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Differential expression of proteins related to smooth muscle cells and myofibroblasts in human thoracic aortic aneurysm

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Summary. Objectives: Increasing knowledge is required for a better comprehension of the etiology of thoracic aortic aneurysm (TAA). The aim of this study was to highlight the modulations in vascular cell phenotypes, including myofibroblasts (MFs), in human TAA specimens compared to healthy aortas.

Methods: histology, RT-PCR and immunohistochemical analysis of a panel of molecules, including ED-A Fibronectin (Fn), smoothelin, CD34 and alpha-smooth muscle actin (alpha-SMA), selected on the basis of their informative potential as markers of smooth muscle cells (SMCs) and MF phenotypic modulation, were performed on all samples.

Results: The media of TAAs was characterized by the absence of smoothelin, the unaltered expression of alpha-SMA accompanied by an alteration of its distribution pattern, and by the activated expression of the ED-A isoform of Fn.

We found a concentration of round-shaped cells exclusively in the adventitia and in the perivascular tissue of TAAs, also rich in *vasa vasorum*, largely expressing alpha-SMA, while a sub-population also expressed ED-A Fn and CD34.

CD34 was expressed by several cells in the intima of TAAs, together with cells expressing cytoplasmatic ED-A Fn and alpha-SMA in comparison to healthy aortas.

Conclusion: TAA specimens show an altered expression and localization of SMC and MF differentiation markers in comparison to healthy aortas, with possible implications on remodeling.

Key words: Thoracic aortic aneurysm, Myofibroblasts, Fibronectin, CD34, Smoothelin, Alpha-smooth muscle actin

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Introduction

The incidence of thoracic aortic aneurysm (TAA) is estimated to be 5.9/100000/year, a rate that is increasing with improvements in screening and with advances in imaging methods (Bickerstaff et al., 1982; Gillum, 1995). While progressive improvements have been achieved in surgical results, increasing knowledge is required for a better comprehension of either the etiology and the pathological features of TAA. Aneurysm development results from the maladaptive remodeling of the extracellular matrix (ECM) in the aortic wall, with an imbalance between proteolysis and protein synthesis in the medial layer, determining the pathological picture defined as medial degeneration (Coady et al., 1999).

Myofibroblasts (MFs) are cells endowed with a contractile apparatus playing a key role in tissue damage repair and in aberrant remodeling (Coen et al., 2011). MFs can derive from a multiplicity of resident cells and/or circulating progenitors, including fibroblasts, smooth muscle cells (SMCs), monocytes and fibrocytes. MF differentiation is associated with the up-regulation of some ECM components, including fibronectin (Fn) and its critical splice variant extra domain-A (ED-A)

(Sandbo and Dulin, 2011). Of interest, ED-A Fn can exert a cooperative function with TGF-ß1 to activate alpha-smooth muscle actin (alpha-SMA) expression in MFs (Serini et al., 1998).

Actually, alpha-SMA has been historically considered an elective marker of MF differentiation (Gan et al., 2007). Nonetheless, in cardiovascular tissues alpha-SMA expression is shared also by SMCs. This is not surprising, since SMCs and MFs have common functional properties, including force development and contraction. This peculiarity makes the univocal identification of MFs in diseased cardiovascular tissues a complex process.

Smoothelin is a structural protein that colocalizes with alpha-SMA in the cytoskeleton (Van Eys et al., 2007), is a specific marker of contractile SMCs (Christen et al., 2001) and, of major interest, is the only marker that differentiates between SMCs and MFs (Maeng et al., 2003). Growing attention to smoothelin expression analysis is related not only to its involvement in SMC contraction, but also to its function as a specific marker in proliferative diseases, thus playing a diagnostic role in specific pathological settings. In this context, recent studies suggested that the extent and pattern of smoothelin expression may help differentiate between benign and malignant mesenchymal tumors of the gastrointestinal tract (Coco et al., 2009; Faragalla et al., 2011) and be a reliable marker in the staging of bladder carcinoma (Council and Hameed, 2009). A similar role could be played by smoothelin in staging of TAAs.

The presence of MFs in human TAA has not yet been clearly established, while studies on murine models suggested a role for this cell type in experimental aneurysm (Jones et al., 2009). The aim of this study was to highlight modulations in cell phenotypes in human TAA specimens compared to aortic samples from organ donors through the combined analysis of a series of molecules, including ED-A FN, smoothelin, CD34 and alpha-SMA, selected on the basis of their informative potential.

Materials and methods

Sample collection

Specimens from the concavity (lesser curvature) and the convexity (greater curvature) of TAA (diameter >5 cm) with tricuspid aortic valve (age 54.7±11.5, n=6 males) were taken from 11 patients during elective surgical intervention. None of the patients had positive family history for aneurysmal disease of the aorta and none had bicuspid aortic valve. None of the patients suffered from connective tissue disease. The measure of the aortic diameter was taken by transthoracic echocardiography at the ascending tract level.

Control specimens (diameter 3.0 ± 0.09 cm) were obtained during multiorgan harvesting from heart donors with no evidence of aneurysmal disease or familiarity (n=12, age 39.7±13.2, n=6 males) all with tricuspid aortic valve, cause of death: trauma in 7 cases, spontaneous cerebral hemorrhage in 5 cases). None of the donors showed evidence of aneurysmal or atherosclerotic disease during multiorgan harvesting.

The study received Institutional Ethic Committee approval and patients gave their informed consent prior to their participation in the study.

Histological analysis

Harvested samples were fixed in 4% buffered formaldehyde, dehydrated and embedded in paraffin. Consecutive 5 μm cross-sections were stained with hematoxylin or with hematoxylin-orcein (Sigma Aldrich) for nucleus and elastic fiber staining, respectively. Image screening and photography were performed using the Leica IM1000 software. Intima thickness was measured through the Leica IM1000 software as the average of measurements at five points for each cross-section. Orcein-stained internal elastic lamina was used as reference.

RNA extraction and RT-PCR analysis

Total RNA was extracted from whole aortic samples stored in RNALater (Qiagen) at -80°C immediately after harvesting, using the RNAeasy minikit (Qiagen). RNA was treated with DNase (Qiagen) to remove DNA contamination, its concentration was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and its integrity was verified by electrophoresis on denaturing 1% agarose gel. Absence of residual DNA was verified by PCR on total RNA without reverse transcription. cDNA was generated from 200 ng of each RNA sample. Reverse transcription was done at 42°C for 1 hr in presence of random examers and Moloney-Murine Leukemia Virus (M-MULV) reverse transcriptase (Finnzymes). GeneBank sequences for human mRNAs and the Primer Express software (Applied Biosystem) were used to design primer pairs for the target genes (Table 1). RT-PCR amplification experiments were performed as previously published (Forte et al., 2012). Each PCR reaction was repeated in quadruplicate. The ChemiDoc and associated software Quantity One (Bio-Rad) were used for densitometric analysis of RT-PCR products after electrophoresis.

Immunohistochemistry

Target proteins were smoothelin, total FN, ED-A FN, α-SMA, CD34, Procollagen type I and collagen type III. Primary antibodies used are listed in Table 2. Consecutive 4% formaldehyde-fixed 5 μm cross-sections were deparaffinized and rehydrated. Antigen retrieval was done in a microwave through incubation in 10mM Citrate buffer pH 6. Endogenous peroxidases were blocked with 4% H_2O_2. Blocking was done in 5% donkey serum, followed by incubation with the primary

antibody at 4°C o.n. After washing, slides were incubated with biotin-conjugated secondary antibody (Santa Cruz, dil. 1:200). Staining was done through incubation with peroxidase-streptoavidin (Vector Laboratories) for 30' at RT, followed by incubation with 3,3'-diaminobenzidine (Vector Laboratories). Primary antibodies were omitted in negative control of the reactions. Nuclei were counterstained with Mayer's hematoxylin (Sigma-Aldrich). Image screening and photography of serial cross-sections submitted to colorimetric immunohistochemical analysis were performed using a Leica IM1000 System. Immunohistochemistry quantification was performed on the central region of the media with Tiffalyzer on digital image files (Matkowskyj et al., 2000), using as control images the cross-sections not exposed to primary antibodies. Data relative to the amount of total chromogen present was given as energy units per pixel. Five consecutive cross-sections for each aortic specimen were considered for quantitative analysis. All the analyses were performed by the same person, blinded to the nature of the aortic sample.

On the basis of our previous observations on aortic samples harvested from healthy donors (Forte et al., 2012), we carefully checked the aortic cross-sections from healthy donors used for immunohistochemical analysis and selected for subsequent analysis only the regions that were free from discrete areas showing an altered structure.

Statistical analysis

The statistical analysis of RT-PCR and immunohistochemical data was performed using the GraphPad software (Prism 4.0). Comparisons between the convexity and the concavity were performed through paired t test. Data are presented as mean ± SEM. A p value <0.05 was considered statistically significant.

Results

Our study focused on specimens from both the concavity and the convexity of the ascending aorta to account for possible differences related to the hemodynamic burden. In fact, we did not reveal any relevant difference at mRNA and protein level between the concavity and convexity of TAAs and of donor healthy aortas, or for the distribution pattern of target proteins and, for this reason, we show here only representative images and data referring to the convexity of the aortic specimens we analysed (Figs. 1-4).

The immunohistochemical qualitative analysis of the ECM components procollagen type I, collagen type III and total Fn (Fig. 1) revealed an altered distribution and organization of these ECM components in the media of TAA specimens in comparison to healthy aortas, thus confirming the pathological nature of the sample set we selected for subsequent analysis of markers of cell phenotype and/or transdifferentiation. Nonetheless, the immunohistochemical quantitative analysis focusing on the central region of the media did not reveal any relevant difference in the expression level of these ECM components in comparison to healthy aortas.

The RT-PCR analysis of alpha-SMA expression highlighted a significant 1.8-fold decrease of its mRNA in TAAs in comparison to healthy aortas ($p<0.05$) (Fig. 2). Qualitative immunohistochemistry revealed a marked expression of alpha-SMA in the media of both TAA and healthy donor aortic samples (Figs. 1a,d, 3c,f, 4i), including the *vasa vasorum* in the outer media (Fig. 4c), but a different, non homogeneous distribution pattern of alpha-SMA was evident in TAAs (Fig. 1d). However, quantitative immunohistochemistry focusing on the central region of the media (Fig 1a,d) did not highlight any significant difference for alpha-SMA between TAA and healthy aortas or between the concavity and the convexity of aortic specimens.

The RT-PCR analysis of the ED-A isoform of Fn revealed a significant 2-fold increase of mRNA in TAAs in comparison to healthy aortas ($p<0.05$) (Fig. 2). This trend was confirmed by the immunohistochemical analysis, revealing a marked expression of ED-A Fn in the media of TAA specimens only (Fig. 1, 3, 4), in contrast to control healthy aortas, where it was expressed exclusively in the intima.

Smoothelin showed an opposite expression profile in comparison to ED-A Fn, as its mRNA significantly decreased in TAAs in comparison to healthy aortas

Table 1. Summary of the RT-PCR primer sequences, position, annealing temperature and PCR product length for each target gene analysed.

GENE	Primer Position	Primer Sequence	Annealing T (°C)	PCR Product Length (bp)
GAPDH	472 799	5'-GCA TCC TGC ACC ACC AAC TG -3' 5'-GCC TGC TTC ACC ACC TTC TT -3'	55	327
Smoothelin	2319 2461	5'-AGC ACC ATG ATG CAA ACC AAG -3' 5'-TCT TTC TTC TTC TCG GCC TGC -3'	62	143
ED-A Fn	5875 5690	5'-ACA ACC ACG GAT GAG CTG -3' 5'-CCA GTG CAC AGC TAT TCC TG -3'	54.8	185
Alpha-SMA	1049 1150	5'-TCC GGA GCG CAA ATA CTC TGT -3' 5'-CCG GCT TCA TCG TAT TCC TGT -3'	61	102

($p<0.05$) (Fig. 2), while the corresponding protein was completely undetectable by immunohistochemistry in the media of TAAs (Fig. 1g,l).

The morphometric analysis of the intima layer revealed a significant increase of its thickness in TAAs in comparison to healthy aortas (750±101 μm vs. 109±19 μm). The immunohistochemical analysis revealed the presence of several $CD34^+$ vascular progenitor cells with heterogeneous shape in the intima of TAAs (Fig. 3d), while only rare $CD34^+$ cells were visible in the intima of donor aortas (Fig. 3a). CD34 was also expressed by some cells in the endothelial layer in TAAs. CD34 expression in the intima of TAAs was accompanied by spindle-shaped cells expressing cytoplasmatic ED-A Fn (Fig. 3e) and by a marked expression of alpha-SMA (Fig. 3f).

Also, the adventitia in TAAs showed morphological alterations in comparison to healthy aortas, appearing as a thin layer, scarcely cellularized and with rare *vasa*

Table 2. Primary antibodies used for immunohistochemical analysis. See text for abbreviations.

Target protein	Ab' type	clone	dilution	Company
Smoothelin	mouse monoclonal	R4A	1:100	Abcam
ED-A Fn	mouse monoclonal	IST-9	1:100	Santa Cruz
Fn	mouse monoclonal	IST-4	1:100	Sigma
α-SMA	mouse monoclonal	1A4	1:200	Sigma
CD34	rabbit polyclonal		1:100	Abcam
Procollagen type I	rabbit polyclonal		1:100	Santa Cruz
Collagen type III	rabbit polyclonal		1:100	Santa Cruz

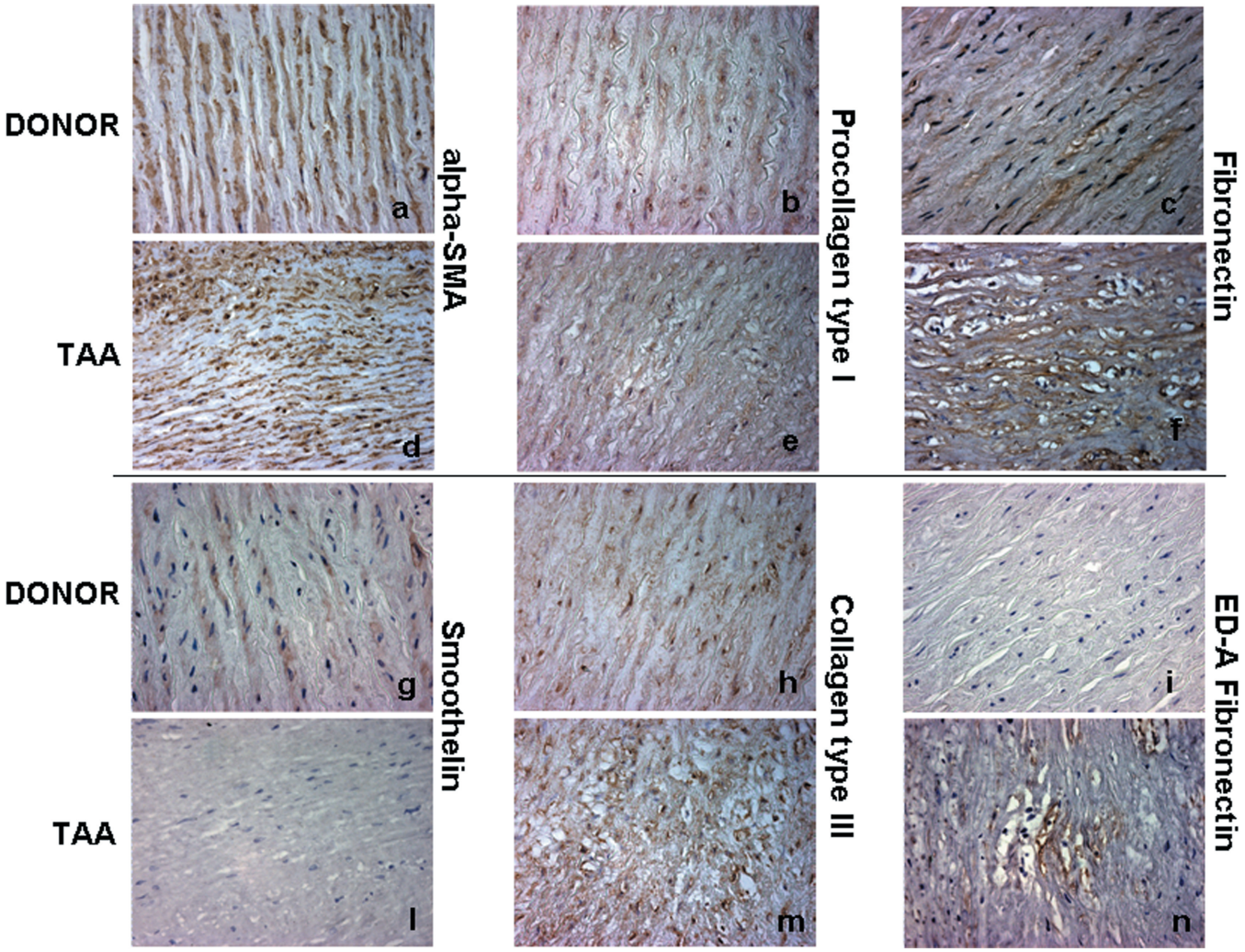

Fig. 1. Representative immunohistochemical analysis of alpha-SMA **(a, d)**, smoothelin **(g, l)**, procollagen type I **(b, e)**, collagen type III **(h, m)**, Fibronectin **(c, f)** and ED-A Fibronectin **(i, n)** in the central region of the media of the convexity of donor aortas **(a-c, g-i)** and TAAs **(d-f, l-n)**. The overall analysis revealed an altered distribution and organization of ECM components (procollagen type I, collagen type III and fibronectin) and of alpha-SMA in TAAs in comparison to donor aortas, together with the loss of smoothelin and the activation of ED-A Fn expression in the media of TAAs. Brown staining corresponds to target protein. Hematoxylin nuclei blue counterstaining. x 40.

vasorum localized in proximity of the outer media in healthy aortas, while in TAAs it was a thick layer, with several *vasa vasorum* of variable diameter and highly cellularized (Fig. 4a-c,g-i), mainly by a high concentration of round-shaped cells (Fig. 4g-n). The immunohistochemical analysis of adjacent consecutive TAA cross-sections revealed that the majority of the round-shaped adventitial cells in TAAs expressed alpha-SMA (Fig. 4i,n), while a sub-population also expressed ED-A Fn (Fig. 4h,m) and CD34 (Fig. 4g,l). A parallel analysis conducted on healthy aortas revealed the presence in the adventitia of rare $CD34^+$ cells (Fig. 4d) and of some alpha-SMA-positive cells around the small *vasa vasorum* in the outer media (Fig. 4c,f), while ED-A Fn was not expressed in the adventitia of healthy aortas (Fig. 4b,e).

Discussion

The absence of relevant differences between the concavity and the convexity of TAA specimens and of healthy aortas differs from previous observations obtained in mildly dilated thoracic aortas and implies that the differences in gene expression between the major and the minor aortic curvatures emerge only in the early phases of aortic dilatation (Della Corte et al., 2008), at least in reference to the panel of molecules we analysed.

Differences in the mean age of patients and of healthy organ donors are unavoidably related to the mean age of TAA onset in patients and to the lower age of organ donors. Nonetheless, to our knowledge, no correlation has been found so far between the genes we

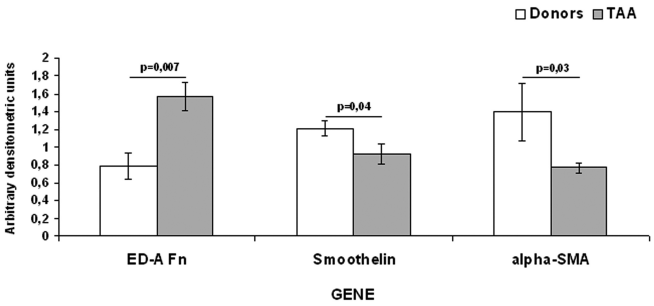

Fig. 2. Histogram showing RT-PCR data (normalized to the expression level of the endogenous GAPDH housekeeping gene) for ED-A Fn, smoothelin and α-SMA obtained on total RNA extracted from the convexity of TAA specimens and of donor aortas. Data are expressed as mean ± SEM. $p<0.05$ was considered statistically significant.

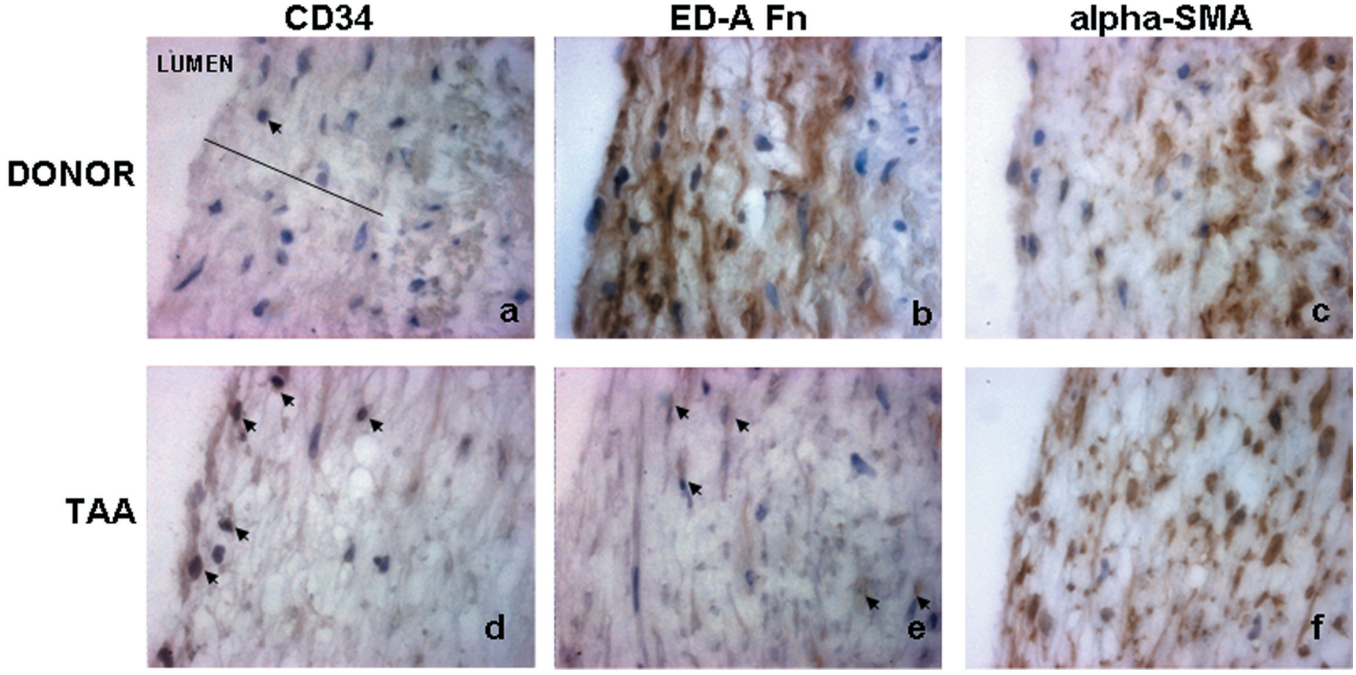

Fig. 3. Representative immunohistochemical analysis of CD34 **(a, d)**, ED-A Fn **(b, e)** and alpha-SMA **(c, f)** in the intima of the convexity of donor aortas **(a-c)** and in **TAAs (d-f)**. Brown staining corresponds to target protein. Several round-shaped $CD34^+$ cells were visible in the intima of TAAs in comparison to rare $CD34^+$ cells detected in healthy aortas (**a, d**, arrows). Fewer spindle-shaped ED-A Fn-positive cells were also visible in the intima of TAAs in comparison to healthy aortas (**b, e**, arrows). An increase in alpha-SMA **(f)** was evident in TAAs in comparison to healthy aortic specimens **(c)**. Solid dark line in **a** indicates the thickness of the intima in donor aortic specimens. Hematoxylin nuclei blue counterstaining. x 100.

analysed (ED-A Fn, smoothelin, alpha-SMA) and the normal ageing process of the aorta.

The immunohistochemical analysis of the ECM proteins procollagen type I and collagen type III revealed an altered distribution and organization of these ECM components in TAAs in comparison to healthy aortas, a well-known characteristic of the aneurysm disease (Jacob, 2003; Cotrufo et al., 2005; Meng et al., 2011). The absence of significant differences in collagen content between the concavity and the convexity of TAA samples is in agreement with others (de Figueiredo Borges et al., 2008). This picture could be linked to the alterations in cell composition and phenotype, including those highlighted by the analysis of the cell markers CD34, smoothelin, ED-A Fn and alpha-SMA, as discussed below.

We revealed for the first time the loss of smoothelin, a specific marker of the SMC contractile phenotype, in human TAA specimens. In more detail, our results highlighted a significant decrease of smoothelin mRNA and the absence of the corresponding protein in TAA samples (Fig. 1l), possibly suggesting a differential regulation of smoothelin at translational level in this setting, and potentially being indicative of the epigenetic state of its gene in SMCs in the TAA pathological setting. Additional experiments would be necessary to demonstrate this hypothesis. On the basis of the expression data obtained in TAAs, additional experiments on aortic specimens with increasing dilatation would also be necessary to highlight a potential role of smoothelin as a marker of TAA staging, as already established in other pathological settings (Coco et al., 2009; Council and Hameed, 2009; Faragalla et al., 2011).

Smoothelin absence in TAAs was accompanied by the decrease of alpha-SMA mRNA expression, in agreement with others (Ailawadi et al., 2009), and by the unaltered expression of alpha-SMA protein (Fig. 1, d), in agreement with Tang et al. (2005). Nonetheless, the imunohistochemical analysis also revealed an altered distribution of alpha-SMA expression in the media of TAAs, with potential consequences for the remodeling

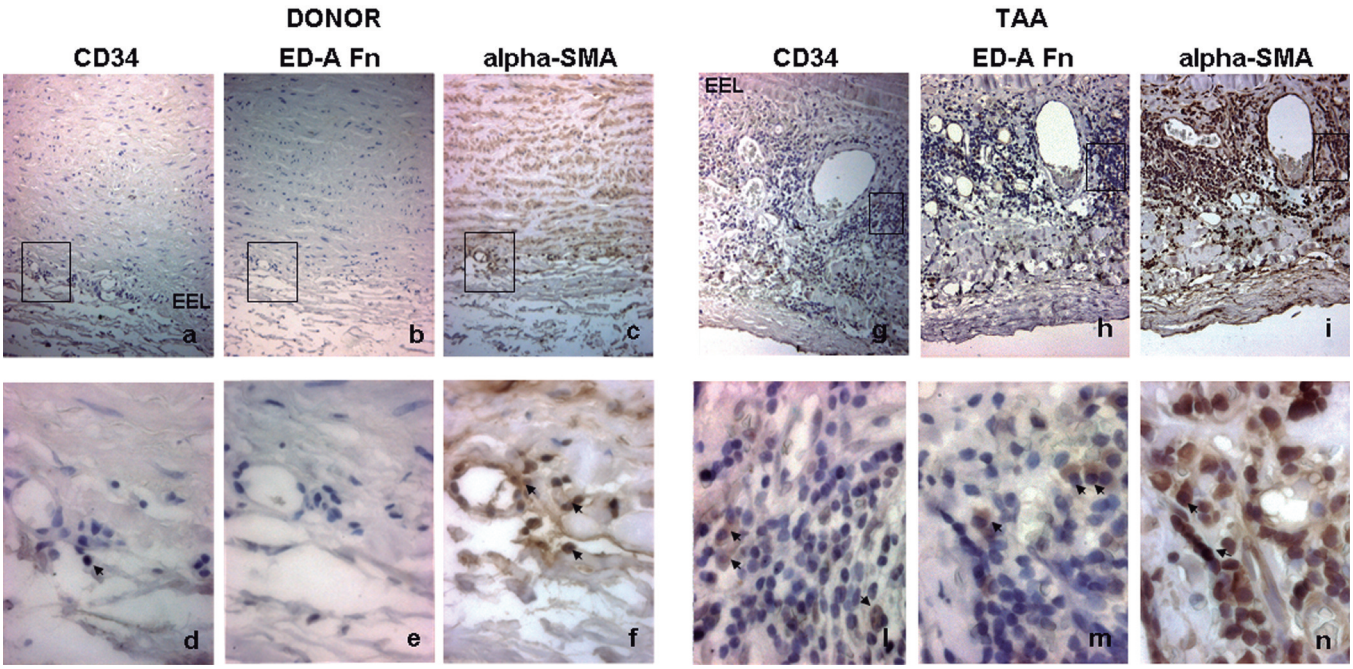

Fig. 4. Representative immunohistochemical analysis of CD34 **(a, d, g, l)**, ED-A Fn **(b, e, h, m)** and alpha-SMA **(c, f, i, n)** in the media and adventitia of the convexity of donor aortas **(a-f)** and in the adventitia of TAAs **(g-n)**. Brown staining corresponds to target protein. While the adventitia in healthy aortas was a thin layer scarcely cellularized and with rare *vasa vasorum* localized in proximity of the outer media, the adventitia in TAA specimens was a thick layer, with several *vasa vasorum* of variable diameter and highly cellularized. The position of the external elastic lamina (EEL) was indicated in **a** and **g** to highlight the different adventitial thickness in healthy aortas and in TAAs. Only rare $CD34^+$ cells were present in the adventitia of donor healthy aortas (arrow, **d**), while the adventitia of TAAs contained several $CD34^+$ cells around *vasa vasorum* (arrows, **l**). ED-A Fn was not expressed in the adventitia of healthy donors **(b, e)**, while it was expressed in the adventitia of TAAs (arrows, **m**). Finally, alpha-SMA was expressed by some cells localized around the small *vasa vasorum* in the outer media of healthy aortas (arrows, **f**), while almost the totality of adventitial cells in TAAs expressed alpha-SMA (arrows, **n**). Immunohistochemistry for CD34, ED-A Fn and alpha-SMA has been performed on consecutive, adjacent aortic cross-sections form donor and TAA specimens. Hematoxylin nuclei blue counterstaining, of the area enclosed in the black perimeter in the upper panel images. a-c, g-i, x 20; d-f, l-n, x 100.

of the aortic wall. Alpha-SMA is known to be transcriptionally regulated by a combination of multiple cis-elements-trans-acting factor interactions (Sobue et al., 1999). However, the differential analysis of alpha-SMA mRNA is not exhaustive, as alpha-SMA has different levels of control of its functions, including polymerization (Albinsson et al., 2004; Yamin and Morgan, 2012). Moreover, a pathological status can alter the cell composition of the aortic tissue and alter the distribution of alpha-SMA in comparison to healthy aortic samples, a phenomenon not evident from the mRNA analysis alone. Our joint immunohistochemical qualitative and quantitative analysis of alpha-SMA and the RT-PCR analysis of mRNA provided a more complete, if not exhaustive, picture of the alpha-SMA alterations in TAA samples in comparison to healthy aortas.

The expression of ED-A Fn exclusively in the intima of healthy aortas is in agreement with previous studies (Glukhova et al., 1989; Forte et al., 2012). The activation of the expression of ED-A Fn in the media of TAAs, in conjunction with the decline of smoothelin expression, could indicate a loss of the contractile phenotype of SMCs, as previously described in intimal thickening and atherosclerotic plaque from human arteries (Glukhova et al., 1989), and/or their differentiation to MFs, in agreement with previous observations reported in a murine model of aneurysm (Jones et al., 2009).

As concerns the adventitia and the perivascular tissue in TAAs, rich in *vasa vasorum*, the concentration of round-shaped cells we found is possibly an inflammatory infiltrate (Fig. 4, g-i), in agreement with previous observations obtained in a murine model of experimental aneurysm (Sho et al., 2004). In particular, on the basis of the immunohistochemical results obtained on this adventitial concentration of round-shaped cells (Fig. 4), we can hypothesize a role for monocyte lineage cells primed to differentiate to MFs, in agreement with the data observed in the vascular remodeling in a rat model of chronic hypoxic pulmonary hypertension (Frid et al., 2006). Of interest, a recent study conducted in a rat model of foreign body reaction suggested that a subpopulation of $CD34^+CD68^+$ cells, also positive to alpha-SMA, are able to differentiate into MFs (Mesure et al., 2010), a picture that could be also compatible with our findings. Our data are of interest also in view of a previous hypothesis suggesting that the perivascular region is a niche of progenitor cells in granulation tissue and a substrate of regulatory mechanisms (perivascular niche hypothesis) (Diaz-Flores et al., 2009). To our knowledge, our data reveal for the first time the presence of cells expressing CD34, alpha-SMA and ED-A Fn in the same adventitial area of human TAA specimens, confirming and integrating the data previously obtained in a murine model of aortic aneurysm (Sho et al., 2004). The possible differentiation of MFs in the adventitia of TAAs can be relevant not only for the consequent alterations in the homeostasis of the ECM, but also in view of the fact that MFs generate a force driving tissue remodelling (Forte et al., 2010). Of note, the mechanisms regulating the force production by MFs are different from those described for SMC contraction, and lead to a slow, irreversible and continuous retractile activity (Hao et al., 2006). Our findings, highlighting a role for the adventitia and the perivascular tissue as a potential source for MFs and their precursors, should be also considered in the light of other studies revealing that the adventitia can act to protect the wall against rupture by keeping stresses below experimentally observed strength waves (Schmid et al., 2011).

The presence of several $CD34^+$ cells also in the intima layer of TAAs (Fig. 3, d) suggests a potential role for circulating early bone marrow progenitor cells in aneurysm progression. The presence of $CD34^+$ cells in the endothelial layer is in agreement with others (Urbich and Dimmele, 2004).

CD34 has been widely used as a marker to highlight the increased microvessel density in the vascular wall both in experimental models and in human abdominal aortic aneurysm (Kobayashi et al., 2002; Måyrånpåå et al., 2009). Other studies focusing on heterogeneous aortic samples from patients affected by Marfan syndrome also revealed the presence of $CD34^+$ microvessels in the adventitia and in the outer media (Nataatmadja et al., 2006). Our data integrate these previous observations and provide additional information about the presence and the nature of different cell populations in the intima and in the adventitia of human TAA specimens, suggesting a bone marrow origin for distinct groups of cells. In particular, we noted three distinct $CD34^+$ cell groups in TAA specimens: 1) clusters of rounded cells in the adventitia around the *vasa vasorum* (Fig. 4); 2) single cells with heterogeneous morphology in the intima (Fig. 3); 3) ECs in adventitial capillaries and in the intima (data not shown). The heterogeneous morphology of ED-A Fn+ and $CD34^+$ cells in TAAs could reflect their different origin. In particular, the cells in the intima of TAA could be fibrocyte-derived myofibroblast-like cells, still expressing low levels of CD34 but already expressing alpha-SMA, collagens and fibronectin (Mattoli et al., 2009).

Several problems are often related to the interpretation of results obtained in experimental models and their extrapolation to the human situation. In this case, our data obtained in human TAAs confirm those obtained in previously mentioned murine models of aortopathy (Sho et al., 2004; Jones et al., 2009) and bring together new data on the expression and the localization of SMC and MF differentiation markers in TAAs in comparison to control healthy aortas. The combined analysis of the genes we selected, together with the assessment of the morphological characteristics of the vascular wall, can lend insight into the role of MFs and of their secretome in human TAA.

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Conflict of interest. The authors confirm that there are no conflicts of interest.

References

Ailawadi G., Moehle C.W., Pei H., Walton S.P., Yang Z., Kron I.L., Lau C.L. and Owens G.K. (2009). Smooth muscle phenotypic modulation is an early event in aortic aneurysms. J. Thorac. Cardiovasc. Surg. 138, 1392-1399.

Albinsson S., Nordström I. and Hellstrand P. (2004). Stretch of the vascular wall induces smooth muscle differentiation by promoting actin polymerization. J. Biol. Chem. 279, 34849-34855.

Bickerstaff L.K., Pairolero P.C., Hollier L.H., Melton L.J., Van Peenen H.J., Cherry K.J., Joyce J.W. and Lie J.T. (1982). Thoracic aortic aneurysms: a population-based study. Surgery 92, 1103-1108.

Christen T., Verin V., Bochaton-Piallat M., Popowski Y., Ramaekers F., Debruyne P., Camenzind E., van Eys G. and Gabbiani G. (2001). Mechanisms of neointima formation and remodeling in the porcine coronary artery. Circulation 103, 882-888.

Coady M.A., Rizzo J.A., Goldstein L.J. and Elefteriades J.A. (1999). Natural history, pathogenesis, and etiology of thoracic aortic aneurysms and dissections. Cardiol. Clin. 17, 615-635.

Coco D.P., Hirsch M.S. and Hornick J.L. (2009). Smoothelin is a specific marker for smooth muscle neoplasms of the gastrointestinal tract. Am. J. Surg. Pathol. 33, 1795-801.

Coen M., Gabbiani G. and Bochaton-Piallat M.L. (2011). Myofibroblast-mediated adventitial remodeling: an underestimated player in arterial pathology. Arterioscler. Thromb. Vasc. Biol. 31, 2391-2396.

Cotrufo M., De Santo L., Della Corte A., Di Meglio F., Guerra G., Quarto C., Vitale S., Castaldo C. and Montagnani S. (2005). Basal lamina structural alterations in human asymmetric aneurismatic aorta. Eur. J. Histochem. 49, 363-70.

Council L. and Hameed O. (2009). Differential expression of immunohistochemical markers in bladder smooth muscle and myofibroblasts, and the potential utility of desmin, smoothelin, and vimentin in staging of bladder carcinoma. Mod. Pathol. 22, 639-50.

de Figueiredo Borges L., Jaldin R.G., Dias R.R., Stolf N.A., Michel J.B. and Gutierrez P.S. (2008). Collagen is reduced and disrupted in human aneurysms and dissections of ascending aorta. Hum. Pathol. 39, 437-43.

Della Corte A., Quarto C., Bancone C., Castaldo C., Di Meglio F., Nurzynska D., De Santo L.S., De Feo M., Scardone M., Montagnani S. and Cotrufo M. (2008). Spatiotemporal patterns of smooth muscle cell changes in ascending aortic dilatation with bicuspid and tricuspid aortic valve stenosis: focus on cell-matrix signaling. J. Thorac. Cardiovasc. Surg. 135, 8-18.

Diaz-Flores L. Jr, Gutierrez R., Madrid J.F., Varela H., Valladares F. and Diaz-Flores L. (2009). Adult stem cells and repair through granulation tissue. Front. Biosci. 14, 1433-1470.

Faragalla H.F., Marcon N.E., Yousef G.M. and Streutker C.J. (2011). Immunohistochemical staining for smoothelin in the duplicated versus the true muscularis mucosae of Barrett esophagus. Am. J. Surg. Pathol. 35, 55-9.

Forte A., Della Corte A., De Feo M., Cerasuolo F. and Cipollaro M. (2010). Role of myofibroblasts in vascular remodelling: focus on restenosis and aneurysm. Cardiovasc. Res. 88, 395-405.

Forte A., Della Corte A., Grossi M., Finicelli M., Bancone C., Provenzano R., Pepino P., Nappi G.A., De Feo M., Galderisi U., Cotrufo M. and Cipollaro M. (2012). Morphological and molecular characterization of healthy human ascending aorta. Histol. Histopathol. 27, 103-112.

Frid M.G., Brunetti J.A., Burke D.L., Carpenter T.C., Davie N.J., Reeves J.T., Roedersheimer M.T., van Rooijen N. and Stenmark K.R. (2006). Hypoxia-induced pulmonary vascular remodeling requires recruitment of circulating mesenchymal precursors of a monocyte/macrophage lineage. Am. J. Pathol. 168, 659-669.

Gan Q., Yoshida T., Li J. and Owens G.K. (2007). Smooth muscle cells and myofibroblasts use distinct transcriptional mechanisms for smooth muscle alpha-actin expression. Circ. Res. 101, 883-892.

Gillum R.F. (1995). Epidemiology of aortic aneurysm in the United States. J. Clin. Epidemiol. 48, 1289-1298.

Glukhova M.A., Frid M.G., Shekhonin B.V., Vasilevskaya T.D., Grunwald J., Saginati M. and Koteliansky V.E. (1989). Expression of extra domain A fibronectin sequence in vascular smooth muscle cells is phenotype dependent. J. Cell Biol. 109, 357-366.

Hao H., Gabbiani G., Camenzind E., Bacchetta M., Virmani R. and Bochaton-Piallat M.L. (2006). Phenotypic modulation of intima and media smooth muscle cells in fatal cases of coronary artery lesions. Arterioscler. Thromb. Vasc. Biol. 26, 326-332.

Jacob M.P. (2003). Extracellular matrix remodeling and matrix metalloproteinases in the vascular wall during aging and in pathological conditions. Biomed. Pharmacother. 57, 195-202.

Jones J.A., Beck C., Barbour J.R., Zavadzkas J.A., Mukherjee R., Spinale F.G. and Ikonomidis J.S.. (2009). Alterations in aortic cellular constituents during thoracic aortic aneurysm development: myofibroblast-mediated vascular remodeling. Am. J. Pathol. 175, 1746-1756.

Kobayashi M., Matsubara J., Matsushita M., Nishikimi N., Sakurai T. and Nimura Y. (2002). Expression of angiogenesis and angiogenic factors in human aortic vascular disease. J. Surg. Res. 106, 239-245.

Maeng M., Mertz H., Nielsen S., van Eys G.J., Rasmussen K. and Espersen G.T. (2003). Adventitial myofibroblasts play no major role in neointima formation after angioplasty. Scand. Cardiovasc. J. 37, 34-42.

Matkowskyj K.A., Schonfeld D. and Benya R.V. (2000). Quantitative immunohistochemistry by measuring cumulative signal strength using commercially available software photoshop and matlab. J. Histochem. Cytochem. 48, 303-312.

Måyrånpåå M.I., Trosien J.A., Fontaine V., Folkesson M., Kazi M., Eriksson P., Swedenborg J. and Hedin U. (2009). Mast cells associate with neovessels in the media and adventitia of abdominal aortic aneurysms. J. Vasc. Surg. 50, 388-395.

Mattoli S., Bellini A. and Schmidt M. (2009). The role of a human hematopoietic mesenchymal progenitor in wound healing and fibrotic diseases and implications for therapy. Curr. Stem Cell Res. Ther. 4:266-280.

Meng Y.H., Tian C., Liu L., Wang L., Liu W.Z. and Chang Q. (2011).

Expression of connective tissue growth factor and pathological remodeling in ascending thoracic aortic aneurysm. Zhonghua Wai Ke Za Zhi. 49, 261-5.

Mesure L., De Visscher G., Vranken I., Lebacq A. and Flameng W. (2010). Gene expression study of monocytes/macrophages during early foreign body reaction and identification of potential precursors of myofibroblasts. PLoS One 5, e12949.

Nataatmadja M., West J. and West M. (2006). Overexpression of transforming growth factor-beta is associated with increased hyaluronan content and impairment of repair in Marfan syndrome aortic aneurysm. Circulation 114, I371-377.

Sandbo N. and Dulin N. (2011). Actin cytoskeleton in myofibroblast differentiation: ultrastructure defining form and driving function. Transl. Res. 158, 181-196.

Schmid H., Grytsan A., Poshtan E., Watton P.N. and Itskov M. (2011). Influence of differing material properties in media and adventitia on arterial adaptation - application to aneurysm formation and rupture. Comput. Methods Biomech. Biomed. Engin. 16, 33-53.

Serini G., Bochaton-Piallat M.L., Ropraz P., Geinoz A., Borsi L., Zardi L. and Gabbiani G. (1998). The fibronectin domain ED-A is crucial for myofibroblastic phenotype induction by transforming growth factor-beta1. J. Cell Biol. 142, 873-81.

Sho E., Sho M., Nanjo H., Kawamura K., Masuda H. and Dalman R.L. (2004). Hemodynamic regulation of $CD34^+$ cell localization and differentiation in experimental aneurysms. Arterioscler. Thromb. Vasc. Biol. 24, 1916-1921.

Sobue K., Hayashi K. and Nishida W. (1999). Expressional regulation of smooth muscle cell-specific genes in association with phenotypic modulation. Mol. Cell. Biochem. 190, 105-18.

Tang P.C., Coady M.A., Lovoulos C., Dardik A., Aslan M., Elefteriades J.A. and Tellides G. (2005). Hyperplastic cellular remodeling of the media in ascending thoracic aortic aneurysms. Circulation 112, 1098-1105.

Urbich C. and Dimmeler S. (2004). Endothelial progenitor cells functional characterization. Trends Cardiovasc. Med. 14, 318-322.

van Eys G.J., Niessen P.M. and Rensen S.S. (2007). Smoothelin in vascular smooth muscle cells. Trends Cardiovasc. Med. 17, 26-30.

Yamin R. and Morgan K.G. (2012). Deciphering actin cytoskeletal function in the contractile vascular smooth muscle cell. J. Physiol. 590 (pt 17), 4145-4154.

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