Supplemental Material

Macrophage-derived TNFα is an early component of the molecular cascade leading to angiogenesis in response to aortic injury

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Expanded materials and methods

Materials

Endothelial basal medium was obtained from Clonetics (San Diego, CA). Recombinant rat TNFα and VEGF were obtained from R&D System (Minneapolis, MN). Recombinant rat macrophage colony-stimulating factor (CSF-1) was from Peprotech Inc. (Rocky Hill, NJ). Neutralizing hamster antibodies that selectively block TNFR1 (55R-170) or TNFR2 (TR75-54) signaling were obtained from Leinco Technologies (St. Louis, MO).[1-3] Goat polyclonal antibodies against rat TNFα and VEGF were purchased from R&D systems. Mouse monoclonal antibodies against rat CD68 (ED1) and CD163 (ED2) were purchased from AbD Serotec (Raleigh, NC). Rat monoclonal antibody against mouse F4/80 (CI:A3-1) was purchased from AbD Serotec. Anti-β-actin rabbit antibody was from Sigma Chemical Company (St. Louis, MO). Peroxidase-conjugated donkey anti-goat and -rabbit antibodies were from Jackson Immunochemicals (West Grove, PA). Alexa Fluor 488- or 568-conjugated donkey anti-mouse or goat anti-rabbit secondary antibodies were obtained from Invitrogen (Carlsbad, CA). Nonimmune IgGs were used as negative control for all antibody neutralization studies and immunocytochemical reactions. The Click-iT EdU (5-ethynyl-2'-deoxyuridine) proliferation assay was obtained from Invitrogen. Collagen was purified from isolated rat tail tendons as described.[4]

Aortic ring cultures

Animal procedures were performed with approval from the Veterans Administration Puget Sound Health care system Institutional Animal Care and Use Committee and according to National Institutes of Health guidelines. Fischer 344 male rats were purchased from Harlan Laboratories (Kent, WA). TNFα-deficient male mice and age matched controls were from Jackson Laboratories (Bar Harbor, ME). Rat or mouse thoracic aortas were dissected to prepare rings as described.[4] Aortic rings were embedded in collagen gels and cultured in serum-free endothelial basal medium, with or without TNFα. Angiogenesis was measured by counting the number of neovessels over time.[4] For VEGF blocking studies, TNFα-treated cultures were supplemented with anti-rat VEGF goat antibody (2μg/ml) or nonimmune goat IgG.

Macrophage ablation

For macrophage ablation experiments, rat aortic rings were pretreated for 24 hr with liposomal clodronate, which selectively kills macrophages,[5] or control PBS-liposomes,[6] washed in EBM, embedded in collagen, and cultured with or without TNFα (5 ng/ml).

qRT-PCR

Total RNA was extracted from freshly cut rat aortic rings or rings embedded in collagen and cultured for 10 min to 24 hrs (RNAEasy Micro kit, Qiagen, Valencia, CA). cDNA was synthesized by reverse transcription (RT).[7] Duplicate reactions lacking Superscript enzyme (Invitrogen) were used as negative controls. The relative expression of TNFα and VEGF was measured by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) using Sybr-Green reagents (Applied Biosystems, Foster City, CA). 1/25 of the RT reaction was used as template in qRT-PCR with gene specific primers (Invitrogen): TNFα: 5'-TCGGGGTGATCGGTCCCAACAA-3' and 5'-GCTACGGGCTT GTCACTCGAGTT-3'; VEGF-A: 5-GGGAGCAGAAAGCCCATGAAGTG-3 and 5'-CCAGGGTCTCAATTGGACGGCAAT-3'; β-Actin: 5'-GGGAAATCGTGCGTGACATT-3' and 5'-GCGGCAGTGGCCATCTC-3'. Relative quantification was carried out in triplicate on an ABI 7500 thermal cycler using Prizm software (Applied Biosystems). The 2-ΔΔCt method was used to calculate ratios of gene expression normalized to β-Actin in the same sample.[8]

Microarray analysis

For microarray studies, total RNA was extracted (RNAEasy Micro kit) from freshly isolated aortic rings or rings cultured in collagen for 24-48 hr without or with TNFα (10 ng/ml). Each group

consisted of triplicate RNA isolates, each from 4-6 samples obtained from independent experiments. All RNA samples were determined to be intact and free of genomic DNA prior to reverse transcription and labeling. Each array probe was generated from 0.2μg of total RNA per group and hybridized to Affymetrix Rat Gene 1.0 ST Arrays (Affimetrix, Santa Clara, CA). All array hybridization procedures were carried out at the University of Washington Environmental and Occupational Health Science Array Center. Gene chips were scanned with an Affimetrix GeneChip 3000 scanner and data were analyzed with Bioconductor software limma package to identify differentially expressed genes between experimental groups ($p<0.05$). Microarray data were deposited into the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) accessible through GEO series accession numbers GSE23152 and GSE23153.

Western blot analysis

Proteins were extracted from rat bone marrow macrophages, aortic endothelial cells, and smooth muscle cells with RIPA buffer (Tris-HCl 50 mM, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl) containing protease inhibitors (Thermo Scientific, Waltham, MA). Protein extracts were diluted in Laemmli buffer, run on 5-18% SDS-polyacrylamide gradient gels (30 μg/lane; Bio-Rad Laboratories, Hercules, CA), and transferred to a nitrocellulose membrane (Bio-Rad Laboratories). Blots were probed with anti-TNFα goat or anti-β-actin rabbit antibodies, washed, and reacted with peroxidase-conjugated donkey anti-goat and -rabbit antibodies. Immunoreactive bands were visualized on X-ray film with the ECL chemiluminescence detection kit (Thermo Scientific).

Immunocytochemistry

Aortic ring cultures or aortic macrophages were fixed in 4% paraformaldehyde and stained by immunoperoxidase or immunofluorescence as described.[9] For immunoperoxidase, cultures were incubated overnight at 4°C with anti-rat CD68, anti-rat CD163 or anti-mouse F4/80 antibody diluted 1:100 in PBS blocking solution containing Tween 20 (0.05%, Sigma-Aldrich). After washing in PBS, the cultures were incubated with biotin-conjugated secondary antibody diluted 1:100, and visualized with the Vectastain ABC kit and diaminobenzidine. Samples were mounted in Aqua Polymount (Polysciences, Warrington, PA) on glass slides. Images were captured with Olympus MicroFire digital cameras. For double immunofluorescence staining, paraformaldehyde-fixed aortic macrophages were reacted with anti-TNFα and anti-CD68

antibodies followed by Alexa Fluorochrome-conjugated secondary antibodies. Immunostained samples were mounted in Aqua Polymount (Polysciences) and examined with a Leica TCS-SP laser-scanning confocal microscope.

Legends of supplemental figures

Supplemental Figure I. Disruption of TNFR1 impairs aortic angiogenesis. (A-C) Photomicrographs of mouse aortic rings treated with nonimmune IgG (A) or blocking antibodies against TNFR1 (B) or TNFR2 (C). **(D, E)**. Quantitative analysis of angiogenic responses of aortic rings treated with nonimmune IgG compared to rings treated with anti-TNFR1 antibody (D) or anti-TNFR2 antibody (E) (N=30; *** $p<0.001$). Note: the angiogenic response is markedly impaired by the anti-TNFR1 antibody. The anti-TNFR2-antibody has a modest but statistically not significant inhibitory effect. Microvessels in A and C are marked by arrows. Magnification bars (A-C) = 500 μm.

Supplemental Figure II. Mechanisms of TNFα-induced angiogenesis in response to injury. TNFα produced by injury-activated aortic macrophages (MΦ) promotes angiogenesis through VEGF-dependent and -independent mechanisms. TNFα uses the VEGF-dependent pathway as its primary mechanism to stimulate angiogenesis; this pathway can be inhibited by VEGF blockade or macrophage ablation. TNFα retains a limited capacity to promote angiogenesis in the absence of macrophages and under conditions of VEGF inhibition.

References

1. Sheehan KC, Pinckard JK, Arthur CD, Dehner LP, Goeddel DV, Schreiber RD. Monoclonal antibodies specific for murine p55 and p75 tumor necrosis factor receptors: identification of a novel in vivo role for p75. *J Exp Med*. 1995;181:607-617.

2. Ruuls SR, Hoek RM, Ngo VN, McNeil T, Lucian LA, Janatpour MJ, Korner H, Scheerens H, Hessel EM, Cyster JG, McEvoy LM, Sedgwick JD. Membrane-bound TNF supports secondary lymphoid organ structure but is subservient to secreted TNF in driving autoimmune inflammation. *Immunity*. 2001;15:533-543.

3. Bruggeman LA, Drawz PE, Kahoud N, Lin K, Barisoni L, Nelson PJ. TNFR2 interposes the proliferative and NF-kappaB-mediated inflammatory response by podocytes to TNF-alpha. *Lab Invest*. 2011.

4. Aplin AC, Fogel E, Zorzi P, Nicosia RF. The aortic ring model of angiogenesis. *Methods Enzymol*. 2008;443:119-136.

5. van Rooijen N, Kors N, ter Hart H, Claassen E. In vitro and in vivo elimination of macrophage tumor cells using liposome-encapsulated dichloromethylene diphosphonate. *Virchows Arch B Cell Pathol Incl Mol Pathol*. 1988;54:241-245.

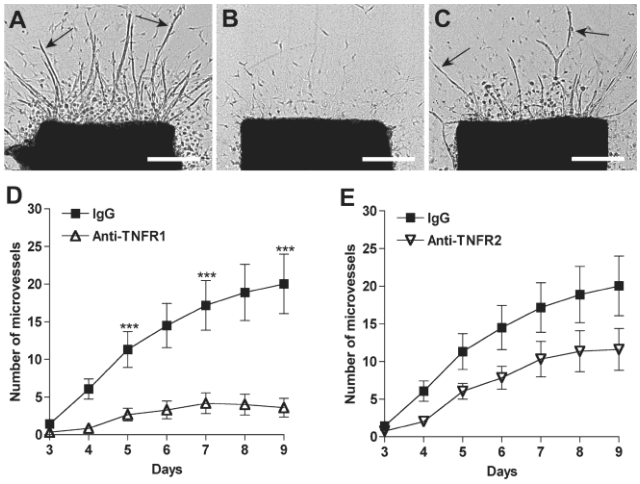
6. Gelati M, Aplin AC, Fogel E, Smith KD, Nicosia RF. The angiogenic response of the aorta to injury and inflammatory cytokines requires macrophages. *J Immunol*. 2008;181:5711-5719.

7. Aplin AC, Gelati M, Fogel E, Carnevale E, Nicosia RF. Angiopoietin-1 and vascular endothelial growth factor induce expression of inflammatory cytokines before angiogenesis. *Physiol Genomics*. 2006;27:20-28.

8. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25:402-408.

9. Zhu WH, Nicosia RF. The thin prep rat aortic ring assay: a modified method for the characterization of angiogenesis in whole mounts. *Angiogenesis*. 2002;5:81-86.

Supplemental Figure I



Supplemental Figure II