Online supplementary methods

Fibronectin splicing variants containing extra domain A promote atherosclerosis in mice through Toll-like receptor 4

Prakash Doddapattar,[1$] Chintan Gandhi,[1$#] Prem Prakash,[1] Nirav Dhanesha,[1] Isabella M. Grumbach,[1] Michael E. Dailey,[2] Steven R. Lentz,[1] and Anil K. Chauhan[1]

[1]Department of Internal Medicine, and [2]Department of Biology,
University of Iowa, Iowa City, IA.
[#]Current address: Center for Vascular and Inflammatory Diseases, School of Medicine,
University of Maryland, Baltimore-MD

[$]These authors contributed equally to the article.

Animals

EDA$^{fl/fl}$ mice are a mutant strain in which the wild-type EDA exon in the FN gene was replaced with a "floxed (loxP-flanked allele)" EDA exon having both the 5′ and 3′ optimized splice sites at splicing junctions to prevent alternative splicing as described.[1] As a result, the EDA exon is included in the FN mRNA, leading to constitutive expression of EDA$^+$-FN in all cells in which FN is normally expressed, including liver (the major source of plasma FN), endothelial cells, and macrophages.[1, 2] *EDA*$^{fl/wt}$ mice were crossed with a CRE-recombinase transgenic mouse (C57BL/6J background) to obtain *EDA*$^{-/wt}$ progeny that were intercrossed to obtain *EDA*$^{-/-}$ mice.[1] To generate *EDA*$^{-/-}$*Apoe*$^{-/-}$ mice, *EDA*$^{-/-}$ mice[1] (backcrossed >15 times to C57BL/6J) were crossed to *Apoe*$^{-/-}$ mice (The Jackson Laboratory, Bar Harbor, ME). *EDA*$^{fl/fl}$ mice[1] (backcrossed >15 times to C57BL/6J) were crossed to *Apoe*$^{-/-}$ mice (The Jackson Laboratory, Bar Harbor, ME) to generate *EDA*$^{fl/fl}$*Apoe*$^{-/-}$ mice. To generate *EDA*$^{fl/fl}$*TLR4*$^{-/-}$*Apoe*$^{-/-}$ mice, *EDA*$^{fl/fl}$*Apoe*$^{-/-}$ mice were crossed to *TLR4*$^{-/-}$*Apoe*$^{-/-}$ mice. Similarly, to generate *EDA*$^{-/-}$*TLR4*$^{-/-}$/*Apoe*$^{-/-}$ mice, *EDA*$^{-/-}$ */Apoe*$^{-/-}$ mice were crossed to *TLR4*$^{-/-}$*Apoe*$^{-/-}$ mice. Whenever possible, littermate control mice were studied; however, due to allelic complexity some groups included animals from multiple congenic and age-matched litters. Mice were genotyped by PCR according to protocols from the Jackson laboratory and as described previously.[1] All the mice used in the present study are on C57BL/6J background. The University of Iowa Animal Care and Use Committee approved all procedures.

Animal diet feeding and preparation of tissues

Both male and female mice (*EDA*$^{fl/fl}$*Apoe*$^{-/-}$, *EDA*$^{-/-}$*Apoe*$^{-/-}$, *Apoe*$^{-/-}$, *EDA*$^{fl/fl}$*TLR4*$^{-/-}$*Apoe*$^{-/-}$, *EDA*$^{-/-}$ *TLR4*$^{-/-}$*Apoe*$^{-/-}$, and *TLR4*$^{-/-}$*Apoe*$^{-/-}$) were fed a high-fat Western diet containing 20% milk fat and 0.2% cholesterol (Harlan Teklad) beginning at 6 weeks of age until they were sacrificed at 5 months of age (i.e., 14 weeks on high-fat Western diet). Blood samples were collected in heparinized tubes by retro-orbital plexus puncture after overnight fasting. Before sacrificing, mice were anesthetized with 100 mg/Kg ketamine/10 mg/Kg xylazine and perfused via the left ventricle with 10 ml PBS followed by 10 ml of 4% paraformaldehyde under physiological pressure. After perfusion, aorta was isolated, dehydrated for 5 min in 70% alcohol and stained with Oil Red O. Hearts containing aortic roots were carefully dissected and fixed overnight in 4% paraformaldehyde prior to embedding in paraffin.

Extent and composition of atherosclerotic lesions
To measure the extent of atherosclerosis, whole aortae were isolated and stained with Oil Red O and *en face* lesion area was measured by morphometry using NIH ImageJ software. To quantify lesions in the aortic sinus, serial cross-sections of 5 μm were cut through the aorta beginning at the origin of the aortic valve leaflets and stained by VerHoeffs/Van Gieson method. Cross-sectional lesion area from each mouse was calculated using the mean value of 4 sections (each 80 μm apart, beginning at the aortic valve leaflets and spanning 320 μm) as described previously.[3] NIH ImageJ software was used for quantification.

Picrosirius red staining for collagen type III and I
To quantify interstitial collagen within the lesions of the aortic sinus, serial cross-sections of 5 μm were stained with Picrosirius red method. Briefly, serial formalin-fixed sections were stained with Weigert's haematoxylin. Sections were then washed in running tap water for 10 minutes followed by incubation for 4 hours in a freshly prepared 0.1% solution of Sirius Red F3B (Sigma-Aldrich, USA) in saturated aqueous picric acid. After rinsing twice-in 0.01 N HCl and distilled water, sections were dehydrated and mounted in Permount (Vector Laboratories). Picrosirius red staining was analyzed by polarization microscopy. NIH ImageJ software with a defined threshold (minimum 100 and maximum 200) was used for quantification. A mean for each mouse was calculated from using the mean value of 4 sections (each 80 μm apart, beginning at the aortic valve leaflets and spanning 320 μm).

Mac3 staining of murine samples
Tissue preparation and histochemical staining were performed as described.[4] Antigen retrieval was performed prior to immunohistochemical staining. Briefly, slides were incubated with blocking reagent followed by rat anti-mouse mac-3 for macrophages [BD Pharmingen] in the presence of 5% rabbit serum overnight at 4°C. The following day, slides were rinsed and stained by biotin-conjugated rabbit anti-rat Ig, avidin-linked enzyme peroxidase complex, and 3, 3′-diaminobenzidine as substrate. Slides were counterstained with hematoxylin, dehydrated, and examined under a light microscope (Zeiss). Incubation without primary antibodies and/or with isotype-matched immunoglobulins was used as a negative control for immunostaining. NIH ImageJ was used for lesion quantification and expressed as the area of positive immunostaining. A mean for each mouse was calculated using the mean value of 4 sections (each 80 μm apart, beginning at the aortic valve leaflets and spanning 320 μm).

Quantification of cellular EDA^+-FN in plasma samples
Cellular EDA^+-FN levels in the plasma were measured by sandwich enzyme-linked immunosorbent assay (ELISA). Briefly, micro titer plates were coated overnight at 4°C with primary antibody for EDA^+-FN (IST-9, 10 μg/mL, Abcam) diluted in 50 mM sodium carbonate buffer. 50 μl of plasma samples (diluted 1:2 in PBS) were incubated for 2 h in the coated wells at RT. After 5 washes biotinylated secondary antibody to FN (2 μg/ml diluted in blocking buffer) was added to wells and incubated for 1 h at RT. Following 5 washes avidin HRP solution (1:1000) in blocking buffer was added to wells and incubated for 30 minutes. Micro titer plates were washed 5 times, before adding 3, 3', 5, 5'-tetramethylbenzidine substrate solution (Sigma) to the wells and the colorimetric reaction was stopped with H_2SO_4 (2M) after 10 min. Results were read in an ELISA microplate reader at A_{450} nm. Human cellular FN (Sigma) was used for standards.

Determination of plasma total cholesterol and lipid levels
Blood samples were collected in heparinized tubes by retro-orbital plexus puncture after overnight fasting. Plasma was separated by centrifugation and analyzed for total cholesterol (Infinity™ Cholesterol, Thermo scientific (#TR13421) and triglyceride (Wako) levels by using enzymatic colorimetric assays according to the manufacturer's instructions.

Bone marrow-derived macrophage (BMDM) culture
Femurs and tibias were removed aseptically from 10-15 week old female mice, bone marrow cavities were flushed, and BM cell suspensions seeded at 2 $X10^6$ cells/ml in DMEM (Dulbecco's modified eagle medium) containing high glucose (25 mM), 4 mM glutamine, 1 MM pyruvate, 10% FCS, 1% penicillin, 1% streptomycin and 10ng/ml macrophage colony stimulating factors. After 7 days, macrophages were washed and recultured in the presence of 0-50 µg/mL cellular fibronectin (#F2518, Sigma) pre-coated tissue culture plates. Medium was collected for ELISA and stimulated macrophages were washed twice with PBS before protein extraction.

Preparation of LDL complexes
We prepared LDL complexes with cFN, denatured collagen and heparin according to protocols as described.[5] Briefly, 100 µl of heparin (Sigma) was added to LDL (355 µg LDL suspended in 3 ml distilled water: Alfa Aesar, MA). To this mixture 500 µl of $CaCl_2$ (0.5 M) was added. The mixture became turbid after addition of $CaCl_2$. To this mixture either collagen (100 µg; Sigma) or collagen (100 µg) + cFN (100 µg; Sigma) was then added and volume adjusted to 3.8 ml and preparation left overnight at 4°C. Insoluble complexes were then centrifuged (1000g for 20 min) and pellets were resuspended in DMEM before addition to macrophage monolayers.

Foam cell formation
Acetylated LDL (100 µg/ml; Alfa Aesar, MA), acetylated LDL (100 µg/ml)+ cFN (10 µg/ml), LDL (50 µg/ml), LDL (50 µg/ml) + cFN (10 µg/ml), and LDL complexes were added to BMDMs for 24 hours. Cells were washed three times with PBS and fixed with 4 % formaldehyde. Lipids were stained using oil red O. Cells were cover slipped and foam cell images were obtained using Olympus BX51TF.

Macrophage lipid parameters
BMDM lipids were extracted with 2 ml of hexane/isopropyl alcohol (3:2, v/v) for 1 h at 4 °C. Lipid extracts were dried and redissolved in 100 µl of 1% Triton X-100 in chloroform. The samples were dried and resuspended in 100 µl of distilled water for 15 min at 37 °C in a water bath. For enzymatic measurements of total cholesterol (Infinity™ Cholesterol, Thermo scientific #TR13421) concentrations, 30 µl of samples were used. Protein was extracted from cells in 2ml of 0.3 M NaOH for 1hour at room temperature and quantified using a Bradford assay (Bio-Rad), using bovine serum albumin as a standard.

Protein extraction and immunoblotting
BMDMs were lysed in ice-cold RIPA buffer. The lysates were spun down at 4°C (14,000rpm) for 15 minutes, and the supernatant was collected. Protein quantification was done by BCA method (Thermo scientific). Protein homogenates (40 µg) were resolved by SDS-PAGE, transferred onto a nitrocellulose membrane. Membranes were blocked with 5% BSA plus 0.1% Tween 20 for 1 hour and incubated with following rabbit monoclonal antibodies NF-κB p65 (1:2000) (D14E12), Phospho-NF-κB p65 (Ser536) (93H1)(1:1000) from cell signaling technologies and polyclonal beta actin antibody (ab8227) (1:5000) from Abcam. Blots were incubated overnight at 4°C. The horseradish peroxidase-conjugated goat anti-rabbit (1:3000) antibodies were

visualized by super signal West Femto Maximum sensitivity substrate (Thermo scientific) on a rpi CLASSIC S-Ray film (248304). Densitometric analysis of the gels was done using ImageJ software.

ELISA assay for TNF-α and IL-1β

Supernatant medium from cFN treated and untreated macrophages were used for determination of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) with commercially available mouse ELISA kits (R&D Systems) according to the kit manufacturer's instructions. Using same kits, plasma TNF-α and IL-1β levels were quantified.

Triple labeling staining of human and mice samples

Human samples: Human coronary artery tissue samples from autopsies of patients with a history of coronary artery disease were procured from the University of Iowa Decedent Center in accordance with guidelines established by the University of Iowa Institutional Review Board. The presence of atherosclerosis was confirmed by gross pathology. Samples were fixed in formalin, processed and paraffin-embedded for immunohistochemistry. Antigen retrieval was performed prior to immunohistochemical staining. Briefly, in the first step, 5-µm serial sections were blocked and incubated with mouse monoclonal antibody specific for EDA^{+}-FN (IST-9, 1:100, Abcam) and rabbit polyclonal antibody specific for TLR4 (H-80, 1:50, Santa Cruz) overnight at 4° C followed by secondary antibodies labeled with anti-mouse Alexa Fluor-568 and anti-rabbit Alexa Fluor-488 (1:400, Abcam) for 1hr at room temperature (RT). In the second step, sections were washed and blocked before incubating with mouse anti-human CD68 Alexa Fluor-647 antibody for macrophage (KP-1, 1:50, Santa Cruz) for 3hrs at RT. Slides were dehydrated, and examined using a Leica SP5 scanning laser confocal microscope at the core facility in the Department of Biology, University of Iowa. Confocal images (1,024 or 2,048 pixel arrays) were collected using a 10x/0.3 Fluotar, 20x/0.8 Plan Apo, or 63x/1.2 water immersion Plan Apo lens, with additional electronic zoom as needed, up to a resolution of 120nm *xy* pixel dimensions. To avoid cross-illumination of fluorophores in triple-labeled samples, images were captured sequentially using the indicated laser lines: Alexa Fluor-488 (Argon 488 nm); Alexa Fluor-568 (HeNe 543 nm); and Alexa Fluor-647 (HeNe 633 nm). Images were then false colored (Alexa Fluor-488, green; Alexa Fluor-568, red; Alexa Fluor-647, blue) and merged using Adobe Photoshop CS. **Murine samples**: Briefly, in the first step, 5-µm serial sections were blocked and incubated with rat anti-mouse monoclonal antibody specific for Mac-3 (CD107b, 1:50, BD Pharmingen) and rabbit anti-mouse polyclonal antibody specific for TLR4 (H-80, 1:50, Santa Cruz) overnight at 4° C. The sections were washed and incubated with secondary antibodies labeled with anti-rat Alexa Fluor-647 and anti-rabbit Alexa Fluor-488 (1:400, Abcam) for 1hr at room temperature (RT). In the second step, sections were washed and blocked before incubating with mouse monoclonal antibody specific for EDA-FN (IST-9, 1:100, Abcam) for 3hrs at room temperature (RT) and after washing incubated with anti-mouse Alexa Fluor- 568 for 1hr at room temperature (RT). Confocal images were taken as described above.

Quantitative colocalization analysis

Leica LAS AF software was used for quantitative analysis of fluorescence intensity and colocalization in confocal images of triple-labeled tissue sections. Three pair-wise comparisons (TLR4-EDA, TLR4-MAC, and EDA-MAC) were made for colocalization analysis in both control and experimental tissue sections. Pixel intensities in 25 × 25 µm regions-of-interest were plotted in 2D scatter plots (cytofluorograms) according to the gray scale values for the two channels being compared. To avoid contamination from autofluorescence and non-specific background staining, threshold and background levels were set to 40% for both channels. Pearson's Correlation was used as a quantitative measure of fluorescence colocalization. A

value of +1 indicates a complete match of the displays in both color channels, and a value of -1 indicates no match. Measurements were obtained from four separate confocal optical sections per tissue. Statistical analysis comparing experimental and control tissues were performed using two-tailed T-tests.

Statistical analysis

Results are reported as mean or mean ± SEM. The number of experimental animals in each group was based on power calculations for the primary parameter with standard deviations taken from pilot data in mice. We used sample sizes of 10 to 12 mice per group with the following assumptions: α=0.05, β=0.2 (power 80%), mean, standard deviation 20% of the mean. For statistical analysis, Prism Graph software package was used. Statistical comparisons were performed using one way analysis of variance followed by Bonferroni's multiple comparison test. For measuring the effect of two factors simultaneously, two ways analysis of variance followed by Holm-Sidak multiple comparison test was used. $P<0.05$ was considered statistically significant.

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