

Original Article

Endothelial MMP-9 drives the inflammatory response in abdominal aortic aneurysm (AAA)

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Received June 21, 2017; Accepted October 15, 2017; Epub December 15, 2017; Published December 30, 2017

Abstract: Progression of abdominal aortic aneurysm (AAA) is typified by chronic inflammation and extracellular matrix (ECM) degradation of the aortic wall. Vascular inflammation involves complex interactions among inflammatory cells, endothelial cells (ECs), vascular smooth muscle cells (vSMCs), and ECM. Although vascular endothelium and medial neoangiogenesis play a key role in AAA, the molecular mechanisms underlying their involvement are only partially understood. In AAA biopsies, we found increased MMP-9, IL-6, and monocyte chemoattractant protein-1 (MCP-1), which correlated with massive medial neo-angiogenesis (C4d positive staining). In this study, we developed an *in vitro* model in order to characterize the role of endothelial matrix metalloproteinase-9 (e-MMP-9) as a potential trigger of medial disruption and in the inflammatory response bridging between ECs and vSMC. Lentiviral-mediated silencing of e-MMP-9 through RNA interference inhibited TNF-alpha-mediated activation of NF-κB in EA.hy926 human endothelial cells. In addition, EA.hy926 cells void of MMP-9 failed to migrate in a 3D matrix. Moreover, silenced EA.hy926 affected vSMC behavior in terms of matrix remodeling. In fact, also MMP-9 in vSMC resulted inhibited when endothelial MMP-9 was suppressed.

Keywords: Abdominal aortic aneurysm, MMP-9 silencing, endothelium dysfunction, inflammatory response, vascular remodeling

Introduction

Abdominal aortic aneurysm (AAA) is a multifactorial degenerative disease characterized by complex interactions among genetic, inflammatory, and hemodynamic factors [1]. The development of AAA is associated with medial degeneration due to disaggregation of the lamellar organization of elastic fibers. This degenerative process is also associated with inflammatory infiltrate in the adventitia and tunica media, degradation of the extracellular matrix (ECM), including elastin fragmentation, apoptosis of vascular smooth muscle cells (vSMCs), medial neoangiogenesis as well as thinning of the tunica media [2].

There are a number of etiological factors of AAA such as hypertension, atherosclerosis, inflammation, oxidative stress, hypoxia, aging, and congenital malformations [3, 4]. With the

exception of congenital malformations, the processes involved in AAA formation are generally associated with upregulation of matrix metalloproteinases (MMPs) activity.

MMPs belong to a family of proteolytic enzymes that degrade several components of the ECM. All MMPs are synthesized as pre-pro-enzymes and secreted as inactive pro-MMPs. The majority of MMPs are able to cleave and activate the pro-forms of other MMPs, thereby acting in protease cascades that can amplify MMP proteolytic activity [5, 6]. Many MMP genes are inducible by a wide array of effectors including growth factors and cytokines.

Matrix metalloproteinase-9 (MMP-9), first termed as 92-kDa type IV collagenase or gelatinase B, belongs to the MMP family and plays a major role in the degradation of the ECM in a large spectrum of physiological and pathophysiological

ological processes involving tissue remodeling. Importantly, MMP-9-mediated proteolysis has been shown to regulate immune cell function in animal model of arthritis, diabetes, and cancer [7]. Among the cytokines able to regulate MMP-9 expression, an important role has been assigned to tumor necrosis factor- α (TNF- α). In this regard, MMP-9 is positively regulated at the transcriptional level by multiple factors including nuclear factor- κ B (NF- κ B) [8]. Before it is secreted, pro-MMP-9 forms a complex with TIMP-1 in the Golgi apparatus [9]. Several ECM proteins are proteolytically processed by MMP-9, including collagen, elastin, fibronectin, and laminin. ECM fragments are known to express bioactive properties and regulate vascular remodeling [10].

The inflammatory response plays a key role in the development of AAA, which also involves the infiltration of several immune cells such as macrophages and T cells [11, 12]. In this regard, monocytes and circulating blood leukocytes, which mediate the inflammatory response, have been implicated in vascular wall remodeling as well as AAA progression [13-15]. MMP-9 is secreted by a wide variety of inflammatory cells including macrophages and neutrophils. Monocytes activity during the inflammatory response is modulated by several mediators such as cytokines, chemokines, and growth factors. In this context, the upregulation of different adhesion molecules on the endothelial layer favors the interactions with circulating monocytes. During this process, pro-inflammatory cytokines such as TNF- α and IL-1 β promote the secretion of MMPs via mitogen-activated protein kinase (MAPK)-mediated pathways [16].

Endothelial cells (ECs) can both synthesize and react to a wide variety of inflammation-related mediators. For example, cytokine-treated ECs express several endothelial-leukocyte adhesion molecules (ELAMs) such as E-selectin as well as integrin-ligands such as intercellular adhesion molecule-1 (ICAM-1). In addition, upregulation of vascular cell adhesion molecule-1 (VCAM-1) expression on activated ECs promotes endothelial-leukocyte interaction. Activated ECs are also a source of chemoattractant cytokines (i.e. chemokines) which contribute to leukocytes adhesion by enhancing their affinity.

In this study, we have investigated the potential contribution of medial neo-angiogenesis during ECM remodeling in AAA with particular regards to the related inflammatory response. *In vitro* model suggests that ECs play a major role in the maintenance of the inflammatory loop *in situ*, which might ultimately result in AAA progression.

Materials and methods

Human aortic samples

Fifteen human samples of AAA were surgically recovered; control aortic tissues were obtained from autopsies. All aneurysm samples were collected from donors correctly informed for the use of excessive pathological material for diagnostic and research purposes according to the local institute's regulations and policies.

Tissue samples were fixed in neutral buffered formalin for a maximum of 24 hours and 5 mm-thick sections were obtained from paraffin-embedded samples. For tissue immunofluorescence, samples were incubated with IL-6 (ab9324; Abcam, Italy) and MCP-1 (ab9669; Abcam, Italy) antibodies. Subsequently, they were incubated with a TRITC or FITC-conjugated secondary antibody (Vector, CA, USA). DAPI (4',6-Diamidino-2-phenylindole, Sigma Aldrich, Italy) was used for nuclear staining. Rehydrated sections were stained with haematoxylin-eosin. Immunohistochemistry with rabbit polyclonal anti-human C4d antibody has been performed by using an automated immunostainer (Ventana, Roche, Italy). All images were acquired using Panoramic MIDI 3DHISTECH and analyzed with Panoramic Viewer software (3DHISTECH, Hungary). For immunohistochemistry, primary antibodies (MMP-9 ThermoFischer, Italy) were bound and detected using the VectaStain Elite Kit. Reveal was performed by the use of diaminobenzidine (Vector, UK). Hematoxylin was used as counter staining (Sigma, Italy).

Fresh tissues were lysed in RIPA buffer (150 mM sodium chloride, 1% Triton X100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM EDTA, 1 mM EGTA, 50 mM TRIS pH = 8) supplemented with protease inhibitors (all from Sigma Aldrich, Italy). Non-reduced protein samples were resolved by SDS-PAGE gels containing gelatin (0.2%, Sigma Aldrich). Briefly, after electrophoresis, gels were incubated with

TRITON X-100 for 3 h at room temperature, and then incubated in a solution of CaCl_2 (1 mM) and NaCl (15 mM), pH 7.4 overnight at 37°C. Subsequently, gels were fixed and then stained with Coomassie Blue. For objective quantification ImageJ software was used.

Cell culture

Human endothelial cells, EA.hy926 (ATCC® CRL-2922™) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) additioned with 10% fetal bovine serum and penicillin (100 U/mL), streptomycin (100 µg/mL), and 2 mM glutamine mixture (all from Euroclone, Italy) at 37°C in humid 5% CO_2 atmosphere. Primary human aortic vSMCs (ATCC® PCS-100-012™) were cultured in Vascular Cell Basal Medium (ATCC® PCS-100-030™) in presence of Vascular Smooth Muscle Cell Growth Kit (ATCC® PCS-100-042™) and Penicillin-Streptomycin-Amphotericin B Solution (ATCC® PCS-999-002™). vSMCs were used up to the 8th passage. U-937 (ATCC® CRL-1593.2™), human monocytes, were used for adhesion assays on endothelial monolayer.

LV production and transduction

MMP-9 silencing was performed in EA.hy926 cells using lentiviral vector (LV) expressing shRNA targeting the human MMP-9. Two different shRNAs DNAs sequences were purchased: shRNA1, TRCN0000373061-CCGGGCCGATCAAACTGGTATTCTCGAG; and shRNA5, TRCN0000051438-CCGGCCACAACATCACCTATTGATCTCGAGATCCAATAGGTGATGTTGTGGTTTTT. Third-generation LVs were generated as previously described [17]. 293T cells were cotransfected by calcium phosphate precipitation with the following four plasmids: pMDLg/RRE packaging plasmid; pMD2.VSV-G envelope-coding plasmid; pRSV-Rev and transfer vector plasmids for MMP-9 silencing as reported above. Thirty hours after transfection, culture supernatants containing the viral particles were collected and concentrated by ultracentrifugation. Collected viral particles were used to transduce 5×10^4 EA.hy926 with two different LVs (shRNA1 and shRNA5). After 3 days of culture, cells were selected with puromycin (2.5 µg/mL) (Sigma, Aldrich, Italy) and used for the experiments.

MMP-9 silencing evaluation

For immunoblotting, cells were lysed in RIPA buffer supplemented with protease inhibitors. Proteins concentration was determined using the bicinchoninic acid assay (Pierce, Rockford, IL, USA). 50 µg total proteins in sample buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 5% β-mercaptoethanol, 0.5% bromophenol blue) were subject to SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK). Membranes were incubated overnight with the MMP-9 antibody at 4°C. Proteins were revealed with secondary antibody-peroxidase conjugates (Perkin-Elmer, anti-rabbit NEF812001EA). Protein bands were visualized using ECL (Perkin-Elmer, Western lightning PLUS-ECL) detection reagents in a chemosensitive visualizer (VersaDoc, BioRad, Italy). Results were normalized according to tubulin expression (DM1A; Millipore, Italy). MMP-9 in the culture media was analyzed by zymography assay as described above.

For immunofluorescent assays, cells ($1 \times 10^4/\text{cm}^2$) were cultured on cover slips in presence of TNF-α (50 ng/mL) enriched medium and TNF-α-free medium as control. Cells were fixed in 4% formalin and incubated with anti-MMP-9. Then, anti-mouse antibody TRITC-conjugated was used. After nuclear staining with DAPI, cells were observed under a fluorescent microscope (DM2500 Leica, Germany).

Cytokines antibody arrays and immunofluorescent microscopy on EA.hy926 cells

Supernatants collected from wild type (WT) or EA.hy926 cells transduced (shRNA5+1) treated with human recombinant TNF-α (50 ng/mL) for 24 h were assayed for a wide panel of cytokines using a human antibody array (Panomics, Italy) following the manufacturer's protocol. Cytokines panel was visualized using enhanced chemiluminescence ECL detection reagents in a chemosensitive visualizer (VersaDoc, BioRad, Italy).

For immunofluorescence, anti-MCP-1 (ab9669, Abcam, UK) and anti-E-selectin (sc-14011, Santa Cruz Biotechnology, USA) CD62E primary antibodies were used following the procedures, as above described.

Table 1. Clinical characteristic of AAA patients

Years (mean ± SD)	75±6
Sex	Male (89%)
DAAA (mean ± SD)	5.1±0.5 cm
Hypercholesterolemia (%)	44%
Smoking (%)	66%
Hypertension (%)	89%
Ischemic cardiomyopathy	56%
COPD	44%
CRF (%)	22% stage 2 22% stage 3
Type 2 Diabetes	0%
B-Blockers	56%
Statins	44% Simvastatin 11% Atorvastatin
Antiplatelet drugs	22% Clopidogrel 44% Acetylsalicylic acid
Oral anticoagulations	11% Warfarin
ACE inhibitors	56%

Leukocyte-endothelium adhesion assay

U937-EA.hy926 cell adhesion assay was performed using Cell Biolabs' CytoSelect™ Leukocyte-endothelium Adhesion Assay. Wild-type or transduced EA.hy926 cells were stimulated with 50 ng/mL TNF- α for 4 h. Subsequently, U937 cells were labeled by the LeukoTracker™ solution. Labeled U937 cells were then incubated with WT or shRNA5+shRNA1-transduced cells. After 1 h of incubation, nonadherent cells were removed by gently rinsing with PBS. Adherent monocytes were counted in three separate fields per well using an inverted fluorescence microscope (DM2500 Leica, Germany).

NF- κ B activation

To characterize the activation of NF- κ B, 1×10^4 ECs were cultured on cover slips and cultured in serum-free media overnight. After 10', 30', and 1 h of TNF- α stimulation, cells were lysed or fixed and subject to immunoblotting or immunofluorescence analysis using an anti-phospho-p65-Ser-536 antibody (sc-33020, Santa Cruz, Biotechnology, USA) or an anti-p65 antibody (F-6, sc-8008, Santa Cruz, Biotechnology, USA) to normalize.

Tube formation assay

Growth Factor-reduced Matrigel (Corning, USA) enriched with VEGF (200 ng/mL), was plated in

a 24 multi-well. After Matrigel jellification (37°C for 30 minutes), EA.hy926 wild-type and transduced cells were trypsinized and seeded (1×10^5 cells/well) in each well with 300 μ l of culture media. Cells were incubated up to 6 h at 37°C. Each well was observed and images were acquired using an inverted microscope with digital camera (DMIL Leica, Germany).

Preparation of conditioned media (CM) and silver staining

2.13×10^4 /cm² endothelial cells (wild-type and transduced cells) were grown to subconfluent density in 6-well culture plates in 10% serum-containing medium. After 16 h, human recombinant TNF- α (50 ng/mL; Sigma Aldrich, Italy) was added to serum-free medium, while wild-type or transduced EA.hy926 cells grown in absence of TNF- α were used to determine MMP-9 basal expression. After 24 h, media were collected as conditioned media (CM) and transferred to human vSMCs (5.4×10^3 cells/cm²) for 24 h. VSMCs cultured in absence of CM were used as negative controls. Silver stain plus (161-0449 Bio-Rad, Italy) was performed on vSMC medium under different conditions described above according to the manufacturer's instructions. A congo red-elastin (5 mg; E0502 Sigma-Aldrich, Italy) (15U of E1250, Sigma-Aldrich, Italy) was used as a positive control of proteolytic digestion.

Statistical analysis

All experiments were performed in triplicate. All data are expressed as mean values \pm standard deviation. Student's t-test was performed to determine the statistical significance. *indicates $p \leq 0.05$ and **indicates $p \leq 0.001$.

Results

MMP-9 and neo-angiogenesis in AAA tissues

Clinic features of AAA patients, such as demographical data and cardiovascular risk, were recorded prospectively in a database (Table 1). Zymography assay of controls and aortic aneurysms showed a significant increase in MMP-9 expression in aneurysmal walls compared to controls (Figure 1A).

As expected, we observed high expression of MMP-9 in serial sections of AAA specimens, but not in healthy aortic tissues (Figure 1B). In

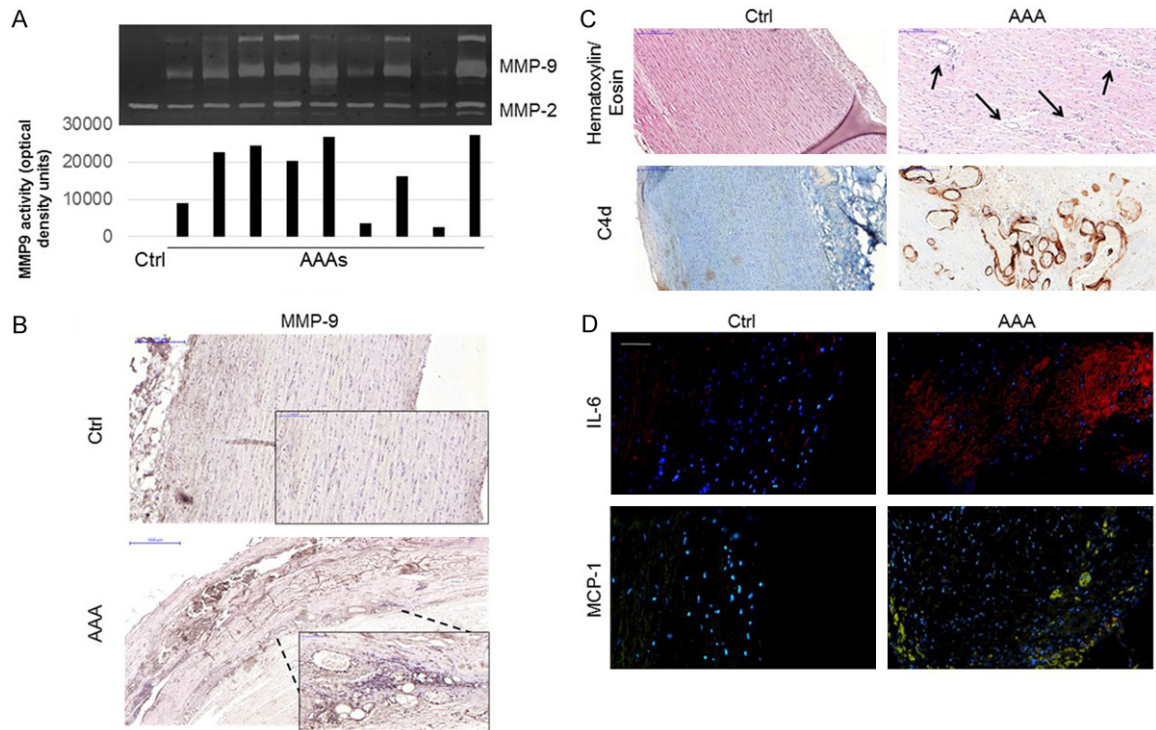


Figure 1. Histological features in human AAA biopsies. A. Zymography assay performed on fresh tissues and relative densitometry values normalized to normal abdominal aorta control (CTRL). N value = 3. B. Representative immunohistochemistry of MMP-9. CTRL scale bar: 200 μ m and 100 μ m; AAA scale bar: 1000 μ m and 100 μ m. C. Representative hematoxylin/eosin (E&E) staining and C4d staining. Arrows show neo-angiogenesis in the medial layer. Scale bar 200 μ m. D. MCP-1 and IL-6 immunostaining on CTRL and AAA samples. Scale bar 250 μ m.

these sections, MMP-9 was readily detected in the AAA inflammatory infiltrate. Vascular resident cells greatly contribute to the secretion of MMP-9. In good agreement with this, we could detect MMP-9 expression related to medial neo-angiogenesis where ECs are present (**Figure 1B**). Furthermore, the aneurysmal vessel wall microstructure resulted significantly altered, and neo-angiogenesis was evident in the medial layer (arrows in **Figure 1C**). Interestingly, we detected strong positive C4d immunostaining, indicating that an inflammatory signal specific for the neo-formed medial endothelial structure was underway (**Figure 1C**). Moreover, by immunofluorescence analysis we observed enhanced levels of both MCP-1 and IL-6 in AAA specimens. MCP-1 (red) was identified in both the endothelium and the medial layer. IL-6 (green) was predominantly detected in the adventitia, in proximity of *vasa vasorum*, and it was barely detectable in the media and intima. Abdominal aorta controls were negative for all these markers (**Figure 1D**).

MMP-9 silencing inhibits angiogenesis in EA.hy926 cells

Since ECs play a key role in wall dysfunction, and previous data on human samples implicated MMP-9 in aneurysm development, we sought to determine whether lack of endothelial MMP-9 could affect EC-mediated matrix remodeling. For this purpose, we silenced MMP-9 gene expression in EA.hy926 cells using lentiviral-transduced shRNA, and assessed the ability of these endothelial cells void of MMP-9 to promote tube formation *in vitro*.

First, we co-transduced EA.hy926 cells with a combination of various LVs: shRNA1+shRNA1; shRNA1+shRNA5; shRNA5+shRNA5, and shRNA5+shRNA1.

Subsequently, we measured MMP-9 protein expression in transduced cells treated with TNF- α (50 ng/mL). To compare the expression of MMP-9 in cell lysates and in supernatants, immunoblotting and zymography assay (**Figure 2A** and **Figure 2B** respectively) were performed.

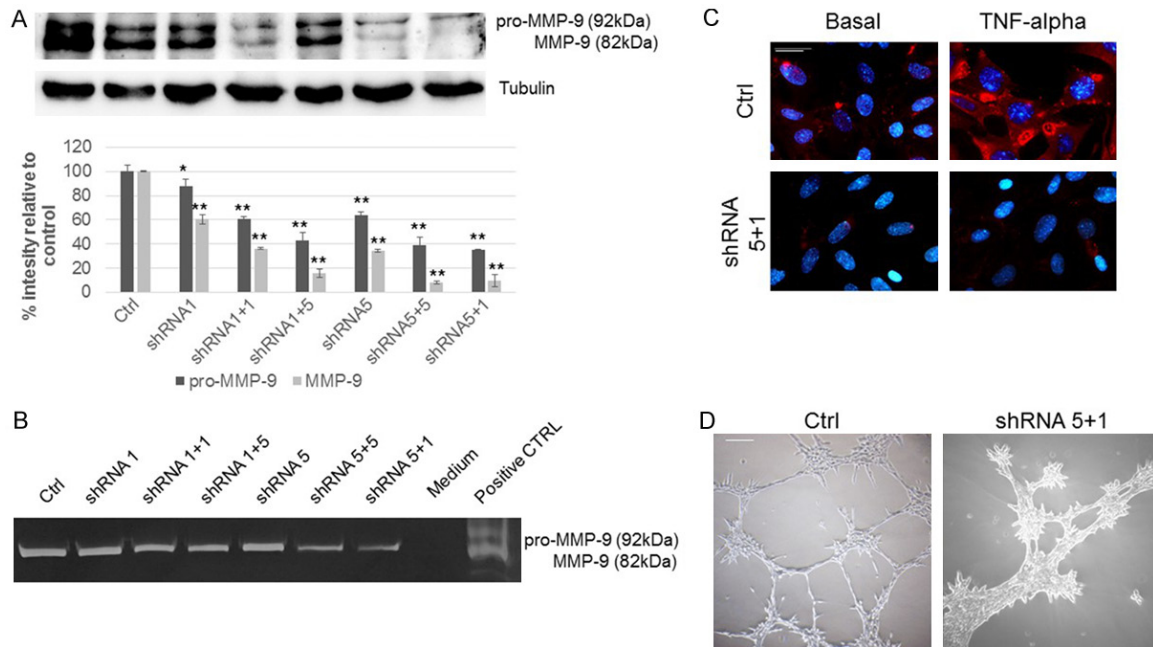


Figure 2. MMP-9 silencing EA.hy926 cells. A. Immunoblotting and relative densitometry of control and shRNA lentiviral-transduced EA.hy926 cells using antibodies against MMP-9 and tubulin. Basal control (without TNF- α treatment) did not express MMP-9 (data not shown). Relative densitometry values obtained from three different experiments are expressed as mean \pm SD. N value = 3. *indicates $p \leq 0.05$ and **indicates $p \leq 0.001$. B. Zymography assay performed on control and transduced EA.hy926 cells stimulated with human recombinant TNF- α (50 ng/mL). Peripheral blood was used as standard. Basal control (without TNF- α treatment) did not express MMP-9 (data not shown). N value = 3. C. Representative immunofluorescence staining for MMP-9 on wild-type and transduced cells. N value = 3. D. Tube formation assay in Matrigel Matrix. N value = 3. Scale bar 100 μ m.

As expected, we observed a significant down-regulation of both proMMP-9 and MMP-9 in lysates of shRNA5- and/or shRNA1-transduced EA.hy926 cells compared to control cells (Ctrl) (**Figure 2A**). Likewise, levels of secreted MMP-9 were significantly down-regulated following MMP-9 gene silencing, albeit to different extents (**Figure 2B**). Moreover, after TNF- α treatment wild-type EA.hy926 showed upregulation of MMP-9, which was strongly inhibited in shRNA5+shRNA1-transduced cells (**Figure 2C**).

When wild-type EA.hy926 cells were placed on growth factor-reduced Matrigel, we observed elongated, cross-linked, and robust cord-like structures upon VEGF stimulation. Remarkably, VEGF-induced tube formation was strongly impaired in EA.hy926 lacking of MMP-9 (**Figure 2D**).

TNF- α -mediated activation of NF- κ B is inhibited in MMP-9-silenced EA.hy926 cells

Since TNF- α induces the inflammatory response through NF- κ B activation in ECs, we sought to

determine whether MMP-9 silencing could affect TNF- α -mediated induction of NF- κ B. As expected, we observed an early and transient upregulation of phospho-p65 protein in lysates from wild-type EA.hy926 cells treated with TNF- α , whereas total p65 remained unchanged. In contrast, in EA.hy926 shRNA5+shRNA1-transduced cells, TNF- α -mediated phosphorylation of p65 was completely inhibited (**Figure 3A**). These results were further confirmed by immunofluorescence assay where a marked nuclear translocation was shown after 10 minutes in wild type cells treated with TNF- α (**Figure 3B**). After 30 minutes, a decrease of nuclear positive staining was observed, while no nuclear p-65 signal was detected after 60 minutes. On the other hand, in MMP-9 silenced cells no p-65 nuclear translocation was observed at any time point (**Figure 3C**).

MMP-9 regulates cytokine production by ECs

We sought to determine whether endothelial MMP-9 could promote a pro-inflammatory auto-crine loop through regulation of cytokines

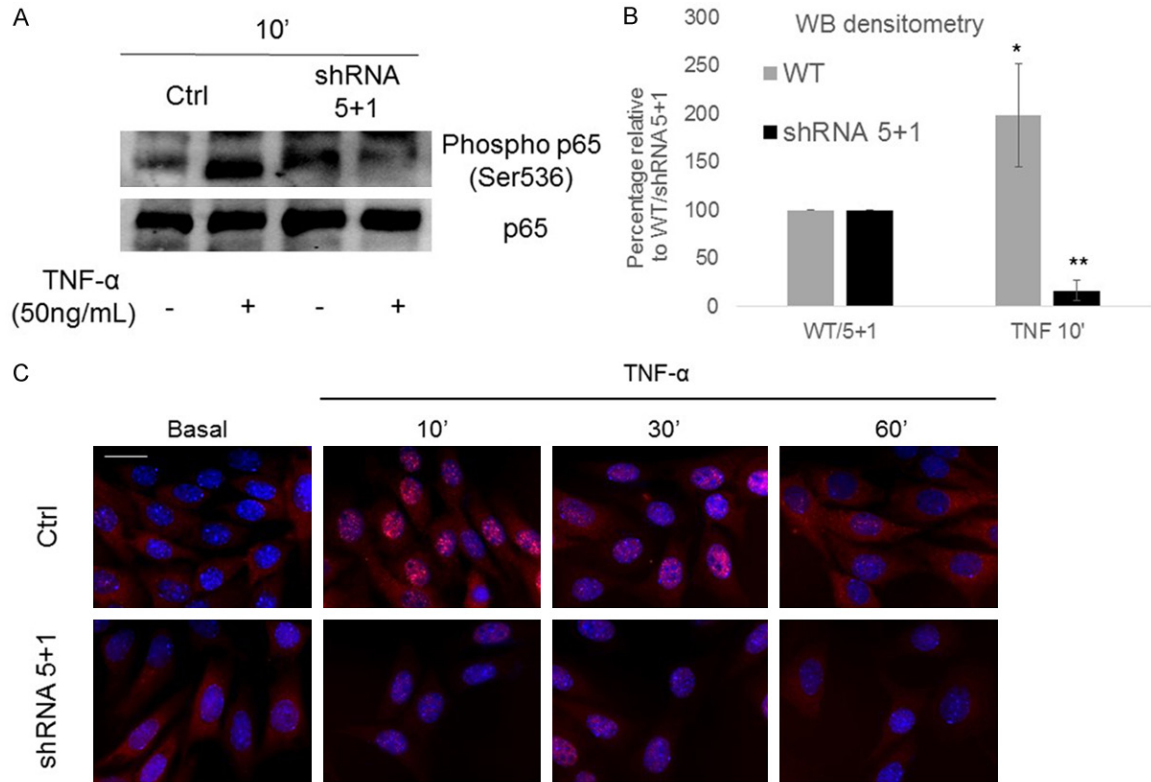


Figure 3. NF-κB activation. A, B. Western blot and relative densitometry for phospho p65 and p65 expression. Relative densitometry values obtained from three different experiments are expressed as mean \pm SD. N value = 3. * indicates $p \leq 0.05$ and ** indicates $p \leq 0.001$. C. Representative immunofluorescence staining for p65. N value = 3.

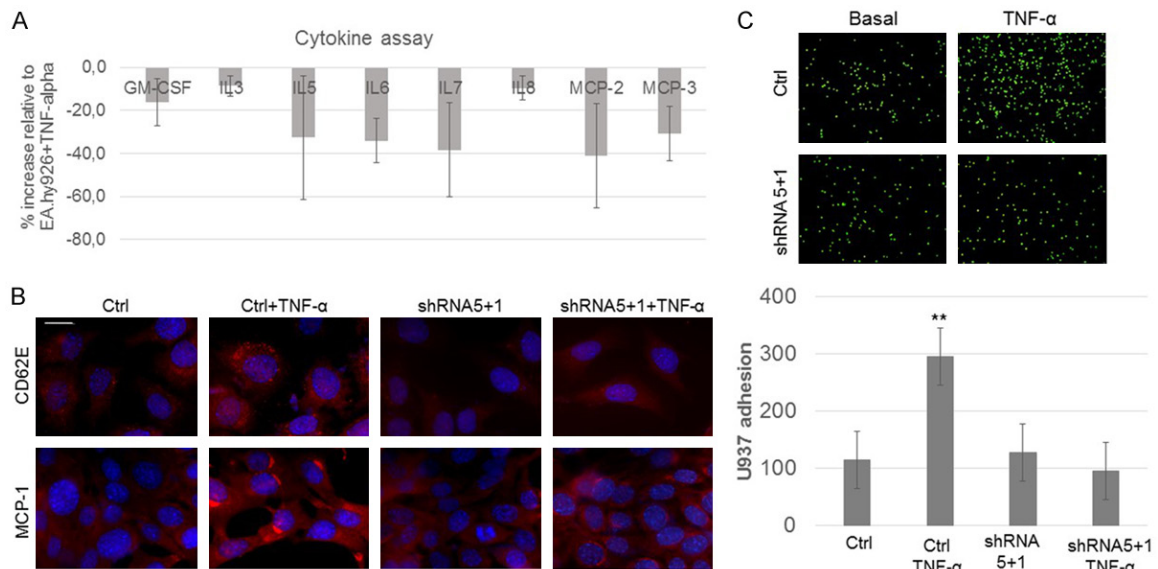


Figure 4. MMP-9 affects the inflammatory response in ECs. A. Cytokine assay performed on conditioned medium (CM) obtained from wild-type and transduced (shRNA5+1) endothelial cells treated with TNF-α in starving conditions. N value = 3. B. Representative immunofluorescence for MCP-1 and E-selectin on wild type and transduced EA.hy926 treated with TNF-α. N value = 3. C. U937 adhesion on ECs. N value = 3.

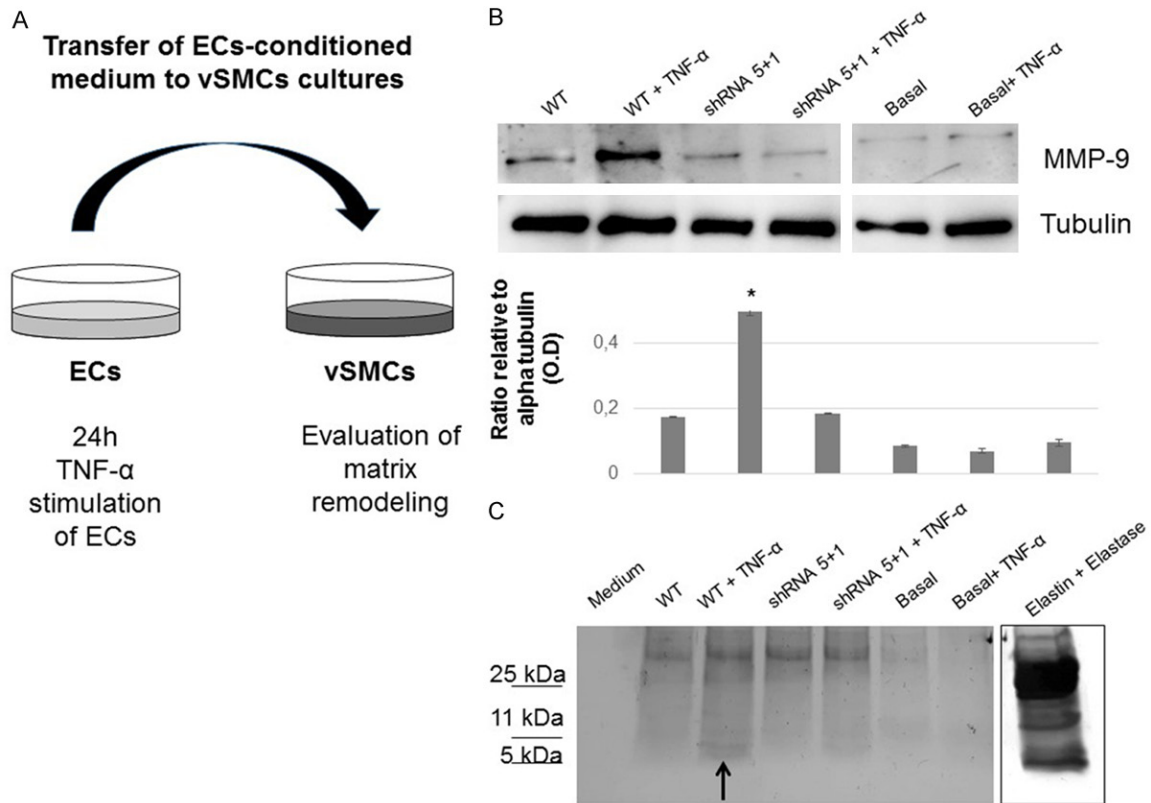


Figure 5. Crosstalk between ECs and vSMCs. **A.** Flowchart of the experimental procedure. **B.** Immunoblotting for MMP-9 and relative densitometry performed on lysates from vSMCs cultured with CM obtained from either wild-type or transduced-EA.hy926 endothelial cells treated or not with 50 ng/mL TNF- α for 24 h. Relative densitometry values obtained from three different experiments are expressed as mean \pm SD. N value = 3. *indicates $p \leq 0.05$. **C.** Representative silver staining of cell culture media. Elastin digested with elastase was used as standard. N value = 3.

expression. In conditioned media derived from MMP-9-silenced EA.hy926 cells, we observed a decrease of several cytokines and growth factors: GM-CSF (16%), IL-3 (8%), IL-5 (32%), IL-6 (34%), IL-7 (38%), IL-8 (9%), MCP-2 (41%) and MCP-3 (30%), where each percentage in bracket represents cytokine expression relative to non-transduced EA.hy926 control cells (**Figure 4A**).

To validate cytokines array data, we performed immunofluorescence assay on cells similarly treated. After TNF- α stimulation, we observed an upregulation of MCP-1 expression in wt-EA.hy926 cells whereas it was significantly down-regulated in transduced cells (**Figure 4B**). Likewise, TNF- α -mediated activation of E-selectin (CD62E), another marker of endothelial inflammatory involvement, was inhibited in MMP-9-silenced EA.hy926 cells, but not in wt-cells. Since CD62E plays an important role in recruiting leukocytes, we next determined

whether lack of MMP-9 would affect U937 cell adhesion. Indeed, MMP-9 silenced cells showed a 3-fold decrease in U937 adhesion in response to TNF- α treatment compared to wild-type cells similarly stimulated (**Figure 4C**).

Effects of endothelial conditioned media on vSMCs

We assessed the effect of conditioned medium (CM) obtained from wt- or shRNA5+1-transduced EA.hy926 cells on vSMC pro-angiogenic functions. First, we measured MMP-9 protein amount in lysates from vSMCs cultured in either wt- or transduced endothelial cell-derived CM. As negative control, lysates from vSMCs grown in non-conditioned medium (basal) were similarly collected (**Figure 5A**). As expected, we failed to detect MMP-9 expression in lysate from vSMCs cultured in basal medium (**Figure 5B**). Interestingly, vSMCs grown in EA.hy926 cell-derived CM displayed enhanced MMP-9

expression as compared to cells grown in the basal medium, suggesting that EA.hy926 cells secrete pro-angiogenic factors able to induce MMP-9 protein expression (**Figure 5B**). Intriguingly, also lysates from vSMCs cultured with shRNA5+1-transduced EA.hy926 cells showed higher MMP-9 expression with respect to basal, showing that this effect is independent of MMP-9 expression in EA.hy926 cells. When we measured MMP-9 expression in vSMCs grown in CM obtained from wt-EA.hy926 cells that had been treated for 24 h with TNF- α , we observed further upregulation of MMP-9 expression (**Figure 5B**). In contrast, when we used CM obtained from MMP-9-silenced EA.hy926 cells, TNF- α -mediated activation of MMP-9 was completely inhibited (**Figure 5B**). Finally, we compared the extent of proteolytic cleavage of elastin in the different conditioned and basal media tested so far. Remarkably, TNF- α treatment induced proteolytic cleavage of elastin in medium from vSMCs cultured with wild-type EA.hy926 cell-derived CM, but not shRNA5+1 transduced cell-derived CM, suggesting that MMP-9 silencing inhibits elastin fragmentation (**Figure 5C**).

Discussion

AAA is partially characterized by an inflammatory response in the aortic wall, which includes dramatic modifications of the endothelial, medial, and adventitial layers [18, 19]. Due to endothelial cell dysfunctions, ECs contribute to AAA progression. However, the specific role of ECs still remains poorly understood. The observation that medial neo-angiogenesis leads to increased vascular permeability and ECM degradation indicates that ECs play a key role in wall dysfunctions [20]. Furthermore, during neo-angiogenesis within the medial layer, human AAA expresses high levels of C4d, a degradation product of the classic complement pathway, which covalently binds to the endothelium and collagen basement membranes [21]. Thus, the accumulation of C4d is a hallmark of EC chronic inflammation.

In AAA, MMP-9 stimulates the inflammatory response to initiate pathogenesis and exacerbate disease progression through proteolytic degradation of proteins in the basal lamina of blood vessels as well as release of the biologically active form of VEGF [22]. Furthermore, MMP-9 serum levels are significantly increased

during several cardiovascular diseases [7] and correlate with aneurysmal tissues [23].

In order to better understand the role of ECs and, in particular, the role of endothelial MMP-9 in AAA progression, we have characterized cell functions in MMP-9-silenced EA.hy926 cells in terms of involvement in medial neo-angiogenesis and inflammatory loop. MMP-9 silencing was carried out through shRNA delivered by lentiviral vectors. Our data clearly show that tube formation activity of MMP-9-silenced EA.hy926 cells is impaired, implying that MMP-9 is required for matrix remodeling and, in particular, for neo-angiogenesis.

Previous data have shown that the recruitment of inflammatory cells is sustained by a chemotactic gradient due to cytokines released through EC activity, and by the increased expression of adhesion molecules able to interact with circulating inflammatory cells [24]. In particular, TNF- α -mediated activation of NF- κ B has been shown to be one of the major pathways contributing to inflammation-mediated tissue damage. Thus, here we asked whether lack of MMP-9 could impair TNF- α /NF- κ B signaling pathway. Our data clearly show that both TNF- α -mediated phosphorylation of p65 and p65 nuclear translocation, hallmarks of NF- κ B activation, were significantly decreased in EA.hy926 cells silenced for MMP-9, but not wt-cells. Thus, MMP-9 plays a role during TNF- α -mediated activation of NF- κ B also related to neo-angiogenesis. Remarkably, MMP-9 silencing showed an autocrine effect on ECs since several cytokines resulted down-regulated, especially IL-6, IL-7, and MCP-1. As it is well-established that TNF- α -mediated upregulation of these cytokine requires an intact NF- κ B signaling, our findings indicate that also MMP-9 levels regulate cytokine production in vascular cells during matrix remodeling.

We also show that lack of MMP-9 affects TNF- α -mediated upregulation of CD62E, a selectin that mediates leukocyte cell adhesion to activated platelets and other leucocytes in response to TNF- α , IL-1 β , or LPS in mice and in humans, thereby initiating multicellular adhesive and signaling events during pathological inflammation processes [25]. Thus, our data indicating the requirement of MMP-9 related to TNF- α -mediated upregulation of CD62E are in line with the observation that MMP-9 released

from ECs represents a fundamental switch able to control in this context, the inflammatory response [26]. In this regard, MMP-9 has been shown to release active TNF- α from cell surface via proteolysis [27]. TNF- α is an activator of NF- κ B which plays a central role in the development of inflammation through further regulation of genes encoding not only pro-inflammatory cytokines, but also adhesion molecules (E-selectin). Furthermore, NF- κ B is also required for cytokine upregulation of MMP-1, -3 and -9 [28].

Since the vascular inflammatory response involves complex interactions among white cells (i.e. neutrophils, lymphocytes, monocytes, macrophages), ECs and vSMCs, we sought to determine whether MMP-9 secreted by ECs could influence vSMCs competence in vessel wall matrix remodeling and weakening. Previous reports have shown that co-culturing of human aortic ECs and vSMCs along with monocytes led to increased accumulation in cell culture medium of matrix remodeling effectors such as fibronectin, collagen, IL-1, and IL-6 [29]. Here, we show that conditioned media (CM) obtained from ECs cultured in presence of TNF- α can upregulate MMP-9 expression in vSMCs, suggesting that this CM contains an adequate amount of inflammatory molecules able to stimulate vSMC functions. Interestingly, treatment of vSMCs with CM derived from MMP-9-silenced EA.hy926 cells, which had been exposed to TNF- α , led to a dramatic inhibition of MMP-9 expression. This finding might be explained, in part, by the assumption that the CM derived from MMP-9-silenced EA.hy926 cells contains lower levels of cytokines, growth factors, and adhesion molecules required for EC-mediated activation of vSMC functions during neo-angiogenesis. Moreover, the CM from silenced-MMP-9 EA.hy926 cell, but not wt-cells, did not display proteolytic activity in terms of elastin degradation in an *in vitro* assay. MMP-9 can directly degrade elastin [30], and elastin fragments promote monocyte/macrophage recruitment *in vivo* [26]. In fact, our data suggest that ECs have a humoral cross-talk with vSMCs that can locally sustain AAA progression, and open new scenarios concerning the role of endothelial MMP-9 in AAA.

Conclusion

Altogether, our data demonstrate that ECs present in the medial layer play a key role in the

progression of AAA *in situ* through regulation of vSMC functions, which ultimately promotes ECM damage. Importantly, our observation that MMP-9-silenced ECs are no longer able to migrate and differentiate in a three-dimensional matrix strongly indicates that MMP-9 regulates proangiogenic EC functions as well.

At this regard, exploring the molecular targets able to restore vSMC phenotypes could lead to novel promising strategies to arrest aneurysm progression.

Acknowledgements

The authors would like to thank Dr. Marina Buzzi for the collaboration, Dr. Maria Luisa Attinà for lentiviral vector production, Dr. Gustavo Oderich for the critical reading of the manuscript and Dr. Marcello Arsura for proof-reading of the work.

Disclosure of conflict of interest

None.

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