ($P < 0.05$) from control, $n = 6$. Cleaved caspase-3 activity in human VSMCs treated with MMP-7 for 24 h (*C*), * indicates a significant difference ($P < 0.05$) from control, $n = 3$. MMP-7 activity in human VSMC conditioned media with or without Fas-L for 4 h (*D*), * indicates a significant difference ($P < 0.05$) from control, $n = 4$. (*E*) Representative western blot for Fc domain of mouse IgG after 30 min incubation of 2 nM MMP-7 with 2 nM recombinant N-cadherin (SNC-Fc).

Supplementary material online, *Figure S1*) or with a caspase inhibitor or γ-secretase inhibitor (data not shown).

3.3 MMP-7 contributes to N-cadherin cleavage and apoptosis

To identify which MMP was responsible for N-cadherin cleavage, we used aortic VSMCs isolated from a selection of MMP knockout mice. We first ensured that we obtained the same effect in wild-type (C57BL/6J) mouse VSMCs as observed in human VSMCs. As seen in human VSMCs, we observed a significant increase in N-cadherin fragment and a decrease in full-length N-cadherin in mouse VSMCs following Fas-L treatment, and this increase was inhibited by BB-94 (*Figure 2B*). Increased N-cadherin fragment and decreased full-length N-cadherin levels were also observed in VSMCs from MMP-3, MMP-9, and MMP-12 knockout mice after treatment with Fas-L (*Figure 2C*). However, this was not observed in VSMCs from MMP-7 knockout mice (*Figure 2C* and *D*). Importantly, we observed that basal levels of full-length N-cadherin in the various cell types used were comparable (wild-type controls: 15.3 ± 3.1, MMP-$3^{-/-}$: 15.9 ± 1.1, MMP-$7^{-/-}$: 14.5 ± 1.6, MMP-$9^{-/-}$: 15.0 ± 3.8, and MMP-$12^{-/-}$: 14.0 ± 2.7, O.D. × mm^2, *Figure 2C*). Additionally, there was no difference in N-cadherin cleavage in the different wild-type VSMCs compared with the C57BL/6J control cells (see Supplementary material online, *Figure S2*). Furthermore, supplementation with BB-94 did not significantly affect the amount of N-cadherin fragment detected after Fas-L treatment of MMP-7 knockout VSMCs (*Figure 2D*).

Cleaved caspase-3 activity was measured 8 h after induction of apoptosis with Fas-L using the Caspase-Glo assay. Cleaved caspase-3 was significantly reduced in MMP-7 knockout VSMCs compared with control VSMCs (*Figure 2E*). In contrast, there was no statistical difference in cleaved caspase-3 in VSMCs from MMP-3, -9, and -12 knockouts compared with controls. Due to a large

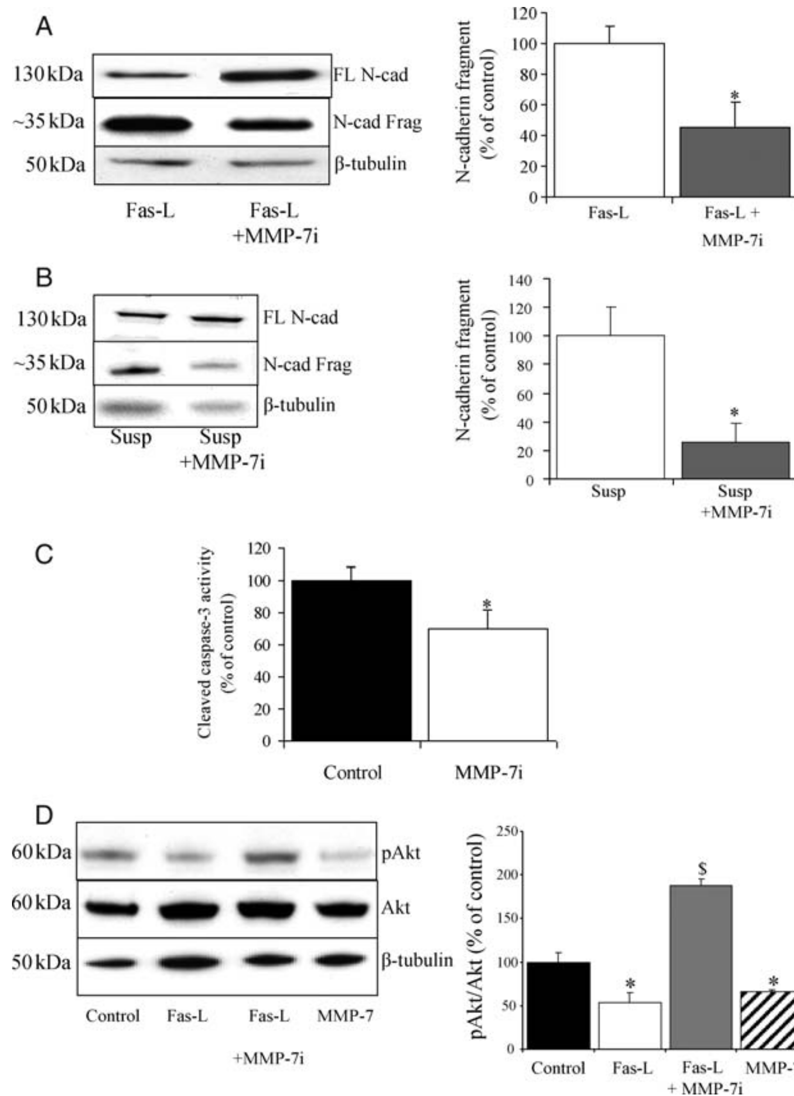

Figure 4 MMP-7 inhibitor (MMP-7i) retards N-cadherin cleavage and VSMC apoptosis. (*A*) Representative western blot ($n = 3$) for full length and ∼35 kDa C-terminal fragment of N-cadherin protein in human VSMCs 4 h after the addition of Fas-L with or without 10 nM MMP-7 inhibitor (MMP-7i). (*B*) Representative western blot ($n = 3$) for full length and ∼35 kDa C-terminal fragment of N-cadherin protein in mouse wild-type VSMCs grown in suspension (Susp) to induce apoptosis and treated with 10 nM MMP-7 inhibitor. Bar charts (*A* and *B*) show densitometric analysis of western blots, data are shown as percent of control and * indicates a significant difference ($P < 0.05$) from Fas-L or suspension culture alone. (*C*) Cleaved caspase-3 activity in human VSMCs treated with Fas-L with or without 10 nM MMP-7 inhibitor for 8 h, * indicates a significant difference ($P < 0.05$) from Fas-L alone, $n = 4$. (*D*) Representative western blots ($n = 3$) for pAkt, and total Akt. Bar chart shows densitometric analysis of western blots, data are shown as percent of control and * and $ indicate a significant difference ($P < 0.05$) from control and Fas-L, respectively. β-Tubulin is shown as loading control. All control samples were supplemented with DMSO to act as a vehicle control.

variation in the response of the MMP-12 knockout VSMCs, we determined the effect of a MMP-12 inhibitor on cleaved caspase-3 activity. No difference in cleaved caspase-3 activity was observed [699316 ± 288179 vs. 690540 ± 258043 luminescent arbitrary units (LAU)].

We investigated whether addition of a human recombinant MMP-7 affected N-cadherin cleavage and apoptosis. Addition of recombinant MMP-7 caused a significant increase in cleavage of N-cadherin, as shown by a reduction in full-length N-cadherin (*Figure 3A*) and production of the ∼35 kDa fragment (*Figure 3B*). Cycloheximide (which was added to all conditions to inhibit replenishment of N-cadherin by protein synthesis) alone had no significant effect on N-cadherin levels compared with control (data not shown). Increased N-cadherin cleavage was associated with significantly augmented apoptosis (*Figure 3C*). To establish whether MMP-7 activity is increased during Fas-L induced apoptosis, we measured MMP-7 in the conditioned media of Fas-L-treated VSMCs. MMP-7 activity was significantly increased in Fas-L treated VSMCs (*Figure 3D*), confirming that MMP-7 activity is elevated during VSMC apoptosis. To establish whether the cleavage of N-cadherin by MMP-7 is direct or indirect, recombinant N-cadherin was incubated with active MMP-7. We observed that cleavage of N-cadherin occurred in the absence of cells and other proteases and therefore is direct (*Figure 3E*).

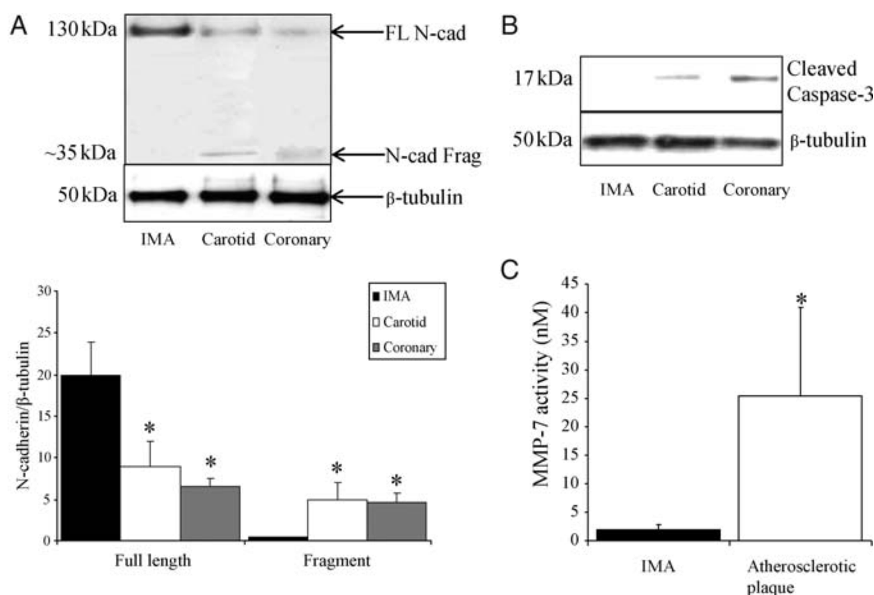

Figure 5 N-cadherin, MMP-7, and apoptosis in human IMA and atherosclerotic plaques. Representative western blot for N-cadherin (*A*) and cleaved caspase-3 (*B*) in IMA and coronary and carotid atherosclerotic plaques, $n = 3$ per group. β-Tubulin is shown as loading control. Bar chart in (*A*) shows densitometric analysis of N-cadherin western blots. (*C*) MMP activity in IMA and atherosclerotic plaques, $n = 15$ and 20. * indicates a significant difference from IMA.

To further study the involvement of MMP-7 in N-cadherin cleavage and VSMC apoptosis, we utilized a MMP-7 inhibitor. Apoptosis was induced in both human and mouse VSMCs using Fas-L and suspension culture, respectively, and in both cases the MMP-7 inhibitor significantly reduced the amount of N-cadherin fragment and increased full-length N-cadherin (*Figure 4A* and *B*). Interestingly, the degree of inhibition of N-cadherin fragment formation observed with the MMP-7 inhibitor was comparable to that seen with BB-94 (*Figure 4B*), which suggests that MMP-7 is the primary MMP involved in N-cadherin cleavage. No further inhibition of N-cadherin cleavage was observed with co-treatment with the MMP-7 inhibitor and the plasmin inhibitor NALME (see Supplementary material online, *Figure S1*). Addition of a MMP-2 inhibitor had no significant effect (data not shown). To ascertain whether reduced N-cadherin cleavage was associated with attenuated apoptosis in human VSMCs, cleaved caspase-3 was measured. As seen in the MMP-7 knockout mouse VSMCs, inhibition of MMP-7 in human VSMCs resulted in a significant reduction in cleaved caspase-3 following addition of Fas-L (*Figure 4C*). Fas-L caused a significant decline in pAkt, whereas significantly elevated levels of pAkt were observed when VSMCs were treated with the MMP-7 inhibitor and Fas-L (*Figure 4D*). In contrast, active MMP-7 reduced pAkt levels (*Figure 4D*).

3.4 N-cadherin, apoptosis, and MMP-7 in atherosclerotic plaques

Human carotid and coronary arterial plaques and IMAs were subjected to western blotting to examine N-cadherin and cleaved caspase-3 protein expression (*Figure 5A* and *B*). Full-length N-cadherin protein was seen in both atherosclerotic plaques and healthy arteries, however significantly lower levels of full-length N-cadherin was detected in plaques compared with IMA (*Figure 5A*). Moreover, cleaved N-cadherin was only detected in the atherosclerotic arteries (*Figure 5A*). Cleaved caspase-3 was also significantly higher in atherosclerotic plaques compared with IMAs (86 ± 33 vs. 4 ± 2 O.D. $\times$ mm^2, *Figure 5B*). Additionally, MMP-7 activity was significantly higher in atherosclerotic plaques than IMAs (*Figure 5C*).

We assessed MMP activity using *in situ* zymography. We observed MMP activity in atherosclerotic plaques while little MMP activity was detected in IMAs (1681 ± 518 vs. 37 ± 12 FAU, *Figure 6A* and *D*). A significant amount of the MMP activity was mediated by MMP-7 since MMP activity was significantly reduced by 70% with the MMP-7 inhibitor (571 ± 218 vs. 1681 ± 518 FAU, *Figure 6A* and *B*). Apoptotic cells were co-located in areas of MMP activity in atherosclerotic plaques, whereas MMP activity and apoptosis were low in IMAs (*Figure 6C–E*).

Apoptosis was quantified in mouse brachiocephalic plaques using ISEL. The percentage of apoptotic plaque cells was significantly lower in atherosclerotic plaques from ApoE$^{-/-}$/MMP-7$^{-/-}$ mice than ApoE$^{-/-}$/MMP-7$^{+/+}$ mice (0.05 ± 0.01 vs. 0.12 ± 0.03%, *Figure 6F–H*).

4. Discussion

This study demonstrates a link between MMP-7, N-cadherin cleavage, and VSMC apoptosis in atherosclerotic plaques. We have shown *in vitro* that during apoptosis, levels of active MMP-7 are elevated and that augmented levels of MMP-7 activity are associated with N-cadherin cleavage and increased apoptosis. Furthermore, we have shown increased MMP-7 activity, apoptosis, and N-cadherin cleavage in atherosclerotic plaques.

Although VSMC apoptosis is rare in normal blood vessels, increased apoptosis is observed in unstable human atherosclerotic plaques[4] and promotes plaque instability in mouse models of atherosclerosis.[5–7] We previously demonstrated that N-cadherin acts as

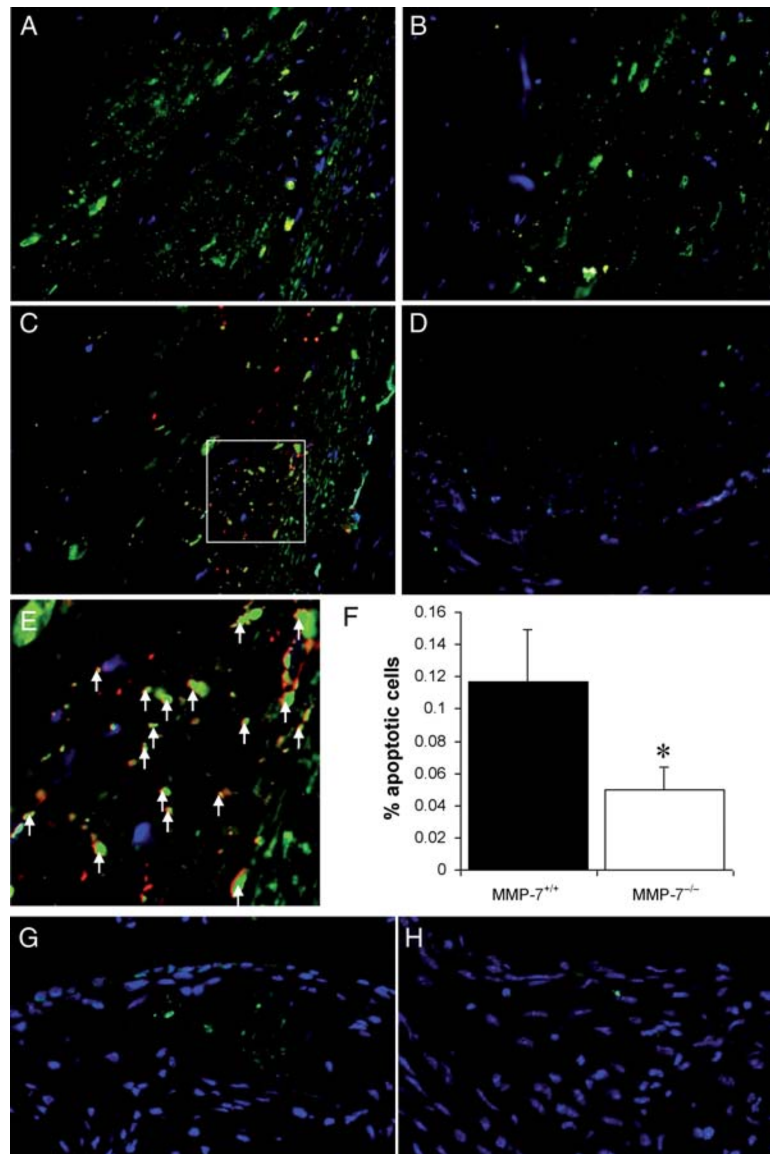

Figure 6 MMP-7 and apoptosis in atherosclerotic plaques. *In situ* zymography of coronary atherosclerotic plaque in the absence (*A*) and presence (*B*) of 10 nM MMP-7 inhibitor. Green colour indicates MMP activity and nuclei are stained blue with DAPI. Dual detection of cleaved caspase-3 (red) and MMP activity (green) in coronary plaque (*C*) and IMA (*D*). (*E*) High power image of marked area in (*C*), arrows indicate co-location of apoptosis and MMP activity. (*F*) Quantification of the percentage of apoptotic (ISEL positive) cells in atherosclerotic plaques from ApoE$^{-/-}$/MMP-7$^{+/+}$ and ApoE$^{-/-}$/MMP-7$^{-/-}$. * indicates a significant difference from ApoE$^{-/-}$/MMP-7$^{+/+}$. ISEL staining of atherosclerotic plaques from ApoE$^{-/-}$/MMP-7$^{+/+}$ (*G*) and ApoE$^{-/-}$/MMP-7$^{-/-}$ (*H*) mice. ISEL positive apoptotic cells are green and nuclei are stained blue (DAPI).

survival factor for VSMCs,[9] and soluble N-cadherin reduces features of plaque instability.[10] In this study, we examined whether proteolytic cleavage of N-cadherin occurs and contributes to VSMC apoptosis. We observed a time-dependent loss of full-length N-cadherin and appearance of a C-terminal fragment of N-cadherin (∼35 kDa) after induction of human VSMC apoptosis with various factors including Fas-L, suspension culture, or TNFα. The appearance of the ∼35 kDa fragment consisting of the intracellular and transmembrane portion of N-cadherin indicates extracellular cleavage. Previously a 38 kDa fragment N-cadherin was reported to be the result of MMP activity and was further processed by presenilin/γ-secretase to 33 kDa.[27] However, we were unable to determine any cleavage by presenilin/γ-secretase. Additionally, we were unable to detect release of the extracellular domain into the conditioned media as seen previously for E-cadherin,[28] presumably due to further proteolytic cleavage of the extracellular fragment of N-cadherin.

Previous studies have highlighted cleavage of cadherins by various proteases including calpain,[29] ADAMs,[27] plasmin,[30] presenilin-1/γ-secretase,[14] and MMPs.[13,28,31–34] During tumour regression, increased apoptosis was accompanied by elevated MMP activity and E-cadherin cleavage.[32] Similarly, Herren *et al.*[13] showed MMP-dependent cleavage of VE-cadherin during endothelial cell apoptosis. Wu and Huang[31] identified that snake venom metalloproteinases cause activation of MMP-2, cleavage of VE-cadherin, and

apoptosis of endothelial cells. Furthermore, N-cadherin cleavage during activated hepatic stellate cell apoptosis is promoted by MMP-2[35] and inhibited by the endogenous inhibitor of MMPs (TIMP-1).[12] In our study, combined treatment with Fas-L and a global non-specific MMP inhibitor significantly reduced N-cadherin cleavage, while plasmin, caspase, and γ-secretase inhibitors had no effect, indicating that MMP activity is involved in N-cadherin cleavage.

Using VSMCs from MMP knockout mice, we identified a role for MMP-7 in the cleavage of N-cadherin during VSMC apoptosis. In support of this, addition of recombinant active MMP-7 caused loss of full-length N-cadherin, appearance of the N-cadherin fragment, and increased apoptosis in human VSMCs while a MMP-7 inhibitor reduced Fas-L-induced N-cadherin cleavage and apoptosis in human VSMCs. Incubation of recombinant N-cadherin with MMP-7 revealed that cleavage of N-cadherin was direct and did not require the presence of other proteases. Interestingly, we also showed that as a result of Fas-L treatment, MMP-7 activity is augmented in human VSMCs. We observed that as a result of N-cadherin cleavage, pAkt levels were reduced, confirming our previous finding that N-cadherin acts as a survival signal via activation of Akt.[9] Together this data clearly indicates that MMP-7 contributes to the cleavage of N-cadherin and the induction of VSMC apoptosis. Although previous studies have shown that MMP-7 cleaves E-cadherin,[28,34] this is the first demonstration that MMP-7 can cleave N-cadherin. Recently, we observed that MMP-9- and MMP-12-dependent cleavage of N-cadherin occurs during VSMC proliferation.[15] Interestingly, in this study, we observed no involvement of MMP-9 and MMP-12 in N-cadherin cleavage during VSMC apoptosis. This indicates that different MMPs are responsible for N-cadherin cleavage during these different cellular processes of proliferation and apoptosis.

Polymorphisms of MMP-7 have been associated with coronary artery disease.[36] Moreover although MMP-7 is absent in control arteries, it is present in atherosclerotic plaques.[37] More specifically, MMP-7 was expressed specifically in lipid-laden macrophages confined to the sites susceptible to rupture; the border between the acellular lipid cores and the fibrous areas.[37] They proposed therefore that MMP-7, specifically expressed in atherosclerotic lesions, could cleave structural proteoglycans including versican, potentially leading to separation of caps and shoulders from lipid cores and plaque instability. Additionally, ApoE[38] and apolipoprotein C-II[39] are substrates for MMP-7 which will affect atherosclerotic plaque formation and stability. We observed elevated MMP-7 activity in atherosclerotic plaques compared with controls, which correlated and co-located with increased apoptosis and N-cadherin cleavage. We therefore suggest that in addition to matrix and apolipoprotein cleavage, MMP-7 further contributes to plaque instability by induction of apoptosis of VSMCs as a result of N-cadherin cleavage. MMP-7 may also exert a pro-apoptotic effect through other proteolytic mechanisms. For example, it has been shown that MMP-7 can cleave Fas-L, releasing soluble Fas-L and thus facilitating Fas-induced apoptosis.[40] Indeed, in our study, we have utilized this well characterized death pathway as an apoptotic stimulus. Moreover, as stated earlier, MMP-7 has numerous other substrates which are present in atherosclerotic plaques and which cleavage of may influence cell survival.

Deletion of MMP-7 in ApoE knockout mice led to a 78% increase in VSMC content of the plaque.[16] We demonstrated in this study that MMP-7 deficiency attenuated apoptosis, indicating that MMP-7 plays a pro-apoptotic role in atherosclerotic plaques *in vivo*. Despite the increased numbers of VSMCs in atherosclerotic plaques of MMP-7 knockout mice however, there was no effect on plaque growth or stability.[16] MMP-7 gene deletion may have other effects on other cell types, apart from VSMCs, within the plaques which are beneficial for plaque stability or may have other effects on arterial physiology that counteract the beneficial changes in VSMC content. For example, MMP-7 has been implicated in the maintenance of arterial tone, and therefore MMP-7 knockout mice may suffer increased tensile force across the fibrous cap as a result of reduced medial tone.[41] Hence, the greater stability conferred on the lesion by increased VSMCs may be negated by increased strain on the fibrous cap.

In summary, we have shown for the first time that MMP-7 activity is up-regulated during apoptosis of VSMCs. Moreover, we have observed that MMP-7 causes cleavage of N-cadherin which is associated with increased VSMC apoptotic rates. We propose that MMP-7 may play a key role in atherosclerotic plaque development and rupture via N-cadherin cleavage and induction of VSMC apoptosis.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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