Protective Aptitude of Annexin A1 in Arterial Neointima Formation in Atherosclerosis-Prone Mice—Brief Report

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Objective—Restenosis as a consequence of arterial injury is aggravated by inflammatory pathways. Here, we investigate the role of the proresolving protein annexin A1 (AnxA1) in healing after wire injury.

Approach and Results—*Apoe*^{−/−} and *Apoe*^{−/−}*Anxa1*^{−/−} mice were subjected to wire injury while fed a high-cholesterol diet. Subsequently, localization of AnxA1 and AnxA1 plasma levels were examined. AnxA1 was found to localize within endothelial cells and macrophages in the neointima. Levels of AnxA1 in the plasma and its lesional expression negatively correlated with neointima size, and in the absence of AnxA1, neointima formation was aggravated by the accumulation and proliferation of macrophages. In contrast, reendothelialization and smooth muscle cell infiltration were not affected in *Apoe*^{−/−}*Anxa1*^{−/−} mice.

Conclusions—AnxA1 is protective in healing after wire injury and could, therefore, be an attractive therapeutic compound to prevent from restenosis after vascular damage. (***Arterioscler Thromb Vasc Biol***. 2017;37:312-315. DOI: 10.1161/ATVBAHA.116.308744.)

Key Words: annexin A1 ■ macrophage ■ neointima formation ■ resolution of inflammation

Atherosclerotic vascular disease is a chronic inflammation of the arterial vessel wall causing a severe socioeconomic burden in western socities.[1] A commonly used interventional technique to widen arteries narrowed by atherosclerotic lesions is angioplasty. In turn, this intervention induces severe arterial damage, possibly leading to the recurrence of stenosis, termed restenosis. Although the incidence of restenosis has declined because of the use of drug-eluting stents and antiplatelet therapy, further optimization of angioplasty remains an important topic.[2]

Restenosis after angioplasty is the consequence of an inflammatory response to arterial damage exaggerating the growth of the neointima. Important determinants of neointima formation are endothelial denudation, leukocyte recruitment, and neointimal hyperplasia, resulting in the thickening of the arterial wall and decreased arterial lumen.[3]

Annexin A1 (AnxA1), a formyl peptide receptor 2 ligand, promotes resolution of inflammation engaging various important pathways.[4] As an example, AnxA1 has been shown to negatively regulate leukocyte trafficking,[5,6] to improve dead cell clearance by macrophages,[7] and to induce polarization of macrophages toward an anti-inflammatory phenotype.[8] All these qualities harbor the potential for AnxA1 to influence vascular repair, and we, therefore, studied the phenotype of a mouse lacking AnxA1 in a model of mechanical wire injury.

Materials and Methods

Material and Methods are available in the online-only Data Supplement.

Results

To identify the source of AnxA1, we stained healthy and injured carotid arteries from *Apoe*^{−/−} mice fed a high-fat diet, for AnxA1 (Figure I in the online-only Data Supplement), along with specific markers for endothelial cells (CD31), smooth muscle cells (α-smooth muscle actin), and macrophages (Mac2). In either case, AnxA1 was found to primarily colocalize with endothelial cells and macrophages (Figure 1A and 1B; Figure II in the online-only Data Supplement). Similar observations were made in human iliac artery sections obtained after percutaneous transluminal angioplasty (Figures II and III in the online-only Data Supplement). Likewise, AnxA1 expression was prominent in cell cultures of mouse and human endothelial cells and macrophages, whereas little staining was found in smooth muscle cells (Figure IV in the online-only Data Supplement). On cell activation, AnxA1 can

be externalized to the cell membrane or secreted into the extra-cellular fluids[9] and can, therefore, be detected in the plasma by ELISA. Interestingly, data from bone marrow transplant studies (Figure VA in the online-only Data Supplement) indicate that plasma AnxA1 may derive from circulating and vessel-resident cells. In fact, no differences in plasma AnxA1 were found between *Apoe*^{−/−} mice reconstituted with *Apoe*^{−/−}*AnxA1*^{−/−} bone marrow and *Apoe*^{−/−}*AnxA1*^{−/−} mice reconstituted with *Apoe*^{−/−} bone marrow. Of note, AnxA1 plasma levels from both groups were lower when compared with *Apoe*^{−/−} mice receiving a high-fat diet for 4 weeks (Figure VA and VB in the online-only Data Supplement), thus, suggesting a contribution of either source to circulating plasma levels. Remarkably, an additional increase of AnxA1 protein level was observed in mice that were both fed a high-fat diet and subjected to wire injury (Figure VB in the online-only Data Supplement).

To study a potential role of AnxA1 during arterial injury, we correlated neointima sizes in *Apoe*^{−/−} mice 4 weeks after wire
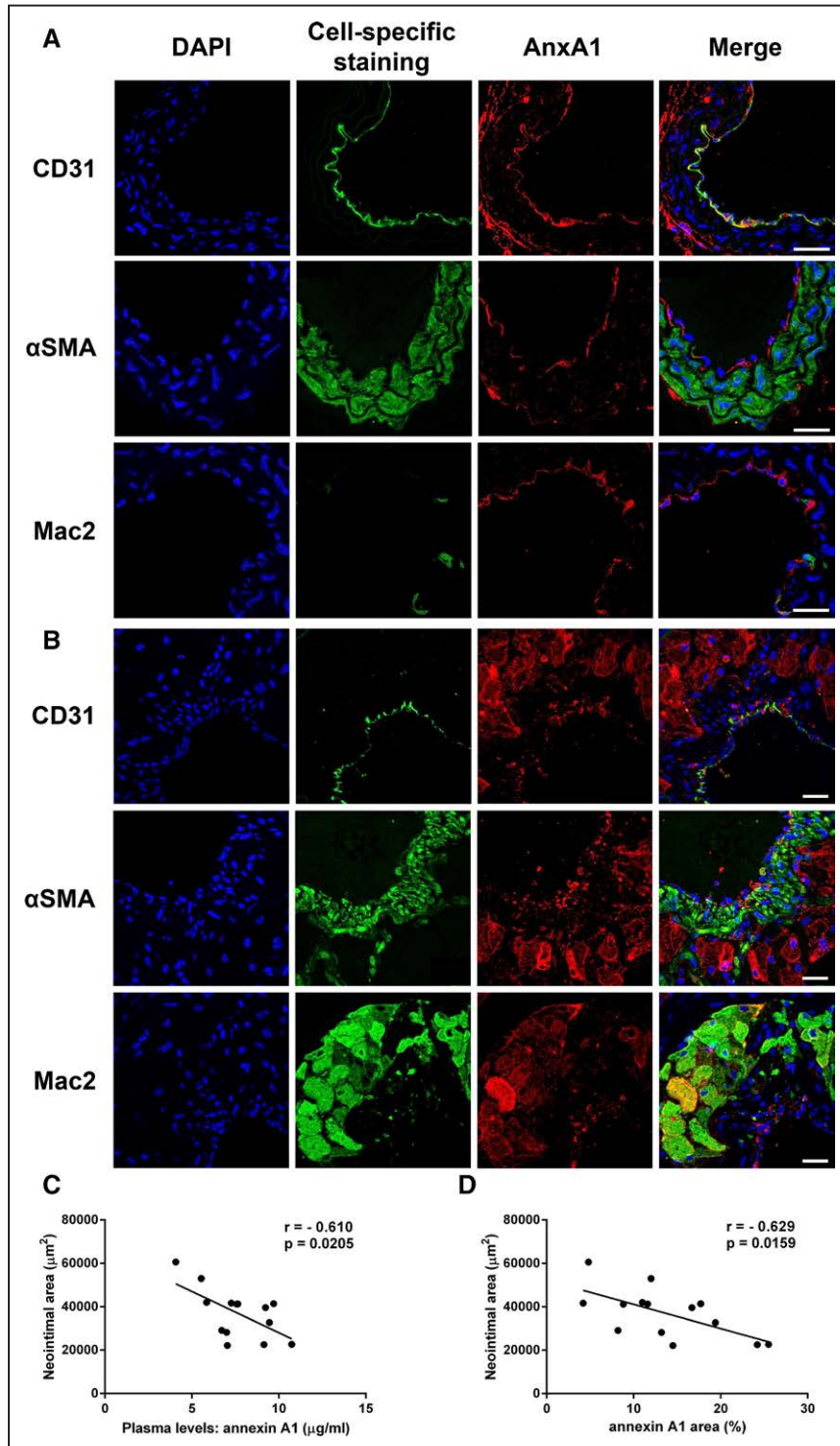

Figure 1. Annexin A1 (AnxA1) negatively correlates with neointima area after arterial injury. Sections of carotid arteries, obtained from *Apoe*^{−/−} mice fed a high-fat diet for 4 weeks, were stained for AnxA1. Stainings were made in undamaged (**A**) or damaged vessels (**B**) in conjunction with antibodies to endothelial cells (CD31), smooth muscle cells (αSMC), or macrophages (Mac2). Scale bars represent 25 μm. **C**, AnxA1 plasma levels, obtained from *Apoe*^{−/−} mice 4 weeks after arterial damage, were correlated with carotid neointima area (Pearson correlation, n=14). **D**, Correlation of neointimal AnxA1 area with carotid neointima size (Pearson correlation, n=14).
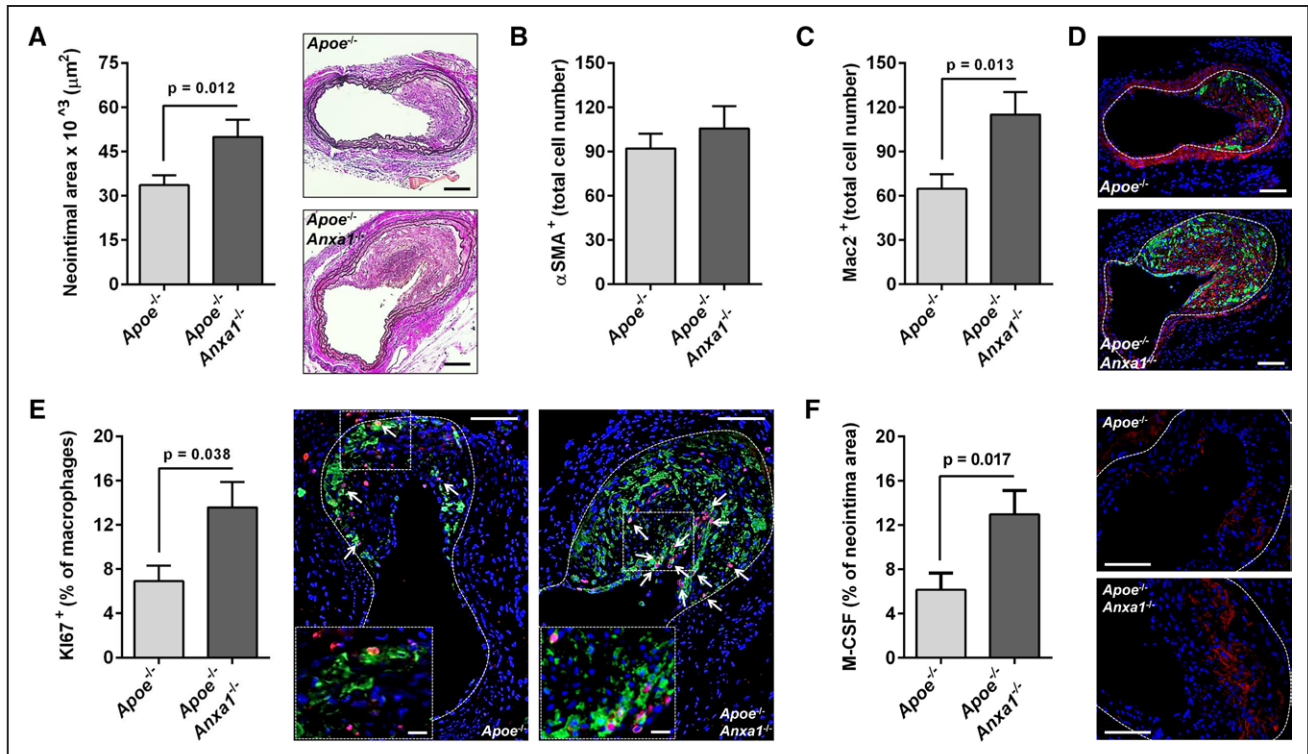
Figure 2. Neointima expansion is aggravated in annexin A1 (AnxA1)–deficient mice. $Apoe^{-/-}$ and $Apoe^{-/-}Anxa1^{-/-}$ mice were subjected to wire injury and fed an atherogenic high-fat diet 1 week prior and 4 weeks after injury. **A**, Quantification of the carotid neointima area in elastic tissue fibers—Verhoeff's van Gieson (EVG)-stained section. **B**, Quantification of α-smooth muscle actin (αSMA⁺) cells in absolute numbers. **C**, Quantification of total Mac2⁺ cells, indicating tissue-resident macrophage accumulation. **D**, Representative images are stained for Mac2 (green), α-SMA (red), and DAPI (blue). **E**, Assessment of macrophage proliferation by colocalization of KI67 within macrophages (Mac2). Representative images are stained for KI67 (red), Mac2 (green), and DAPI (blue). White arrows indicate proliferating macrophages (Mac2⁺/KI67⁺ cells) **F**, Assessment of presence of M-CSF (macrophage colony-stimulating factor; %) in the neointima area. Scale bars represent 100 or 25 µm (**right**, panel **D** zoom). All data are presented as mean±SEM. Data were analyzed by unpaired *t* test. n=9 to 10. *P* values <0.05 were considered significant.

injury with AnxA1 plasma levels measured by ELISA (Figure 1C) or the degree of lesional AnxA1 expression (Figure 1D). In both analyses, a negative correlation was perceived suggestive of a protective effect of AnxA1 in healing after injury.

To establish a causal contribution of AnxA1 in arterial healing, $Apoe^{-/-}$ and $Apoe^{-/-}Anxa1^{-/-}$ mice were subjected to wire injury while fed a high-cholesterol diet. No significant differences were observed in myeloid cell counts in the blood, bone marrow, and spleen (Table I in the online-only Data Supplement). In sections obtained 4 weeks after the instigation of injury, $Apoe^{-/-}Anxa1^{-/-}$ mice showed a marked increase in neointima size, corroborating a protective effect of AnxA1 (Figure 2A). To reveal the cell type that is responsible for the aggravation of neointima growth, the cellular composition of the neointima was characterized. No changes were observed in the number of smooth muscle cells present in the neointima (Figure 2B). However, in line with the predominant expression of AnxA1 in macrophages, we evidenced an expansion of these cells in neointimal lesions obtained from AnxA1-deficient mice (Figure 2C and 2D; Figure VI in the online-only Data Supplement), which can possibly be attributed to a larger fraction of proliferating macrophages in these mice (Figure 2E). In addition, more M-CSF (macrophage colony-stimulating factor) staining was found in neointima sections from $Apoe^{-/-}Anxa1^{-/-}$, which could explain an increase in macrophage proliferation

(Figure 2F). Interestingly, an inhibitory effect of AnxA1 on the expression (Figure VIIA in the online-only Data Supplement) and release (Figure VIIB in the online-only Data Supplement) of M-CSF from macrophages was observed in vitro. Of note, the endothelial lining was equally restored in AnxA1-deficient mice (Figure VIII in the online-only Data Supplement).

Discussion

In the present study, we examined the role of AnxA1 in healing after arterial injury. We witnessed a prominent expression of AnxA1 in endothelial cells and macrophages residing in the neointima. Lesional AnxA1 and plasma AnxA1 levels negatively correlated with neointima area, suggestive of a reparative role of AnxA1. In AnxA1-null mice, neointima development was mainly aggravated by the accumulation of proliferating macrophages in the injured tissue.

Inflammatory macrophages are histologically identified within neoatherosclerosis in human coronary implants and stents.[10] Arterial macrophage accumulation is controlled by different mechanisms, including apoptosis, recruitment, and proliferation.[11] In previous work, it has been shown that AnxA1 and its N-terminal fragment Ac2-26 counteract chemokine-induced integrin activation by inhibition of Rap1 activation in monocytes and, therefore, restrains adhesion to large arteries.[5] Consequently, enhanced recruitment of monocytes

in AnxA1-deficient mice may contribute to the phenotype observed in the present study. Moreover, macrophage proliferation instigated by modified lipids and proinflammatory meditators in atherosclerotic lesions is a denominator of arterial macrophage accumulation.[12] Herein, we found an increase in the number of proliferating macrophages in AnxA1-null mice, suggesting that AnxA1 acts as negative regulator of macrophage proliferation. In line with those findings, an elevated M-CSF staining in the neointima of AnxA1-deficient mice was found, as well as an inhibitory effect of recombinant AnxA1 on the expression and release of M-CSF by macrophages in vitro. By which mechanism AnxA1 controls M-CSF production in macrophages and if this is of relevance also during advanced stages of atherosclerosis remain to be defined. Taken together, the phenotype observed in our model likely originates from a combination of augmented monocyte recruitment and the increased ability of arterial macrophages to proliferate in the absence of AnxA1 (Graphic Abstract in the online-only Data Supplement).

Therapeutic intervention with AnxA1 or its bioactive peptide Ac2-26 have proven to be beneficial in mouse models of arterial vascular disease.[5,13] Of note, it has been demonstrated that arterial-specific nanodelivery of Ac2-26 could be a promising tool to prevent atheroprogression, a strategy that may also be applicable to treatment of angioplasty-related side effects.[14] In line with previously successfully used FPR2 ligands,[3] stents coated with AnxA1 protein could potentially be suitable to prevent lumen loss and inflammation after arterial injury.

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Disclosures

None.

References

1. Mozaffarian D, Benjamin EJ, Go AS, et al; American Heart Association Statistics Committee; Stroke Statistics Subcommittee. Heart disease and stroke statistics-2016 update: a report from the American Heart Association. *Circulation*. 2016;133:e38–360. doi: 10.1161/CIR.0000000000000350.
2. Katz G, Harchandani B, Shah B. Drug-eluting stents: the past, present, and future. *Curr Atheroscler Rep*. 2015;17:485. doi: 10.1007/s11883-014-0485-2.
3. Soehnlein O, Wantha S, Simsekyilmaz S, et al. Neutrophil-derived cathelicidin protects from neointimal hyperplasia. *Sci Transl Med*. 2011;3:103ra98. doi: 10.1126/scitranslmed.3002531.
4. Ortega-Gómez A, Perretti M, Soehnlein O. Resolution of inflammation: an integrated view. *EMBO Mol Med*. 2013;5:661–674. doi: 10.1002/emmm.201202382.
5. Drechsler M, de Jong R, Rossaint J, Viola JR, Leoni G, Wang JM, Grommes J, Hinkel R, Kupatt C, Weber C, Döring Y, Zarbock A, Soehnlein O. Annexin A1 counteracts chemokine-induced arterial myeloid cell recruitment. *Circ Res*. 2015;116:827–835. doi: 10.1161/CIRCRESAHA.116.305825.
6. Lim LH, Solito E, Russo-Marie F, Flower RJ, Perretti M. Promoting detachment of neutrophils adherent to murine postcapillary venules to control inflammation: effect of lipocortin 1. *Proc Natl Acad Sci U S A*. 1998;95:14535–14539.
7. Arur S, Uche UE, Rezaul K, Fong M, Scranton V, Cowan AE, Mohler W, Han DK. Annexin I is an endogenous ligand that mediates apoptotic cell engulfment. *Developmental Cell*. 2003; 4:587–598.
8. Li Y, Cai L, Wang H, Wu P, Gu W, Chen Y, Hao H, Tang K, Yi P, Liu M, Miao S, Ye D. Pleiotropic regulation of macrophage polarization and tumorigenesis by formyl peptide receptor-2. *Oncogene*. 2011;30:3887–3899. doi: 10.1038/onc.2011.112.
9. de Jong RJ, Leoni G, Drechsler M, Soehnlein O. The advantageous role of annexin A1 in cardiovascular disease. *Cell Adh Migr*. 2016;18:1–14. doi: 10.1080/19336918.2016.1259059.
10. Nakazawa G, Otsuka F, Nakano M, Vorpahl M, Yazdani SK, Ladich E, Kolodgie FD, Finn AV, Virmani R. The pathology of neoatherosclerosis in human coronary implants bare-metal and drug-eluting stents. *J Am Coll Cardiol*. 2011;57:1314–1322. doi: 10.1016/j.jacc.2011.01.011.
11. Drechsler M, Duchene J, Soehnlein O. Chemokines control mobilization, recruitment, and fate of monocytes in atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2015;35:1050–1055. doi: 10.1161/ATVBAHA.114.304649.
12. Robbins CS, Hilgendorf I, Weber GF, et al. Local proliferation dominates lesional macrophage accumulation in atherosclerosis. *Nat Med*. 2013;19:1166–1172. doi: 10.1038/nm.3258.
13. Kusters DH, Chatrou ML, Willems BA, De Saint-Hubert M, Bauwens M, van der Vorst E, Bena S, Biessen EA, Perretti M, Schurgers LJ, Reutelingsperger CP. Pharmacological treatment with annexin A1 reduces atherosclerotic plaque burden in LDLR^{-/-} mice on western type diet. *PLoS One*. 2015;10:e0130484. doi: 10.1371/journal.pone.0130484.
14. Fredman G, Kamaly N, Spolitu S, Milton J, Ghorpade D, Chiasson R, Kuriakose G, Perretti M, Farokhzad O, Farokzhad O, Tabas I. Targeted nanoparticles containing the proresolving peptide Ac2-26 protect against advanced atherosclerosis in hypercholesterolemic mice. *Sci Transl Med*. 2015;7:275ra20. doi: 10.1126/scitranslmed.aaa1065.

Highlights

- Annexin A1 is expressed by endothelial cells and macrophages in large arteries.
- Local and systemic annexin A1 levels negatively correlate with neoimtima sizes.
- Lack of annexin A1 exacerbates neointima formation by enhanced macrophage accumulation.
- Annexin A1 suppresses M-CSF (macrophage colony-stimulating factor) production and release.