CHAPTER III

TIE1 ATTENUATION DECREASES ATHEROSCLEROSIS IN A DOSE
DEPENDENT AND LOCATION SPECIFIC MANNER

Introduction

In the preceding chapter, we showed that Tie1 expression is augmented in the aortic sinuses and bifurcations of vessel branches, all areas of the vasculature subjected to disturbed flow and low shear stress. Similarly, Porat et al had also previously shown that Tie1 expression is augmented at the ostia of microvascular branches under normal physiological conditions, and also in the endothelium overlying atherosclerotic lesions (Porat *et al.*, 2004). Notably, these locations experiencing atherogenic shear stress are known to develop atherosclerotic lesions. *In vitro* studies have also confirmed that flow profiles simulating atherogenic shear stress upregulates Tie1 expression (Chen-Konak *et al.*, 2003; Porat *et al.*, 2004). However, the nature of Tie1 function at in the progression of atherosclerosis at these sites has not been investigated.

Whereas Tie2 activation promotes endothelial integrity and inhibits apoptosis (Kwak *et al.*, 1999; Papapetropoulos *et al.*, 2000; Harfouche *et al.*, 2002), Tie1 inhibits its ability to maintain endothelial quiescence. Hence, Tie1 may play a role in endothelial cell activation. Shahrara et al recently showed an increase in expression of Tie1 in tissue samples from patients with diagnoses of inflammatory conditions such as rheumatoid arthritis and osteoarthritis (Shahrara *et al.*, 2002). Overexpression of Tie1 in cultured endothelial cells upregulated the inflammatory markers VCAM-1, ICAM-1 and E-

selectin (Chan *et al.*, 2008). In contrast, Tie1 siRNA had the opposite effect whereby transfected endothelial cells decreased expression of inflammatory markers such as ICAM-1, VCAM-1, E-selectin, TLR2 and IL-1β, but did not alter Tie-2, eNOS or transforming growth factor (TGF)-β expression (Chan & Sukhatme, 2009). Hence, the pro-inflammatory effect observed in Tie1 overexpressing cells may be due to excessive inhibition of Tie2 whereas the reduced pro-inflammatory molecules in Tie1 downregulated cells may conversely be due to unrestricted Tie2 signaling.

To circumnavigate the early embryonic lethality of Tie1 knockout mice, we capitalized on Cre-Lox technology for spatial and temporal regulation of genetic deletion. Cre is a 38 kDa product of the *cre* (cyclization recombination) gene of bacteriophage P1 (reviewed in (Sauer, 1998)). Cre recognizes a 34 base pair sequence on the P1 genome called loxP (locus of X-over of P1) and catalyzes the reciprocal conservative DNA recombination between two loxP sites. Each loxP site consists of two 12 base pair inverted repeats flanking a core region that provides an overall directionality to the recombination. Cre-mediated recombination between two flanking loxP sequences results in the excision of the DNA between them. LoxP sequences were inserted to flank the transcription start site and exon 1 of the *tie1* (Qu *et al.*, 2010). In conjunction with Tie1-floxed alleles, tamoxifen-inducible estrogen receptor (ER^T)-linked Cre, driven by the stem cell leukemia (SCL, also known as Tal1) endothelial specific promoter (Sinclair *et al.*, 1999) was bred into the mouse background. Upon ligand binding with tamoxifen, the estrogen receptor will translocate to the nucleus, facilitating Cre-mediated recombination (Feil *et al.*, 1996). Gothert et al generated the SCL-ER^T-Cre transgenic mice and bred them with ROSA26 mice expressing the LacZ reporter. They showed that tamoxifen

treatment induced LacZ expression in the endothelia of pre-natal and adult mouse organs, and tumor vasculature (Gothert *et al.*, 2004). Tie1$^{flox/flox}$:SCL-ER^T-Cre mice were then bred to the ApoE knockout background. ApoE deficient mice are one of the most commonly used models of atherosclerosis, they develop spontaneous atherosclerotic lesions similar to human type III hyperlipidemia (reviewed in (Fazio & Linton, 2001)). While on regular diet, fatty streaks develop by 10 weeks, intermediate lesions are visible by 15 weeks, and advanced fibrous plaques form by 20 weeks. Hence, we used these Tie1$^{flox/flox}$:SCL-ER^T-Cre:ApoE$^{-/-}$ mice to evaluate our hypothesis.

Experimental Procedures

Genotyping

At three weeks of age, tail samples from offspring were digested in 100 mM Tris pH 8.5, 5 mM EDTA, 0.2% sodium dodecyl sulfate, 200 mM NaCl, 100 µg/ml of Proteinase K overnight at 55°C. Mice were genotyped by polymerase chain reaction with REDExtract-N-Amp PCR Reaction Mix (Sigma) using the following primers,

cre: 5'- TCC GGG CTG CCA CGA CCA A -3' and 5'- GGC GCG GCA ACA CCA TTT TT -3';

apoE: 5'- GCC TAG CCG AGG GAG AGC CG -3' and 5'- TGT GAC TTG GGA GCT CTG CAG C -3' and 5'- GCC GCC CCG ACT GCA TCT -3';

tie1: 5'- TGC CCC CCC TTC CAG AGA CTT CC -3' and 5'- GCA AAG AGG ATC CCC ACC AGA CCA TAC T –3' and 5'- GGG GAT GTG CTG CAA GGC GAT TAA G -3';

tie-flox: 5'- TCG GGC GCG TTC AGA GTG GTA T -3' and 5'- ATG CCT GTT CTA TTT ATT TTT CCA G -3';

ROSA26R: 5'-GCG AAG AGT TTG TCC TCA ACC-3' and 5'-GGA GCG GGA GAA ATG GAT ATG-3' and 5'-AAA GTC GCT CTG AGT TGT TAT-3'

Animal Breeding and Tamoxifen Injections

SCL-ER^T-Cre mice (Gothert *et al.*, 2004) were kind gifts from Dr. Gothert. Tie1$^{+/-}$:ApoE$^{-/-}$, Tie1$^{flox/\,flox}$:SCL-ER^T-Cre and Tie1$^{-/flox}$:SCL-ER^T-Cre:ApoE$^{-/-}$ mice were bred in our laboratory. All mice used in this study were bred onto a pure C57BL/6 background and were maintained in microisolator cages on a rodent chow diet (Purina Mills Inc) and autoclaved water ad libitum. Animal care and experimental procedures were performed according to the regulations of Vanderbilt University's Animal Care and Use Committee. Tamoxifen injections were performed on 6 weeks-old mice. Intraperitoneal injections of 2mg tamoxifen (T5648, Sigma) dissolved in 10% ethanol and 0.1ml sunflower oil (S5007, Sigma) was administered once every 2 days (7 injections total).

Generation of Tie1 mutant alleles

To generate Tie1$^{flox/flox}$ mice a loxP site and a neomycin resistance cassette were introduced within the first intron of Tie1, and another loxP site just upstream of the minimal promoter region. This strategic placement of loxP sites would allow excision of the Tie1 minimal promoter and exon 1 upon Cre activation (Qu *et al.*, 2010).

Analysis of Aortic Lesions

The extent of atherosclerotic lesions was evaluated by two alternate methods: analysis of cross-sections of the aortic sinus, and the whole aorta pinned out en face (Babaev *et al.*, 2005). Aortas were flushed through the left ventricle, and the entire aorta was dissected and pinned out for en face analysis as described (Babaev *et al.*, 2005). In addition, to quantify atherosclerotic lesions in the aortic sinus, the heart was embedded in optimal cutting temperature (OCT) medium and frozen. 6μm cryosections of the aortic valve region was collected and processed for oil red O staining. Lesions in the aorta were analyzed using Imaging System KS 300 (Kontron Electonik GmbH). The mean atherosclerotic lesion area for each animal was determined by taking the average of 15 alternate sections for each mouse. To quantify atherosclerotic plaques in the aorta en face, the aorta was fixed in 4% paraformaldehyde and dissected free of fat. The aorta was stained in Sudan IV and analyzed using the Imaging System KS 300. Lesions in the descending aorta were evaluated in two ways. First, the percent lesion area for the whole aorta was calculated as follows: total lesion area/total surface area. Second, lesions were assessed according to three regions: (1) lesser curvature of the aortic arch, (2) greater curvature of the aortic arch including the brachiocepahlic trunk, left common carotid and left subclavian arteries, (3) descending aorta including the thoracic, abdominal and lumbar regions. For each region, the lesion area was calculated as a percentage of total aorta surface area.

Serum Lipids and Lipoprotein Distribution Analysis

The serum total cholesterol and triglyceride levels were determined on samples obtained from mice fasted for 4 hours as described (Fazio *et al.*, 1997). Fast performance liquid chromatography (FPLC) was performed on an HPLC system model 600 (Waters, Milford, MA) using a Superose 6 column (Pharmacia, Piscataway, NJ).

Protein Isolation and Western Blotting

Tissue samples or cells were lysed on ice using a lysis buffer (50mM Tris, 50mM NaCl, 1% Triton X-100, 1mM EGTA, 1mM Na_3VO_4, 5mM NaF, 1x β-glycerophosphate) supplemented with 1x Complete protease inhibitor cocktail (Roche). After centrifugation at 12000 rpm at 4^oC, the supernatant was collected. Protein concentrations were determined with Protein Assay Reagent (Bio-Rad Laboratories). The nuclear pellet was rinsed three times in lysis buffer and incubated at 100^oC with 2x sample buffer to extract nuclear proteins. Lysates were resolved by SDS-PAGE electrophoresis on a 7% gel, and transferred onto Hybond ECL nitrocellulose membranes (GE Healthcare). Blots were blocked with 5% non-fat milk and probed with rabbit antibody to Tie1 (Santa Cruz, cat# sc342) and mouse antibody to β-actin (Sigma cat# A5316) overnight at 4^oC. After washing with 0.1% Tween-20 supplemented phosphate buffered saline (PBST), blots were incubated with secondary antibodies IRDye 800CW goat anti-rabbit IgG and IRDye 680CW goat anti-mouse IgG for 1 hour at room temperature with gentle agitation. Blots were washed and scanned on the Odyssey Infrared Imaging System (LI-COR Biosciences) and densitometry analyses were performed using Odyssey Software.

RNA Extraction

RNA from tissue or cells was isolated using TriReagent (Molecular Research Center, Cincinnati, OH, USA) following the manufacturer's instructions. RNA concentration and purity were analyzed on a NanoVue spectrophotometer (Thermo Scientific). Samples with 260/280 ratio > 1.8 (1.9-2.1 preferrably) and 260/230 ratio > 1.5 were deemed of sufficient quality for qRT-PCR. 2 μg of total RNA was reverse-transcribed into cDNA using SuperScript III First-Strand System (Invitrogen) in 20 μl as per manufacturer's instructions.

Quantitative real-time Polymerase Chain Reaction (qRT-PCR)

All qRT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad) on the iQ5 Real Time PCR Detection System (Bio-Rad). Experiments were performed in triplicate using glyceraldehyde-3-phosphate dehydrogenase as the housekeeping gene to normalize the data. The relative change in gene expression was determined using the critical threshold (C_t) and the equation Fold Induction = $2^{\wedge}(-\Delta\Delta C_t)$ (Schmittgen & Livak, 2008). Primers used were as follows, Tie1: 5'-GTGCCACCATTTTGACACTG-3' and 5'-CAGGCACAGCAGGTTGTAGA-3'; glyceraldehyde-3-phosphate dehydrogenase: 5'-CACTGGCATGGCCTTCCGTG-3' and 5'-AGGAAATGAGCTTGACAAAG-3

Statistical Analyses

All statistical differences in this study were determined by a 2-tailed, unpaired, student's t-test with 95% confidence intervals, using Prism 4.0 software (Graphpad Inc).