Lack of Fibronectin-EDA Promotes Survival and Prevents Adverse Remodeling and Heart Function Deterioration After Myocardial Infarction

Fatih Arslan, Mirjam B. Smeets, Paul W. Riem Vis, Jacco C. Karper, Paul H. Quax, Lennart G. Bongartz, John H. Peters, Imo E. Hoefer, Pieter A. Doevendans, Gerard Pasterkamp, Dominique P. de Kleijn

Rationale: The extracellular matrix may induce detrimental inflammatory responses on degradation, causing adverse cardiac remodeling and heart failure. The extracellular matrix protein fibronectin-EDA (EIIIA; EDA) is upregulated after tissue injury and may act as a "danger signal" for leukocytes to cause adverse cardiac remodeling after infarction.

Objective: In the present study, we evaluated the role of EDA in regulation of postinfarct inflammation and repair after myocardial infarction.

Methods and Results: Wild-type and EDA<sup>−/−</sup> mice underwent permanent ligation of the left anterior coronary artery. Despite equal infarct size between groups (38.2±4.6% versus 38.2±2.9% of left ventricle; P=0.985), EDA<sup>−/−</sup> mice exhibited less left ventricular dilatation and enhanced systolic performance compared with wild-type mice as assessed by serial cardiac MRI measurements. In addition, EDA<sup>−/−</sup> mice exhibited reduced fibrosis of the remote area without affecting collagen production, cross-linking, and deposition in the infarct area. Subsequently, ventricular contractility and relaxation was preserved in EDA<sup>−/−</sup>. At tissue level, EDA<sup>−/−</sup> mice showed reduced inflammation, metalloproteinase 2 and 9 activity, and myofibroblast transdifferentiation. Bone marrow transplantation experiments revealed that myocardium-induced EDA and not EDA from circulating cells regulates postinfarct remodeling. Finally, the absence of EDA reduced monocyte recruitment as well as monocytic Toll-like receptor 2 and CD49d expression after infarction.

Conclusions: Our study demonstrated that parenchymal fn-EDA plays a critical role in adverse cardiac remodeling after infarction. Absence of fn-EDA enhances survival and cardiac performance by modulating matrix turnover and inflammation via leukocytes and fibroblasts after infarction. (Circ Res. 2011;108:582-592.)

Key Words: myocardial infarction ■ remodeling ■ inflammation ■ heart failure ■ immune system

Heart failure (HF) is becoming a major socioeconomic burden for Western societies, because both incidence and prevalence numbers are increasing. Improved reperfusion strategies have caused a decline in death rates after acute myocardial infarction (MI). However, because more patients do survive the initial infarction, infarct-related morbidity increases. The extent of tissue loss in the acute phase after MI is a major determinant of the degree of adverse remodeling. However, chronic processes like extracellular matrix (ECM) turnover, fibrosis, and inflammation are key mediators of post-MI cardiac repair and adverse remodeling. Despite pharmacological advances (eg, β-blockers, renin–angiotensin–aldosterone system inhibitors)<sup>1,4</sup> incidence for HF is increasing and mortality remains very high (5-year mortality, 30% to 70%).<sup>2</sup> Inflammation is the first critical step in tissue repair responses after MI. Many interventions have been studied to modulate the inflammatory reaction to enhance heart function and attenuate structural changes leading to HF. However, interfering within the innate immune system is not without danger and necessitates understanding the temporal, spatial, and (patho)physiological role of the target.<sup>2,6</sup> Endogenous “danger signals” have gained much interest recently, because they are released after tissue

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injury and do not play a biological role under normal conditions. Danger signals (eg, cardiac myosin\(^2\) and HMGB1\(^1\)) are released on ECM degradation or cardiomyocyte death and can activate leukocytes, thereby inducing the detrimental responses seen after MI. Toll-like receptors (TLRs) are postulated as one of the main targets for endogenous ligands released after infarction.\(^9\) On recognition of endogenous ligands, TLRs exert the same detrimental inflammatory processes via cytokine and chemokine expression.

Cellular fibronectin is a multifunctional adhesive glycoprotein present in the ECM and is produced by cells (eg, fibroblast, endothelial cells) in response to tissue injury. It contains an alternatively spliced exon encoding type III repeat extra domain A (EIIIA; EDA) that act as an endogenous ligand for both TLR2 and -4; in vitro, EDA repeat extra domain A (EIIIA; EDA) that act as an endogenous ligand for both TLR2 and -4; in vitro, EDA also acts as a ligand for several integrins (eg, VLA-4/CD49d) regulating cell adhesion and proliferation.\(^12,13\) The presence of EDA is also crucial for myofibroblast phenotype induction and function.\(^14\)

In summary, EDA is able to activate leukocytes and cause an upregulation of cytokines and chemokines. One might hypothesize that on degradation of the ECM and/or de novo synthesis after infarction, EDA could cause detrimental inflammatory responses. In the present study, we show, in vivo, that the lack of EDA indeed prevents adverse cardiac remodeling and enhances cardiac function after acute MI.

Methods

Animals and Experimental Design
EDA\(^{−/−}\) mice were kindly provided by Dr John H. Peters and back-crossed into a Balb/C background for 8 generations. Male Balb/C wild-type (WT), EDA\(^{−/−}\), and chimeric mice (10 to 12 weeks, 25 to 30 g) received standard diet and water ad libitum. Myocardial infarction was induced by left coronary artery ligation, just below the left atrial appendage. All animal experiments were performed in accordance with the national guidelines on animal care and with prior approval by the Animal Experimentation Committee of Utrecht University.

Online Data Supplement
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org and contains detailed information regarding MI in vivo, protein and RNA isolation, flow cytometry, the generation of chimeric mice, infarct size calculation, MRI measurements, invasive left ventricular (LV) pressure measurements, immunohistochemistry, polymerase chain reaction, zymography, caspase 3/7 activity, elastase activity, myofibroblast culture, fibronectin-EDA Western blotting, and statistical analysis.

Results

Lack of EDA Promotes Survival and Prevents Heart Function Deterioration, As Well As Maladaptive Remodeling, After Myocardial Infarction
EDA synthesis is stimulated after infarction in both infarct and remote myocardium, reaching a peak at 7 and 3 days, respectively (Online Figure I). Baseline MRI assessment of cardiac function and dimensions revealed no differences between EDA\(^{−/−}\) and WT mice. Microscopic analyses did not show any alterations in cellularity and matrix composition in EDA\(^{−/−}\) mice (Online Figure II).

The extent of endangered myocardium (area at risk/LV) determined at 2 days after MI was similar between the groups. Infarct size as percentage of LV was also similar between groups (infarct size/LV 38.2±1.2%, \(P=0.985\); Figure 1A). Kaplan–Meier survival analysis showed a significant survival benefit in EDA\(^{−/−}\) mice over WTs (Figure 1B). Most deaths occurred after day 6 and were not caused by cardiac rupture (only 2 ruptures in the WT and 1 rupture in EDA\(^{−/−}\) mice were observed during 28 days follow-up). In line with increased mortality, WT mice had greater LV dimensions and reduced systolic performance compared with EDA\(^{−/−}\) mice (Figure 1C; Table 1). These significant differences were already present 7 days after infarction, and continued to deteriorate till 28 days after MI. EDA\(^{−/−}\) mice were relatively protected against remodeling and exhibited better systolic function after MI (Figure 1C; Table 1). The protective effect seen in EDA\(^{−/−}\) was not attributable to changes in the extent of viable tissue, because infarct size did not differ between WT and EDA\(^{−/−}\) mice 28 days after MI (33.7±2.3% versus 34.3±3.5%, respectively; \(P=0.818\)). However, wall thickness of the infarct area did not decline in EDA\(^{−/−}\) mice as much as in WTs. At day 28 postinfarction, the entire infarct area was replaced by a dense collagen network in both groups. At day 7, however, there was reduced granulation of the infarct as shown by delayed degradation of acellular matrix in EDA\(^{−/−}\) mice (Figure 1D).
Lack of EDA Decreases Endogenous MMP-2, -9, and Elastase Activities

Enhanced MMP2 and -9 activities are detrimental for cardiac performance and geometry during the postinfarct healing process. We performed zymography to study whether the protection against adverse remodeling seen in EDA−/− mice is also attributable to changes in proteinase activity. In line with the reduced matrix degradation in the knock-out animals at day 7 observed in histology, both active forms of MMP-2 and -9 in infarct areas were reduced in EDA−/− mice, 7 days postinfarction (Figure 2). Analysis of the remote area after 7 days infarction was not possible because of very low...
Table 1. Cardiac Function and Geometry After Infarction

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<th>Baseline</th>
<th>7 Days MI</th>
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<td>EDA KO BM</td>
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<td>EF (%)</td>
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<td>Stroke work</td>
<td>15.6 ± 2.3</td>
<td>15.6 ± 2.3</td>
<td>15.6 ± 2.3</td>
<td>NS</td>
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**EDA** /-/- Mice Exhibit Less Postinfarct Fibrosis

Collagen deposition occurs in the infarct area on degradation of the matrix and in the remote myocardium on changes in wall stress. Myofibroblasts are the primary source of de novo collagen synthesis. In our study, collagen deposition in both the infarct and remote area was similar between the groups at day 7 after MI (Online Figure IV). After 28 days, collagen content was again similar in the infarct area, suggesting that scar formation is not negatively affected in EDA /-/- mice (Figure 3A). However, the remote myocardium now contained less collagen fibers in EDA /-/- mice compared with WT animals (Figure 3B; lower pixel count in the small-size gray value spectrum). These findings were supported at the mRNA level. Both procollagen-1 and -3 are reduced in the remote myocardium of EDA /-/- mice (Figure 3C). Within the infarct, collagen synthesis in EDA /-/- mice was again comparable to WT animals. There was no difference in lysyl-oxidase and TIMP-2 production between the groups, suggesting no differences in collagen cross-linking and protease inhibition, respectively (Online Figures V and VI). The reduced fibrosis in the remote myocardium at 28 days after MI is preceded by a significantly decreased myofibroblast transdifferentiation in EDA /-/- mice, in both remote and infarct areas 7 days after infarction (Figure 3D and 3E). Periostin is described as a maturation factor of cardiac fibroblasts. In our study, periostin-positive area was reduced as well in EDA /-/- mice compared with WT animals (Figure 3F; Online Figure VII). To study whether WT and EDA /-/- myofibroblasts differed in their matrix synthesis activity and MMP expression profile, we cultured postinfarct myofibroblasts and stained for myofibroblast markers and procollagen III. In vitro, there were no differences between the 2 genotypes (Online Figure VIII). In addition, zymography was done using the supernatants of the cells and showed also no differences in MMP2 and -9 activity (Online Figure IX).

Lack of EDA Results in Enhanced Inotropy and Lusitropy

Altered fibrotic processes in EDA /-/- mice indicate that diastolic function could be affected as well. Table 2 shows the results from the invasive LV pressure assessments. Contractility, as indicated by dP/dt_{max}, was much higher in EDA /-/- mice after 28 days infarction. This confirmed our previous MRI findings, that EDA /-/- mice exhibit enhanced systolic performance (Table 1). Increase of LV end-diastolic pressure and tau is detrimental for heart function and is caused by increase of EDV and/or fibrosis, one of the hallmarks of heart failure. Compared with WT animals, diastolic performance signal intensity. Finally, we studied whether elastase activity was also affected by the absence of EDA. In line with reduced MMP2 and -9 activity, elastase activity was also reduced in EDA /-/- mice 7 days after infarction (Online Figure III).
was also significantly enhanced in EDA−/− mice after 28 days infarction. Both parameters were significantly lower in EDA−/− mice compared with WT animals, providing evidence that the improved survival in EDA−/− mice is a consequence of both systolic and diastolic functional improvements.

EDA Regulates Post-MI Inflammation
Lack of EDA should result in a decreased inflammatory status, because EDA is considered as a ligand for TLR2 and -4. Neutrophils are the first leukocyte subset migrating on tissue injury and are known to be associated with the extent of damage. Neutrophil count in the infarct area was not different between the groups (Figure 4A). Hereafter, macrophages clear cell debris (e.g., necrotic neutrophils and cardiomyocytes) and, more importantly, initiate the remodeling process after infarction.2 In our study, the number of macrophages was highly reduced in EDA−/− mice 7 days postinfarction (Figure 4B). There were no cells detectable after 28 days infarction in both groups (data not shown). In concordance with the reduced macrophage influx, levels of tumor necrosis factor (TNF)α, RANTES, granulocyte/macrophage colony-stimulating factor (GM-CSF) (responsible for recruitment, differentiation and maturation of macrophages) and interleukin-10 were highly reduced in EDA−/− mice, 7 days after infarction (Figure 4C through 4F). In contrast, monocyte chemoattractant protein (MCP)-1 levels were increased in EDA−/− mice compared with WT animals at protein and mRNA level (Online Figure X).

Parenchymal EDA Mediates Postinfarct Survival and Maladaptive Remodeling
We generated chimeric mice to differentiate between the contribution of the blood and parenchymal compartments to the observed effects after MI. Interestingly, WT/EDA KO BM had similar survival rates and cardiac performance compared with WT/WT BM animals. In contrast, EDA KO/WT BM were similar to EDA KO/EDA KO BM animals and showed higher survival rates and exhibited less adverse remodeling after MI, compared with WT/EDA KO BM (Figure 5A and 5B; Table 1). These data indicate that postinfarct parenchymal EDA expression drives maladaptive remodeling. From a danger model perspective, we may postulate that EDA expression as a danger signal can have profound effects on circulating cells which are responsible for postinfarct repair responses.

EDA Mediates Both Integrin-α4 and Toll-Like Receptor Signaling in Circulating Monocytes
EDA is a known ligand for integrin-α4β1 (VLA-4)12 and TLR2 and -4.10 Because parenchymal EDA mediates adverse remodeling, we hypothesized that EDA from the heart may serve as an endogenous activator of circulating cells after infarction. EDA−/− mice showed a significant reduction in peripheral monocytes 3 days after infarction, whereas after 7 days the numbers were similar between the groups (Figure 6A). TLR2 expression on monocytes was significantly altered in the absence of EDA, whereas TLR4 did not show any difference in expression levels after MI between the groups (Figure 6B and 6C). Integrin-α4 (CD49d) expression was also significantly reduced on monocytes of EDA−/− mice after infarction (Figure 6D). In addition, there was a subgroup of monocytes that showed a significant higher expression level of CD49d. EDA−/− mice showed again a reduced CD49d expression in this subgroup, 7 days postinfarction (Figure 6E).

Discussion
HF is already considered as an epidemic of the 21st century and its socioeconomic burden of HF is likely to increase.16 Mortality after acute MI declines because of
Figure 3. EDA\(^{-/-}\) mice exhibit less fibrosis after MI. Histograms give an overview of the entire spectrum of collagen intensity. The larger the gray value, the more intense the collagen staining. Gray values below 30 are considered as background signal of the intensity image acquired after polarized light microscopy. A, Collagen intensity in the infarct area, 28 days post-MI. B, Collagen intensity in the remote myocardium, 28 days post-MI. *\(P=0.042\) area under the curve. C, mRNA levels in infarct and remote areas during infarct development. Procollagen-1 and -3 mRNA levels in the remote area are significantly reduced in EDA\(^{-/-}\) mice. *\(P=0.004\), †\(P=0.008\), ‡\(P=0.007\) compared with WT animals. D, Positive a-SMA fraction in LV wall during cardiac repair. Vessels are not taken in the positive area fraction calculation. *\(P=0.014\). E, Positive a-SMA fraction in infarct and remote area 7 days postinfarct. *\(P=0.009\), †\(P=0.047\). F, Periostin-positive area within infarct and border zone. *\(P=0.018\), †\(P=0.045\). Representative images of collagen intensity using white and polarized light microscopy. Each bar represents mean±SEM, \(n=6/group/time\) point. LV indicates left ventricular.
pharmacological and technical advances facilitating early reperfusion and survival. However, morbidity increases because of the excessive tissue loss and detrimental chronic processes during infarct healing. Inflammation is necessary for adequate wound healing and interfering with antiinflammatory agents may be devastating.17 However, postinfarct recovery is characterized by an autodestructive inflammatory response causing a vicious circle. Hereby, the same inflammatory response influences cardiac performance and geometry in a detrimental way. This adverse healing process causes changes in ventricular structural design and dimension, referred as adverse cardiac remodeling. Despite the therapeutic advances for patients experiencing acute MI, there is a window of opportunity for adjunctive interventions. In particular, therapeutic targets within our innate immune system hold great promise for future application for cardiomyocyte salvage after acute MI.18,19 Ideally, the optimal therapy for patients experiencing acute MI encompasses both cardiomyocyte salvage and attenuating adverse remodeling, without affecting a proper scar formation.

The discovery of TLRs has not only provided us with great insight into pathophysiological mechanisms in HF but may also hold therapeutic value. We and others have shown that deficient TLR2 and -4 activation has therapeutic effects in murine models of atherosclerosis10,20 and cardiac ischemia.19,21–23 The idea that pattern-recognition receptors also induce detrimental inflammatory responses in noninfectious settings has resulted in the search for endogenous ligands, so called danger signals. These danger signals are thought to be released and/or produced during tissue injury and cell stress. From this “danger model” perspective, EDA may be a critical mediator of adverse remodeling after MI.

We have shown that EDA is indeed upregulated after acute MI. This is in line with previous reports that EDA is produced in response to tissue injury (reviewed in Ref.12). EDA mRNA levels peaked at day 7 after MI in the infarcted wall and returned to baseline levels after 28 days. Immunohistochemistry suggested similar if not greater expression of EDA in the remote myocardium. The discrepancy between mRNA and protein levels and EDA staining could potentially reflect sampling of EDA pools of differing solubility. Experiments with cultured cells have shown that, after synthesis and secretion, soluble fibronectin dimers first appear at the cell surface but, over time, incorporate into less soluble multimeric aggregates in the ECM.23 Immunohistologic analysis has the capacity to detect such insoluble fibronectin aggregates in the ECM of tissues, whereas immunoblot analysis is apt to preferentially detect soluble extractable fibronectins.25 Potentially, EDA that is produced after the modest increase in EDA transcription in remote myocardium could preferentially incorporate into an insoluble fibronectin pool, thereby becoming relatively more evident by immunohistologic than immunoblot analysis (Online Figure I). Despite similar infarct size, EDA−/− mice showed enhanced survival compared with WT animals. In addition, WT animals exhibited a severely affected cardiac function and profound expansive remodeling during 28 days of recovery. In contrast, EDA−/− mice showed preserved LV geometry, better systolic performance and less LV bulging during systole. Interestingly, this protective effect was already significant 7 days after infarction and continued to be apparent in the long-term follow-up. The detrimental changes were associated with the EDA mRNA peak seen at day 7.

We explored several cellular and molecular mechanisms to explain the protective effect of EDA absence after MI.

First, we showed that EDA−/− mice have a normal scar formation (normal collagen content in infarct area), whereas remote fibrosis was decreased. This was also supported at the mRNA level for both procollagen-1 and -3, showing only decreased expression in the remote area. Furthermore, granulation of the infarct area was delayed and wall thickness did not decline in EDA−/− mice as much as in WT animals. In addition, myofibroblast transdifferentiation in the postinfarct ventricular wall was highly suppressed in EDA−/− mice. These observations suggest that EDA does not mediate fibrosis directly, because there is selective attenuation of fibrosis in the remote myocardium and not in the scar area in EDA−/− mice. We may hypothesize the following mechanisms: because of delayed matrix degradation in EDA−/− after infarction, infarct thinning is also delayed. The thicker wall of the infarct area in EDA−/− mice results in less wall stress and thus end-diastolic volume is limited. In addition, the decreased wall stress in EDA−/− mice may explain the decreased LV pressure overload in these mice. Subsequently, reduced ventricular pressure may result in reduced fibrosis of the remote myocardium, as observed in our EDA−/− animals 28 days after infarction. Invasive pressure measurements indeed revealed that WT animals have nearly 90% increase of both LV end-diastolic pressure and LV relaxation time compared with EDA−/− mice. We studied whether apoptosis in the remote myocardium could explain the functional impairment in WT animals, but did not observe a difference in caspase 3 and 7 activity between the groups during follow-up (Online Figure XI).

Secondly, we studied the inflammatory status in the postinfarct hearts. The absence of EDA did not affect neutrophil count, suggesting that the extent of tissue injury was similar between the groups. This is supported by the observation that infarct size did not differ between the
groups. In contrast, macrophages (known as key players in adverse cardiac remodeling) migrated less in both the infarct and remote myocardium of EDA−/− mice. In addition, lack of EDA protected the animals against the production of TNFα, RANTES, and GM-CSF: all known detrimental (chemotactic) cytokines in acute MI and HF, negatively affecting cardiomyocyte survival and function and ECM remodeling.2,18 In contrast, MCP-1 is increased in EDA−/− mice. There are several possible explanations for reduced migration of mononuclear cells in EDA−/− mice despite increased MCP-1 levels. First, decreased CD49d expression seen in our experiments results in reduced migration capacity of monocytes (Figure 6).26 It is likely that MCP-1 fails to cause effective migration because of reduced CD49d expression on monocytes. The complexity of the role of MCP-1 in adverse remodeling is also shown by the fact that several studies have reported contradictory results. Both cardiac-specific MCP-1 over-
expression and MCP-1 inhibition have shown to prevent adverse remodeling and fibrosis after infarction.\(^{27-29}\)

Thirdly, we studied MMP and elastase secretion because MMP2, -9 and elastase have been described as mediators of adverse cardiac remodeling.\(^{30}\) Both endogenous MMP2, -9 and elastase activities were decreased in EDA\(^{-/-}\) mice compared with WT animals. These findings corroborate our MRI observations, in which wall thickness of the infarcted free wall is thicker in EDA\(^{-/-}\) mice, already at 7 days after MI (Table 1). Our observation of decreased endogenous MMP2 and -9 activities in the absence of EDA is supported by Okamura Y et al. They showed that EDA mediates MMP9 production via TLR4 activation.\(^{31}\) Despite the fact that they did not see any effect on MMP2 production in vitro, we did show that MMP2 was affected as well in vivo. One possible explanation is that the in vitro setting used by Okamura Y et al does not represent the spatial and temporal dependence of MMP production; infarct and remote myocardium have their own MMP signature during the post-MI period.\(^{30}\) In addition, we examined whether EDA is a direct inhibitor of MMP2 and -9. Adding recombinant EDA to MMP2 and -9 did not have an inhibitory effect (Online Figure XII), suggesting that the reduced MMP2 and -9 activity observed in vivo is likely via reduced secretion.

Finally, we found that EDA expression in resident myocardial cells mediate LV remodeling after infarction. Moreover, we have shown that circulating monocytes are significantly affected in the absence of EDA. Besides the fact that monocyte numbers are temporarily decreased after infarction, the absence of EDA also downregulates TLR2 and CD49d expression in vivo.

Taking these novel findings into account, we can hypothesize the following on the mode of action of EDA: (1) both leukocytes and cardiac fibroblasts, critically involved in cardiac remodeling, are activated by the danger signal EDA produced in the heart after MI; and (2) leukocytic integrins (eg, \(\alpha_4\beta_1\)) have higher binding affinity to the ECM in the presence of EDA,\(^{32}\) resulting in increased migration and/or differentiation of leukocytes after MI. It is very likely that both fibroblast transdifferentiation and leukocytic activation and migration contribute equally to the observed effects in our study.
In summary, we have shown that EDA is upregulated in response to the infarction and returns to normal levels after long-term survival. In the absence of EDA, postinfarct adverse remodeling is highly attenuated with concomitant survival benefit. EDA−/− mice are relatively protected against the profound expansive remodeling. In addition, both systolic and diastolic performance is preserved in EDA−/− mice via decreased fibrosis and altered inflammation.

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Disclosures
None.

References

Figure 6. EDA alters peripheral monocyte characteristics after infarction. A, Circulating monocyte count at baseline and 3 and 7 days after infarction. *P*<0.006. B and C, TLR2 (B) and TLR4 (C) expression at baseline and 3 and 7 days after infarction. *P*=0.01, †*P*=0.02. D and E, CD49d expression after infarction. *P*=0.004, †*P*=0.028. Each bar represents means±SEM (n=8/group per time point). MFI indicates mean fluorescence intensity.

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Methods

Mice
Mice were generated as described previously by Tan et al.\textsuperscript{1}

Myocardial infarction in vivo
Mice were anesthetized with a mixture of Fentanyl (Jansen-Cilag) 0.05 mg/kg, Dormicum (Roche) 5 mg/kg and medetomidine 0.5 mg/kg through an intraperitoneal injection. Core body temperature was maintained around 37°C during surgery by continuous monitoring with a rectal thermometer and automatic heating blanket. Mice were intubated and ventilated (Harvard Apparatus Inc.) with 100% oxygen. The left coronary artery (LCA) was permanently ligated using an 8-0 vicryl suture. Ischemia was confirmed by bleaching of the myocardium and ventricular tachyarrhythmia. In sham operated animals the suture was placed beneath the LCA without ligating. The chest wall was closed and the animals received subcutaneously Antisedan (Pfizer) 2.5 mg/kg, Anexate (Roche) 0.5 mg/kg and Temgesic (Schering-Plough) 0.1 mg/kg. Mice were terminated at 3, 7 and 28 days after infarction for tissue harvesting.

Protein and RNA isolation
Total RNA and protein was isolated from snap frozen infarcted heart sections (infarct and remote area separated) using 1 ml Tripure\textsuperscript{TM} Isolation Reagent (Roche, Woerden, the Netherlands) according to the manufacturers' protocol. A 40 mM Tris solution (pH 7.5) was used for protein isolation from samples harvested 7 days after infarction for zymography and caspase 3/7 activity (online Data Supplement).

Flow Cytometry
Tumor necrosis factor (TNF)-α, RANTES, IL10, MCP-1 and granulocyte macrophage-colony stimulating factor (GM-CSF) levels in isolated tissue protein samples were measured by flow cytometry (Cytomics FC500, Beckman Coulter) using the Th1/Th2 customized multiplex kit (Bender MedSystems, Vienna, Austria). The protein samples were diluted 1:1 in assay buffer, and the protocol is further followed according to the manufacturer’s instructions. TLR2, TLR4, CD49d expression was assessed on circulating monocytes of EDTA anticoagulated blood by flow cytometry. Whole blood was stained for TLR2 (FITC, eBioscience, San Diego, Calif), TLR4 (PE, eBioscience, San Diego, Calif), CD49d (Alexa Fluor 488, Serotec, Oxford, UK) and F4/80 for monocytes (Alexa Fluor 647, Serotec, Oxford, UK).

Generation of chimeric mice
We generated chimeric mice to study the relative contribution of EDA expression in blood and parenchymal cells to LV remodeling. Donor bone marrow (BM) cells were collected from WT and EDA\textsuperscript{-/-} mice by flushing humerus, femurs and tibiae with RPMI-1640 medium. Recipient mice received 5x10\textsuperscript{6} BM cells after receiving a single dose of 10 Gy to irradiate host bone marrow. Mice recovered for 6 weeks to ensure stable engraftment of the donor bone marrow cells. Hereafter, DNA was extracted from peripheral blood samples and used for genotyping with quantitative polymerase chain reaction (qPCR). Successful chimization (>95% circulating donor cells) was achieved in all mice (data not shown). Irradiated WT mice with EDA\textsuperscript{-/-} bone marrow are referred as WT/EDA KO BM, and EDA\textsuperscript{-/-} mice with WT bone marrow as EDA KO/WT BM. WT/WT BM and EDA KO/EDA KO BM mice served as appropriate controls for functional and statistical comparison.

Infarct size
Digital photos of infarcts were encrypted before being analyzed by the researcher. Infarct size (IS) as a percentage of the left ventricle (LV) was determined using Evans’ blue dye injection and TTC staining, 2 days after infarction (n=6/group). By assessing infarct size in the acute phase (at 2 days), one can determine whether differences are present between WT and EDA-/- mice in myocardial perfusion. Hence, 4% Evans blue dye was injected via the thoracic aorta in a retrograde fashion. By doing so, one can demarcate the area-at-risk (AAR), the extent of myocardial tissue that underwent ischemia (i.e. endangered myocardium). Hearts were rapidly explanted, rinsed in 0.9% saline and put in -20ºC freezer for 1 hour. Hereafter, hearts were mechanically sliced into four 1-mm cross sections. Heart sections were incubated in 1% triphenyltetrazolium chloride (Sigma-Aldrich) at 37ºC for 15 minutes before placing them in formaldehyde for another 15 minutes. Viable tissue stains red and infarcted tissue appears white. Heart sections were digitally photographed (Canon EOS 400D) under a microscope (Carl Zeiss®). IS, AAR and total LV area were measured using ImageJ software (version 1.34). Infarct size was corrected for the weight of the corresponding heart slice.

After 28 days, IS/LV was determined using hematoxylin-eosin stained cross sections.

**Magnetic resonance imaging**

Heart function and geometry assessment was done by a technician blinded to genotype. Forty-two mice without bone marrow transplantation (n=15/group in ischemic and n=6/group in sham operated mice) and 44 mice (n=11/group) in the chimeric mouse experiments underwent serial assessment of cardiac dimensions and function by high resolution magnetic resonance imaging (MRI, 9.4 T, Bruker, Rheinstetten, Germany) under isoflurane anesthesia before, 7 and 28 days after MI. Long axis and short axis images with 1.0 mm interval between the slices were obtained and used to compute end-diastolic volume (EDV, largest volume) and end-systolic volume (ESV, smallest volume). The ejection fraction (EF) was calculated as 100*(EDV-ESV)/EDV. Wall thickness (WT) and systolic wall thickening (SWT) were assessed from both the septum (remote myocardium) and free wall (infarct area). All MRI data are analyzed using Qmass digital imaging software (Medis, Leiden, The Netherlands).

**LV pressure measurements**

In a subset of mice, invasive assessment of cardiac performance and LV pressure development was performed 28 days after infarction. A Millar 1.4F pressure catheter (model SPR-839) was inserted in a retrograde fashion via the right common carotid artery. Systolic function was assessed by dP/dt\text{max}, whereas diastolic function by LV end-diastolic pressure and tau (time constant of LV relaxation). Tau was determined from the regression of dP/dt versus LV pressure.

**Immunohistochemistry**

Upon termination, hearts were excised and fixed in 4% formaldehyde and embedded in paraffin. Paraffin sections were stained for Ly-6G (for neutrophils; rat anti-mouse Ly-6G 1:100, Abcam, Cambridge, United Kingdom), MAC-3 (for macrophages; rat anti-mouse MAC-3 1:30, BD Pharmingen, Breda, the Netherlands), α-SMA (for myofibroblasts; rabbit anti-human α-SMA 1:50, Acris antibodies, Herford, Germany), periostin (maturation factor; rabbit-anti human periostin 1:50, Sigma, Zwijndrecht, the Netherlands) and fibronectin-EDA (mouse anti-human 1:150, Abcam, Cambridge, United Kingdom)

Quantification of collagen density was performed using Picrosirius Red staining of 4% formalin fixated and paraffin embedded heart sections.

Sections were stained by overnight incubation with the first antibody at 4°C for MAC-3 and periostin or by 1 hour incubation at RT for Ly-6G and ½ hour incubation at RT for α-SMA. Before staining, sections were deparaffinized and endogenous peroxidase was blocked by 30 minutes incubation in methanol containing 1.5% H2O2. Antigen retrieval was performed
by 20 minutes boiling in citrate buffer (MAC-3, Ly-6G and peristin), 10 min boiling in citrate buffer for fibronectin-EDA or 15 minutes at 37°C in pepsin buffer (α-SMA).

For MAC-3, α-SMA and peristin staining, sections were pre-incubated with normal goat serum and incubated with the primary antibody (MAC-3, 1:30 overnight at 4°C; α-SMA, 1:50 for 30 minutes at RT; peristin, 1:50 overnight at 4°C). Sections were then incubated for 1 hour at RT with a biotin labeled secondary antibody, followed by 1 hour incubation with streptavidin-horseradish peroxidase at RT and developed with AEC.

For Ly-6G, sections were incubated with the primary antibody (1:100 for 1 hour at RT). Sections were then incubated for 30 minutes with a secondary antibody followed by 30 minutes incubation with Powervision poly-HRP anti-rabbit IgG (ImmunoVision Technologies, Daily City, USA). The staining was immediately visualized with Vector NovaRED™ substrate kit following the manufacturer's instructions (Vector Laboratories Inc., Burlingame, USA).

For Fibronectin-EDA, sections were pre-incubated with bovine serum albumin and incubated with the primary antibody (1:150 overnight at 4°C). Next day, sections were incubated with a secondary antibody (rabbit anti mouse, Dako, Glostrup, Denmark) followed by incubation for 1h with AB complex (Vector Laboratories Inc., Burlingame, USA). The staining was immediately visualized with DAB (Dako, Glostrup, Denmark).

All sections were counterstained with Mayer's hematoxylin stain.

Collagen density analysis was done with circularly polarized light after conversion into grey values and digital image microscopy. Histograms were generated of the entire image, in which the number of pixels with a certain grey value was represented. Grey values below 30 were considered as background signal of the image. Myofibroblasts influx and peristin expression were calculated as the positive α-SMA and peristin area fraction. Vessels were excluded from the analysis.

**Polymerase chain reaction**

RNA was isolated using Tripure reagent according to manufacturer’s protocol (Roche). After DNase treatment, 500 ng total RNA was used for cDNA synthesis using the iScript™ cDNA synthesis kit (Bio-Rad). Amplification was performed using 10 µl iQ™ SYBR Green supermix and 10 µl cDNA. Quantities are determined by comparison with known quantities of cloned PCR products. All mRNA expression levels were corrected for the amount of p0.

Primers were designed using Beacon Designer 4.0 (Premier Biosoft): collagen-1 (forward: 5’-tcaaggtctactgaacatgg-3’; reverse: 5’-aatccatggcatgtctct-3’), collagen-3 (forward: 5’-cgtaagcactggattgacagtcc-3’; reverse: 5’-gcaaatcacaacgcatctctcagg -3’), EDA (forward: 5’-actgtgattgtttggtctgc-3’; reverse: 5’-tggaatgacatccacatcag-3’), and p0 (forward: 5’-gagcccgagaacctctttc-3’; reverse: 5’-gcacatcactcagatgtct-3’), lysyl-oxidase (forward: 5’-cgcaaaagttgaacatcag-3; reverse: 5’-ggcatacagctgctcagc-3’), TIMP-1 (forward: 5’-gatggacacataagatcag-3; reverse: 5’-tgtgaaggtggtgtgagtc-3’), and TIMP-2 (forward: 5’-tacccgcaacgctttt-3’; reverse: 5’-ttctctcaacgtccagcga-3’)

**Zymography**

Tris isolated protein samples (5 µg) were separated on a sodium dodecyl sulfate–polyacrylamide gel containing 1 mg/ml gelatin (Sigma) in the 8% running gel. After running, the gel was washed 2×15 min in 2.5% Triton X-100 and incubated overnight at 37°C in Brij solution (0.05 M Tris–HCl pH 7.4, 0.01 M CaCl₂, 0.05% Brij 35 (Sigma)). The gel was then stained with Coomassie blue (25% methanol, 15% acetic acid, 0.1% Coomassie blue) for 1 h at room temperature (RT), followed by a destaining in 25% methanol/15% acetic acid for approximately 30 min. Active MMP-2 and -9 were identified by size and in co-migration with its recombinant protein.

**Caspase 3/7 activity**

The level of apoptosis was assessed with the Caspase-Glo 3/7 assay kit (G8091, Promega, Madison, Wisconsin) according to the manufacturer’s instructions using 25 µg of Tris isolated protein samples.

**Elastase activity**
Elastase activity was measured in 100 µg Tris-protein extracts using EnzCheck Elastase Assay kit (E12056, Invitrogen, Breda, Netherlands) according to the manufacturer’s instructions. Aliquots of the DQ elastin substrate (with a final concentration of 25 µg/ml) were added to the samples and incubated for 1-3 hours at 37 °C. Background fluorescence from a no-enzyme control reaction had been subtracted from each value.

**Myofibroblast culture**
Hearts from WT and EDA−/− mice were flushed with saline and explanted 5 days after infarction. Right ventricle, atria and valves were removed. Hearts were cut into submillimeter pieces and treated for 1 hour with collagenase (2 mg/mL, Roche) at 37°C. Single cell suspensions were obtained through cell strainers. Myofibroblasts were selected based on their ability to proliferate in vitro. Myofibroblasts were expanded using DMEM culture medium (Gibco), supplemented with 10% FCS (HyClone, Logan, Utah, VS) and 1% penicillin/streptomycin (Sigma). For MMP expression profile, myofibroblast were cultured overnight in 0.1% FCS to minimize background MMP2 and -9 activity on zymography. Immunocytochemistry was performed on cells at passage 3, seeded on coverslips and cultured for 3 days, fixed in 4%. For the staining, coverslips were washed, permeabilized with 0.1% Triton-X100 and blocked in a 2% BSA (Roche), 0.1% saponin solution in PBS, and incubated for 1 hr with primary antibodies in PBS. Antibodies used: αSMA (1:400, Sigma, A2547, stock concentration (sc): 4.5 mg/mL), vimentin (1:400, ab20346, VI-10, Abcam, sc: 1.0 mg/mL), desmin (1:50, MA1-46394, ThermoScientific) and procollagen type III (1:50, BP8034, Acris, sc: 1.0 mg/mL). Control stainings were performed by omitting primary antibody. The used secondary antibodies were: AlexaFluor-555-labeled goat-anti-rabbit (1:400, invitrogen, A21429, sc: 2mg/mL) and AlexaFlour-488-labeled goat-anti-mouse (1:400, invitrogen, A11001, 2mg/mL). Cell nuclei were stained in 1ng/mL Hoechst dye for 5 minutes. Coverslips were mounted with a 10% mowiol-solution (w/v) (25% glycerol, 50% Tris-HCl, pH 8.5). Human adult cardiomyocytes were added later as a positive control to confirm negative desmin-staining. Cells were viewed by fluorescence microscopy (Olympus, BX60).

**Fibronectin-EDA Western Blotting**
Tris isolated protein samples (5 µg) were separated on a 4-12% sodium dodecyl sulfate–polyacrylamide gel (Invitrogen) and blotted to nitrocellulose. The blot was incubated with R191A, an immunopurified rabbit anti-EDA antibody (1:200 dilution), then horseradish peroxidase labeled goat anti-rabbit, and detected with ECL (Sigma). R191A was prepared by immunizing a rabbit with bimonthly intradermal injections of 100 micrograms of the 19 amino acid synthetic peptide ELFPAPDGEEDTAELQGGC from the human EDA (except a C-terminal cysteine for solid phase linkage), synthesized and conjugated to keyhole limpet hemocyanin as described, with the first injection in complete adjuvant and subsequent injections in incomplete adjuvant. Serum antibody titers specific for the peptide were confirmed by enzyme-linked immunosorbent assay and antibodies were immunopurified from antiserum on a column of the 19 amino acid peptide conjugated to cyanogen bromide-preactivated Sepharose as described for goat anti-EDA peptide antibodies. The immunopurified antibodies were subsequently observed to detect EDAPos “cellular” but not EDAneg “plasma” fibronectin in western blot analysis. Confirmation of specific immunoblot staining of myocardial ~250 kDa FN-EDA subunits in immunoblot analysis in this study was confirmed in experiments in which preincubation of R191A with the 29 amino acid EDA peptide TYSSPEDGIHELFPAPDGEEDTAELQGGC, the 19 C-terminal amino acids of which are identical to the immunizing 19 amino acid peptide (at a final concentration of 400 micrograms/ml) abolished staining of the band.

**Statistical analysis**
Data are represented as Mean±SEM. One-way ANOVA with post-hoc LSD test was used for comparison >2 groups. Non-parametric t-test (abnormally distributed data) and independent-samples t-test (for normally distributed data) for 2 group comparisons. Kaplan-Meier survival
analysis with log-rank test was used to evaluate mortality differences between groups. All statistical analyses were performed using SPSS 15.1.1. and $p<0.05$ was considered significant.

Figure 1. A, EDA mRNA levels in the heart during infarct development (corrected for p0). B, biochemical assessment of fn-EDA in WT hearts 7 days after infarction. Representative images from WT hearts stained for EDA protein. Heart section from EDA$^{-/-}$ remains negative for EDA protein staining. SI=signal intensity, *$p=0.009$
Figure II. Baseline HE & Picosirius Red staining. Representative images from baseline hearts sections of WT and EDA\(^{+/−}\) mice. The matrix and cellularity is comparable between groups. Histograms give an overview of the entire spectrum of collagen intensity. The larger the grey value, the intenser the collagen staining. Grey values below 30 are considered as background signal of the intensity image acquired after polarized light microscopy.
Figure III. Elastase activity within the infarcted myocardium. *p=0.032 compared to WT. RLU=relative light unit.
**Figure IV.** Collagen intensity in A, infarct and B, remote area 7 days after MI. Histograms give an overview of the entire spectrum of collagen intensity. The larger the grey value, the intenser the collagen staining. Grey values below 30 are considered as background signal of the intensity image acquired after polarized light microscopy.
Figure V. Lysyl-oxidase mRNA levels in infarct area corrected for p0.

Figure VI. TIMP-2 mRNA levels in infarct area corrected for p0.
Figure VII. Periostin staining (red). Note the increased red staining in WT hearts.
Figure VIII. Cultured myofibroblasts from post-infarct WT and EDA−/− hearts.
Figure IX. MMP2 and -9 activity in supernatants of cultured myofibroblasts from WT and EDA-/- hearts (n=4/group). MMP9 activity is nearly absent in the supernatant of cultured myofibroblasts. FCS=fetal calf serum.
**Figure X.** A, MCP-1 protein levels; *p=0.001 compared to WT. B, MCP-1 mRNA levels corrected for p0; *p<0.001 compared to WT.
Figure XI. Apoptosis in the remote myocardium after infarction.
Figure XII. MMP2 and 9 activity with and without EDA. There is no direct inhibitory effect of EDA on MMP2 and 9.

References

