Soluble N-Cadherin Overexpression Reduces Features of Atherosclerotic Plaque Instability

Cressida A. Lyon, Jason L. Johnson, Helen Williams, Graciela B. Sala-Newby, Sarah J. George

Objectives—Vascular smooth muscle cell (VSMC) apoptosis contributes to atherosclerotic plaque instability and myocardial infarction. Consequently, reducing VSMC apoptosis may be beneficial for reducing plaque instability and acute coronary events. We previously demonstrated that N-cadherin, a cell–cell adhesion molecule, reduces VSMC apoptosis in vitro. In this study, we examined whether a soluble form of N-cadherin (SNC) affected VSMC apoptosis and plaque stability.

Methods and Results—SNC significantly inhibited VSMC apoptosis in vitro by \( \approx 50\% \) via activation of fibroblast growth factor receptor, phosphoinositide-3 kinase, and Akt signaling. SNC also significantly reduced macrophage and foam cell–macrophage apoptosis in vitro by \( >50\% \), without affecting monocyte invasion or macrophage proliferation. Elevation of plasma levels of SNC in male apolipoprotein E–deficient mice with existing atherosclerosis via adenoviral delivery significantly reduced VSMC and macrophage apoptosis in brachiocephalic artery plaques by \( \approx 60\% \). Additionally, SNC promoted plaques of a more stable phenotype by elevating VSMC:macrophage ratio and presence of VSMC-rich fibrous cap, as well as attenuating macrophage number and incidence of buried fibrous caps (a surrogate plaque rupture marker).

Conclusions—In summary, this study demonstrates that SNC suppressed plaque instability by attenuation of apoptosis, suggesting that SNC may have a therapeutic potential for retarding plaque instability. (Arterioscler Thromb Vasc Biol. 2009;29:195-201.)

Key Words: apoptosis ■ atherosclerosis ■ cell adhesion molecules ■ smooth muscle ■ macrophages

Plaque rupture is the precipitating event in the majority of clinical events of acute coronary syndrome patients presenting with unstable angina, acute myocardial infarction, and sudden coronary death.\(^1\) Accelerated vascular smooth muscle cell (VSMC) apoptosis contributes to fibrous cap thinning and therefore is vitally important in plaque stability.\(^2\)

In normal blood vessels VSMC apoptosis is rare. However, increased apoptosis is observed in unstable human atherosclerotic plaques.\(^3\) Interestingly, apoptotic cells are located in the fibrous cap\(^2\) and associated with fibrous cap thinning.\(^4\) 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.

Homophilic cell–cell contacts provide a survival signal in many cell types. In VSMCs homophilic cell–cell contacts are mediated by N-cadherin.\(^8\) We previously demonstrated that N-cadherin mediated cell–cell adhesion provides a survival signal in VSMCs and is equally effective as cell–matrix contact.\(^9\) Homophilic interaction of N-cadherin causes activation of the PI-3 kinase/Akt pathway.\(^10\)–\(^12\) Interestingly, N-cadherin can associate with the fibroblast growth factor receptor (FGF-R)\(^13,14\) and as a result causes activation of the FGF-R.\(^14\)–\(^17\) Although it has been demonstrated that association of FGF-R and N-cadherin provides a survival signal,\(^16,18\) the mechanism remains undefined.

Previous studies have shown that the extracellular domain of cadherins acts as a mimic.\(^19\)–\(^21\) We therefore determined whether a 90-kDa soluble fragment of N-cadherin composed of the extracellular domain (soluble N-cadherin, SNC) affects VSMC apoptosis and plaque instability. This soluble form of N-cadherin not only facilitates in vivo studies but has therapeutic potential. First, we determined the effect of SNC on VSMC apoptosis and the mechanism of action in vitro. Second, we examined whether SNC affected monocyte, macrophage, and foam cell–macrophage behavior in vitro. Third, we determined the effects of elevated plasma levels of SNC on apoptosis and plaque composition using adenoviral infection in fat-fed ApoE\(^{-/-}\) mice.

Methods

Expanded methods are provided in the supplemental materials (available online at http://atvb.ahajournals.org).
VSMC Culture
Human saphenous vein VSMC at passage 4 to 8 were generated as described\(^6\) (Research Ethics Committee #04/Q2007/6). Each experiment was carried out with VSMCs from at least 3 different segments of vein.

Purification of SNC-Fc and Fc
CHO cells were infected with 50 pfu/cell of RAd Fc or RAd SNC-Fc, the conditioned media was collected at 66 and 138 hours after infection. Conditioned media was pooled, and purification of SNC-Fc or Fc was achieved with protein A columns (Amersham Biosciences). Protein concentration was determined using the Bradford Protein assay (Sigma).

Inducing VSMC and Macrophage Apoptosis
VSMC apoptosis was induced by culturing in 10% (v/v) FCS in 10% (w/v) agarose-coated wells (suspension culture) or serum-free media with 200 ng/mL Fas-L, or 50 ng/mL tumor necrosis factor (TNF-\(\alpha\)) and 2 \(\mu\)g/mL cycloheximide, in the presence of 20 pmol/L SNC-Fc or Fc for 24 hours. The effect of SNC was compared with 20 pmol/L (\(\sim 2\, \text{ng/mL}\)) IGF-1. VSMCs were also treated with 30 \(\mu\)mol/L SUS4502 (Calbiochem), 20 nmol/L Wortmannin (Calbiochem), 10 \(\mu\)mol/L Akt inhibitor (Calbiochem) to inhibit FGF-R signaling, PI-3K, and Akt, respectively. HAV peptides, generously provided by Dr Orest Blaschuk (Adherex, Durham, NC), were used at 500 \(\mu\)g/mL as previously described,\(^9\) to assess the role of the HAV binding domain in the prosurvival effect. HAV peptide (Ac-Cys-His-Ala-Val-Cys-NH\(_2\)) is a nonspecific HAV inhibitory peptide, inhibiting all classical cadherins and other HAV motif containing proteins including FGF-R,\(^2,22,23\) HAVDI peptide (N-Ac-Cys-His-Ala-Val-Asp-Cys-NH\(_2\)) is a N-cadherin specific peptide,\(^24\) HGV (Ac-Cys-His-Gly-Val-Cys-NH\(_2\)) and HGVDI (N-Ac-Cys-His-Gly-Val-Asp-Cys-NH\(_2\)) were used as control peptides. Mouse macrophage and rabbit foam cell–macrophage apoptosis was induced by culture in serum-free media for 72 hours in the presence of 20 pmol/L SNC-Fc or Fc.

Immunocytochemistry
Apoptosis was assessed by cleaved caspase-3 immunocytochemistry as previously.\(^9\) Phosphorylated Akt (pAkt) was detected by immunocytochemistry using rabbit anti-pAkt antibody (Cell Signaling) diluted 1:25.

In Vivo Experiments
Homozygous C57BL/6;129 male ApoE\(^{-/-}\) mice (strain background 71% C57BL/6 and 29% 129) were bred within the Animal Unit of the University of Bristol. 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. To induce formation of complex atherosclerotic lesions, male 8-week-old apoE\(^{-/-}\) mice were fed a high-fat rodent diet containing 21% (w/w) fat from lard supplemented with 0.15% (w/w) cholesterol (Special Diets Services) for a period of 8 weeks. Mice were given an intravenous dose of \(8\times10^{10}\) viral particles of an empty adenovirus (RAd66) as a predose 4 hours before administration of 2.25\(\times10^{6}\) viral particles of RAd SNC-Fc (\(n=13\)) and Fc (\(n=13\)), or vehicle control PBS (\(n=15\)), as previously described.\(^{25}\) Mice were then maintained on a high-fat diet for 28 days to induce hepatic expression of transgene. Plasma was assayed for lipid and SNC and brachiocephalic arteries were analyzed as described in the supplemental materials.

Statistical Analysis
Values are expressed as mean±SEM. Data were analyzed by ANOVA for multiple comparisons, and the Student Newman Keuls test was used. For experiments with 2 groups, the paired \(t\) test was used. Differences were considered significant when \(P<0.05\).

Results
Effect of SNC-Fc on VSMC Apoptosis
The effect of purified SNC-Fc and Fc (the control for SNC-Fc) on VSMC apoptosis was analyzed by staining for cleaved caspase-3, an effector caspase involved in the apoptotic pathway. SNC-Fc significantly reduced VSMC apoptosis in a dose-dependent manner (20, 100, and 200 pmol/L) when apoptosis was induced by suspension culture. SNC at 20 pmol/L also significantly reduced VSMC apoptosis induced by Fas-L or TNF\(\alpha\) by approximately 50%. VSMC apoptosis was not affected by Fc (Figure 1A). Interestingly, the survival effect of SNC-Fc on Fas-L–treated VSMCs was as potent and as effective as the well documented survival factor,\(^{26}\) IGF-1 (SNC-Fc inhibited apoptosis by 57±15% versus 51±15% with 20 pmol/L IGF-1, \(n=4\)). No additional benefit was obtained by combined treatment (results not shown).
Mechanism of Action of SNC

SNC significantly reduced both the ability of single VSMCs to aggregate (18.5±2.7 versus 8.4±2.7% cells in aggregates, n=3, P<0.05) and form cell–cell adhesions (14.8±1.3 versus 10.5±1.2% adhesion, n=3, P<0.05), indicating SNC acts as a mimic interfering with cell–cell association. In contrast SNC had no effect on adhesion or aggregation of VSMCs with established cell–cell contacts.

Inhibitors were used to investigate the involvement of FGF-R1 (SU5402), PI-3 kinase (Wortmannin) and Akt (Akt inhibitor 1) in the prosurvival effect of SNC-Fc. All inhibitors resulted in complete ablation of the prosurvival effect of SNC-Fc, suggesting that all 3 factors are essential (Figure 1B). After 24 hours, siRNA knockdown of FGF-R was 85±7% (n=3, P<0.05) by quantitative polymerase chain reaction (PCR) and confirmed by immunocytochemistry (data not shown). Knockdown of FGF-R with siRNA confirmed the requirement for FGF-R in the prosurvival effect of SNC-Fc, suggesting that all 3 factors are essential (Figure 1C). Significantly increased phosphorylation of Akt was demonstrated by Western blotting after 10 minutes of SNC-Fc treatment (120±1% versus 100±1%, n=3, P=0.0037; Figure 2A). SNC-Fc treatment also increased (and thereby inactivated) phosphorylated Bad (serine 136) after 30 minutes, providing a direct link to cell survival (Figure 2B). Immunocytochemistry after 24 hours of SNC-Fc treatment confirmed the Western blot results (Figure 2C) and importantly, the increased nuclear pAkt was significantly attenuated by the PI-3 kinase and FGF-R inhibitors (Figure 2D), indicating that SNC-Fc caused activation of Akt via FGF-R and PI-3 kinase signaling.

To explore the mechanism by which SNC-Fc activated the FGF-R, we used peptides to inhibit the interaction between SNC-Fc and N-cadherin or FGF-R, as HAV is a common binding motif in N-cadherin and FGF-R (supplemental Figure I). Two HAV peptides were used to inhibit these interactions: HAV, a generic peptide preventing binding of SNC-Fc to both full-length N-cadherin and FGF-R, and HAVDI, a specific peptide preventing binding of SNC-Fc to N-cadherin, but permitting FGF-R binding. Treatment with HAV completely ablated the prosurvival effect of SNC-Fc compared to the control (HGVDI) peptide, whereas treatment with the HAVDI peptide reduced but did not completely ablate the prosurvival effect of SNC compared to the control (HGVDI) peptide. In fact, the effects of HAV and HAVDI were significantly different, suggesting that, although interaction of SNC-Fc with N-cadherin was required, binding through an alternative HAV containing molecule such as FGF-R is essential for the prosurvival effect of SNC-Fc.

The involvement of β-catenin in the prosurvival effect of SNC-Fc was investigated using TOPGAL aortic VSMCs, which have the β-galactosidase gene under the control of a minimal promoter containing β-catenin responsive elements. Beta-galactosidase activity was quantified using a chemiluminescent assay called Galactolight. No change in β-catenin signaling was observed after addition of SNC-Fc (Fc: 3.3±0.8, SNC-Fc 3.2±0.7, arbitrary units, n=3).

Effect of SNC-Fc on Monocyte/Macrophage Behavior In Vitro

Macrophages express N-cadherin mRNA at a similar level to VSMCs, whereas expression in monocytes is lower (supplemental Figure II). FGF-R1 mRNA was also detected in monocytes and macrophages but at lower levels than in VSMCs. Immunohistochemistry confirmed expression of both N-cadherin and FGF-R1 in macrophages in atherosclerotic plaques (supplemental Figure II). Apoptosis of macrophages and foam cell–macrophages was significantly attenuated by SNC-Fc by >50% in vitro (11.3±0.7 versus 23.6±2.3% and 1.8±0.6 versus 8.5±3.5%, respectively). However, addition of SNC-Fc did not affect monocyte invasion compared to Fc (8.6±1.0 versus 8.0±0.7 cells per field, n=4, P>0.05). Similarly, addition of SNC-Fc had no effect on macrophage invasion.
proliferation compared to Fc control (16.6 ± 1.8 versus 15.7 ± 1.8 cells per field, n = 3, P < 0.05).

**Effect of SNC-Fc on Plaques**

Plasma concentrations of SNC-Fc were significantly increased in mice infected with RAd SNC-Fc compared to control mice (PBS control mice or mice infected with RAd Fc) at 2, 6, and 8 days postinfection (Figure 3). Importantly, there were no significant differences in the plasma levels of low-density lipoprotein between groups (PBS: 10.5 ± 0.3 mmol/L, Fc: 8.1 ± 1.6 mmol/L, SNC-Fc: 10.6 ± 0.2 mmol/L). Additionally, no adverse effects in the mice and no alteration to the histological appearance of the thoracic aorta or carotid arteries in which atherosclerosis is absent were observed (data not shown).

Fluorescent immunohistochemistry for cleaved caspase-3 and in situ end labeling (ISEL) showed that apoptosis was significantly reduced by ≈60% in brachiocephalic artery plaques from mice infected with RAd SNC-Fc compared to the controls (Figure 4; supplemental Table I). Dual immunohistochemistry for ISEL and α-smooth muscle cell actin or Mac-2 revealed SNC-Fc significantly reduced the percentage of apoptotic VSMCs and macrophages compared to the controls (Figure 4D through 4I and 4M). Additionally the amount of pAkt was significantly increased in plaques from animals infected with RAd SNC-Fc compared to controls (Figure 4J through 4L).

Interestingly, although RAd SNC-Fc infection had no effect on brachiocephalic artery atherosclerotic plaque area, it...
did affect composition (Figure 5; Table). Increased plasma SNC-Fc levels significantly elevate the ratio of smooth muscle cells to macrophages and the proportion of the lumen covered in VSMC-rich fibrous cap (supplemental Table I). Additionally, it reduced the number of buried layers (a surrogate marker of plaque rupture) and the percentage of the plaque occupied by macrophages (Table). Together these findings are indicative of a more stable plaque phenotype. Infection with RAd SNC-Fc caused no significant change in any of the other parameters measured (Table).

Discussion

VSMC apoptosis is a major contributor to atherosclerotic plaque rupture and therefore myocardial infarction. Consequently, strategies to reduce VSMC apoptosis have potential for plaque stabilization. We previously showed that N-cadherin, the predominant cadherin expressed by VSMCs, inhibits VSMC apoptosis. In this study we examined whether a soluble form of N-cadherin acts as a mimetic, providing a survival signal for VSMCs. In addition, we evaluated whether reduction of VSMC apoptosis by SNC promotes atherosclerotic plaque stability.

VSMC apoptosis was stimulated in vitro by 3 inducers that are relevant to atherosclerosis: Fas-L or TNF-α (extrinsic apoptotic pathway inducers) and culture in suspension (activator of intrinsic and extrinsic apoptotic pathways). SNC significantly reduced VSMC apoptosis by all inducers. Although we previously showed that full-length N-cadherin reduces VSMC apoptosis, these findings with SNC are important as this small soluble molecule is more versatile for in vivo and clinical studies.

We also investigated the mechanism underlying the pro-survival effect of SNC. SNC significantly reduced the formation of cell–cell interactions, the opposite of full-length N-cadherin. This indicates SNC acts as a mimetic reducing the ability to form homophilic cell–cell interactions and activating signaling pathways, a concept suggested previously. We investigated 3 potential signaling pathways: FGF-R signaling, PI-3 kinase signaling, and β-catenin signaling. Interaction between N-cadherin and FGF-R has been previously proposed via HAV binding motifs. We found that inhibiting or siRNA knockdown of FGF-R1 prevented the prosurvival effect of SNC, suggesting it is the major pathway. Further support was obtained from the HAV peptide experiments, which suggested that an additional HAV containing molecule other than N-cadherin (ie, FGF-R) was required for the prosurvival effect.

Previous research in various cell types showed N-cadherin based adhesive contacts recruit PI-3 kinase and stimulate membrane recruitment of Akt. Interaction between N-cadherin and FGF-R has been previously proposed via HAV binding motifs. We found that inhibiting or siRNA knockdown of FGF-R1 prevented the prosurvival effect of SNC, suggesting it is the major pathway. Further support was obtained from the HAV peptide experiments, which suggested that an additional HAV containing molecule other than N-cadherin (ie, FGF-R) was required for the prosurvival effect.

Table. Effect of SNC-Fc on Plaques

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle Control (PBS)</th>
<th>Fc</th>
<th>SNC-Fc</th>
</tr>
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<tbody>
<tr>
<td>Cleaved-caspase-3, % total cells</td>
<td>11.5±0.8%</td>
<td>12.8±2.7%</td>
<td>5.0±0.8%*</td>
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<tr>
<td>VSMCs, % total plaque area</td>
<td>25.8±3.8%</td>
<td>28.7±4.1%</td>
<td>28.8±3.4%</td>
</tr>
<tr>
<td>Fibrous cap coverage, % total plaque area</td>
<td>35.8±7.3%</td>
<td>35.7±6.4%</td>
<td>79.4±3.4%*</td>
</tr>
<tr>
<td>Macrophages, % total plaque area</td>
<td>10.0±3.1%</td>
<td>11.9±3.8%</td>
<td>2.6±1.0%*</td>
</tr>
<tr>
<td>VSMC:Macrophage ratio</td>
<td>1.4±0.5</td>
<td>2.2±0.6</td>
<td>9.4±3.6*</td>
</tr>
<tr>
<td>No. of buried layers</td>
<td>0.9±0.1</td>
<td>0.9±0.1</td>
<td>0.3±0.1*</td>
</tr>
<tr>
<td>Plaque area, (\times 10^3 \text{ mm}^2)</td>
<td>76.9±20.8</td>
<td>57.6±16.9</td>
<td>52.6±15.4</td>
</tr>
<tr>
<td>Collagen, % total plaque area</td>
<td>Polarized</td>
<td>8.6±1.8%</td>
<td>15.4±6.0%</td>
</tr>
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<td></td>
<td>Brightfield</td>
<td>31±5.0%</td>
<td>25±5.6%</td>
</tr>
<tr>
<td></td>
<td>Elastin content, % total plaque area</td>
<td>19.8±6.4%</td>
<td>6.3±2.5%</td>
</tr>
<tr>
<td></td>
<td>Lipid content, % total plaque area</td>
<td>51.9±5.6%</td>
<td>58.7±6.6%</td>
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<tr>
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<td>Necrotic core, % total plaque area</td>
<td>19.7±3.3%</td>
<td>21.9±3.7%</td>
</tr>
<tr>
<td></td>
<td>Endothelial coverage, %</td>
<td>78±14%</td>
<td>71±14%</td>
</tr>
<tr>
<td></td>
<td>PCNA, % total cell No.</td>
<td>30±9.5%</td>
<td>28.9±8.4%</td>
</tr>
</tbody>
</table>

*Significant difference from PBS and Fc.
The involvement of β-catenin signaling in the prosurvival effect of SNC-Fc was also investigated. Interestingly, although SNC-Fc mimics cell–cell junctions, the prosurvival effect of SNC-Fc is β-catenin signaling independent.

We investigated whether SNC-Fc affected the stability of advanced brachiocephalic artery atherosclerotic plaques in fat-fed ApoE−/− mice. The direct effect of therapeutic potential of reducing VSMC apoptosis on plaque instability has remained relatively unstudied. Adenoviral infection of hepatocytes caused a transient increase in transgene plasma levels, which was detectable until day 8 as previously observed, and significantly reduced apoptosis in the plaque. Immuno-histochemistry detected abundant N-cadherin and FGF-R in the plaques, providing a potential mechanism of action for SNC-Fc. Dual immunohistochemistry for ISEL and α-smooth muscle cell actin or Mac-2 revealed that SNC-Fc significantly reduced VSMC and macrophage apoptosis, complementing our in vitro findings. Additionally pAkt was increased in plaques from animals infected with RAd SNC-Fc, supporting our in vitro mechanism data.

RAd SNC-Fc infection also significantly increased the VSMC:macrophage ratio and the presence of a VSMC-rich fibrous cap, while significantly reducing the number of macrophages and buried layers (a surrogate marker of plaque rupture). These properties are all indicative of increased plaque stability. This highlights for the first time the involvement of cadherins in atherosclerosis, and provides the first evidence that inhibiting VSMC apoptosis increases plaque stability.

Increased coverage of the lumenal surface with a VSMC-rich fibrous cap was observed in the RAd SNC-Fc–infected mice, which is indicative of a more stable phenotype. However, although SNC-Fc reduced VSMC apoptosis, the number of VSMCs was not increased. We suggest that this is attributable to reduced plaque disruption. In control plaques we speculate that increased growth of VSMCs is necessary for new fibrous cap formation after disruption, consequently loss of VSMC number attributable to apoptosis is counter-balanced. Although in SNC-Fc–treated animals plaque disruption and therefore formation of a new fibrous cap is ablated, the reduction in VSMC apoptosis does not increase VSMC content.

Interestingly we also observed a significant reduction in macrophage content in the plaques. Our in vitro data, however, indicate that the reduction in macrophage number is not attributable to altered monocyte invasion or macrophage proliferation. We propose that the observed decrease in macrophage content is as a result of reduced inflammation. This occurs through reduced VSMC and macrophage death and retarded plaque disruption, therefore suppressing macrophage infiltration and proinflammatory cytokines production. However, we did not detect a reduction in the size of the necrotic core, which may be because of the short time course of our studies and that a longer period may be required to detect a decrease in the necrotic core size.

Additionally, SNC-Fc did not affect size or composition of plaques from proximal aorta and aortic sinus (data not shown). We propose that as plaque rupture has not been reported at these sites, perhaps VSMC apoptosis is not as prevalent and therefore SNC-Fc has no detectable effect.

In summary, this study has illuminated SNC-Fc as a successful prosurvival factor for VSMCs in vitro via FGF-R, PI-3 kinase, and Akt signaling. Interestingly, the effect of SNC-Fc may not be restricted to VSMCs but may also have beneficial effects on macrophage and foam cell–macrophage survival, which we hope to investigate more thoroughly in the near future. Most importantly, in the ApoE−/− mouse model of atherosclerosis, RAd SNC-Fc infection reduced apoptosis in the plaques formed in the brachiocephalic artery, and increased plaque stability, suggesting that it may have therapeutic potential in the treatment of atherosclerosis.

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Disclosures
None.

References
12. Tran NL, Adams DG, Vaillancourt RR, Heimark RL. Signal transduction from N-cadherin increases Bcl-2: regulation of the phosphatidylinositol


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Supplementary material

Supplementary Methods

Adenovirus production
The Fc domain, containing mutations in the IgG-R and complement binding domains, was amplified by PCR from an IL-10-Fc fusion plasmid (generously provided by Dr Terry Storm, Harvard University, USA). The extracellular domain of mouse N-cadherin was cloned with the Fc domain into pDC515 (Microbix). The secretion signal was cloned from full length N-cadherin onto the Fc fragment and cloned into pDC515. Both were recombined with the adenovirus genomic plasmid by co-transfection into 293 cells. The resultant adenoviruses were termed RAd SNC-Fc and RAd Fc (control). This chimeric molecule comprised of SNC and an antibody constant domain, enables protein A binding, as well as extending plasma half-life\(^1\)\(^-\)\(^3\) and possibly increasing receptor–ligand interaction. Such immunoadhesins have use in vivo as potential therapeutic agents\(^4\).

Purification and culture of mouse blood monocytes
Mouse peripheral blood monocytes were purified by Ficoll-Hypaque gradient (Ficoll-Paque Plus: Amersham Biosciences), followed by differential adherence and culture in 20ng/ml M-CSF for 7-10 days to induce differentiation into macrophages.

Purification of rabbit foam cell-macrophages
New Zealand White rabbits (Harlan, UK) fed a 1% cholesterol-supplemented diet had sterile sponges placed under the dorsal skin to generate foam cell macrophages, as described\(^5\).

siRNA Knockdown
Two HP validated silencing RNA oligonucleotides (siRNA) for FGF-R1 and control (Allstars Negative control siRNA) were purchased from Qiagen (catalogue numbers SI02224677, SI02224684 and 1027281). VSMCs (8x10\(^5\)) were subjected to Amaxa nucleofection with
250pmol of FGF-R1 or control siRNAs using the VSMC kit and U-25 program following the manufacturer's instructions (Amaxa, Inc., Cologne Germany). Treated cells were analysed 24 hours after nucleofection. Knockdown of FGF-R was estimated as 85±7% (n=3, p<0.05) by quantitative PCR and was confirmed by immunocytochemistry (data not shown).

**Western blotting**

SDS lysed cell extracts were subjected to Western blotting as described previously. Blots were incubated overnight at 4°C with primary antibodies diluted in Starting block (Pierce, Chester, UK). Antibodies were used at the following concentrations: total and pAkt (Cell Signalling, 1:1000). Bound antibodies were detected by rabbit anti-mouse horseradish peroxidase conjugated antibodies (Dako, High Wycombe, UK) and enhanced chemiluminescence (Amersham International, Little Chalfont, UK).

**Galactolight assay**

TOPgal transgenic mouse VSMCs were grown from aortic explants, as described previously. These VSMCs contain the β-galactosidase gene under the control of the β-catenin promoter. TOPgal VSMCs were treated with 0.01% (w/v) trypsin, 1 mM CaCl₂ in PBS) and seeded into a 24 well plate in the presence of SNC-Fc or Fc and Fas-L. β-galactosidase activity was quantified using a chemiluminescent assay called Galactolight as described by the manufacturer's instructions (Tropix).

**Aggregation and adhesion assays**

Cell aggregation and cell-cell adhesion were determined as previously.

**Invasion of monocytes and proliferation of macrophages**

Mouse peripheral blood monocyte invasion and macrophage proliferation were assessed as previously.
**Quantitative PCR**

Total RNA was isolated by the RNAeasy kit (Qiagen) was reverse transcribed and subjected to quantitative PCR for N-cadherin and FGF-R using Quantitect primers (Qiagen, QT00102837 and QT00198548, respectively) as described by the manufacturer.

**In vivo experiments**

*Quantification of plasma SNC-Fc and lipoprotein levels*

Plasma samples were taken at 2, 6, 8, 14 and 28 days after RAad administration and levels of SNC-Fc were analysed by ELISA as described previously\(^6\). Plasma lipid profiles were analyzed in terminal plasma samples as previously described\(^11\).

**Immunohistochemistry**

VSMCs, macrophages, and proliferating and apoptotic cells were identified by immunohistochemistry for α-smooth muscle cell actin, Mac-2, proliferating cell nuclear antigen (PCNA) and CC-3 as described previously\(^10\). Fluorescent immunohistochemistry for pAkt was performed using rabbit anti-pAkt antibody (Cell Signalling) diluted 1:25. Fluorescent dual immunohistochemistry for Mac-2 and N-cadherin or FGF-R1 was performed on control atherosclerotic plaques using 5 μg/ml rat anti-Mac-2 (Cedar Lane) and 2 μg/ml rabbit anti-N-cadherin (Santa Cruz) or rabbit anti-FGF-R1 (Cell Signalling) diluted 1:12.5.

*In situ end labelling and actin dual immunohistochemistry*

Apoptotic cells were identified by *in situ* end labelling (ISEL), performed as previously described\(^12\). This was followed by the smooth muscle α-actin immunohistochemistry protocol outlined above.
Identification of buried fibrous caps

Serial sections stained for elastin and α-smooth muscle cell actin were examined for the presence of structures rich in elastin and VSMCs and these were identified as buried fibrous caps, a surrogate marker of previous plaque instability, as previously described13.

References


Supplementary Figure Legends

Supplementary Figure I: The pro-survival effect of SNC requires the HAV motif.
Percentage of apoptotic VSMC (CC-3 ICC) 24 hours after FasL treatment with peptide (n=3).*significant difference from Fc control, §significant difference from HAV.

Supplementary Figure II: Expression of N-cadherin and FGF-R1 in monocytes and macrophages.
Table shows quantitative PCR results for N-cadherin and FGF-R1 in monocytes, macrophages and VSMCs expressed as copy number of mRNA.
Images show representative dual immunohistochemistry for N-cadherin (A, D) or FGF-R1 (B, E) in green and macrophages in red in PBS control atherosclerotic plaque. Arrowheads indicate macrophages expressing N-cadherin or FGF-R. Scale bar in panel A represents 50 μm and applies to panels A-C. Scale bar in panel D represents 15 μm and applies to panels D-F. Non-immune IgG is shown as negative control (C, F). Nuclei are stained blue with DAPI.

Supplementary Figure III: Schematic diagram of the mechanism of action of SNC
SNC may interact with full length N-cadherin which in turn binds to FGF-R or it may bind directly with FGF-R and full length N-cadherin. These interactions activate PI3-kinase and thereby activate Akt. Active Akt phosphorylates Bad which inhibits the interaction with Bcl-2, released Bcl-2 then provides a survival signal for VSMCs, inhibiting apoptosis.
Supplementary Figure II

<table>
<thead>
<tr>
<th>Copy number of mRNA</th>
<th>N-cadherin</th>
<th>FGF-R1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monocytes</td>
<td>13.7±13.7</td>
<td>4.2±2.3</td>
</tr>
<tr>
<td></td>
<td>(n=4)</td>
<td>(n=3)</td>
</tr>
<tr>
<td>Macrophages</td>
<td>1104±449</td>
<td>6.0±4.5</td>
</tr>
<tr>
<td></td>
<td>(n=4)</td>
<td>(n=3)</td>
</tr>
<tr>
<td>VSMCs</td>
<td>1707±871</td>
<td>168±76</td>
</tr>
<tr>
<td></td>
<td>(n=6)</td>
<td>(n=3)</td>
</tr>
</tbody>
</table>

N-cadherin  FGF-R  IgG