CHAPTER II

TIE1 IS SPECIFICALLY EXPRESSED IN REGIONS OF THE ADULT AORTA EXPERIENCING ATHEROGENIC DISTURBED FLOW WITH LOW SHEAR STRESS

Introduction

Tie1 is the last receptor tyrosine kinase expressed during embryonic vascular development and ablation of Tie1 expression results in early embryonic lethality due to severe edema, hemorrhages and loss of microvessel integrity (Puri *et al.*, 1995; Sato *et al.*, 1995; Qu *et al.*, 2010). It is an orphan receptor tyrosine kinase that is expressed in endothelial cells and cells of hematopoietic lineage (Partanen *et al.*, 1992). At embryonic day 13.0, Tie1-/- embryos have a normal vascular network suggesting that Tie1 is not essential for vasculogenesis (Puri *et al.*, 1995; Sato *et al.*, 1995). Tie1-/- embryos display increased vascular density and hyperactive endothelial cells (Patan, 1998), suggesting that Tie1 inhibits endothelial cell stretching and promotes cell maturation. During embryonic development Tie2 expression precedes Tie1 and previous studies have suggested that Tie2 phosphorylation and downstream signaling enhances endothelial cell activation (Patan, 1998). A putative role of Tie1 may be to counteract Tie2 activity, thus alleviation of inhibition by Tie1 prevents maturation of endothelial cells. These results suggest that Tie1 is not required for vasculogenesis, but it plays a requisite role during embryonic development for the integrity and survival of vascular endothelial cells.

Tie1 expression varies in different organs pre- and post-natally (Taichman *et al.*, 2002). During mouse embryonic development, Tie1 is expressed in the heart, kidneys, liver, lungs and brain. Postnatal cardiac expression of Tie1 remains the same, however, renal, hepatic and cerebral expression is almost eliminated. Interestingly, pulmonary expression of Tie1 is dramatically increased consistent with a role of Tie1 in formation and maturity of the pulmonary vascular bed. Hence, transcription of Tie1 is increased during pre- and post-natal angiogenesis.

Tie1 expression is also increased during pathological conditions (Korhonen *et al.*, 1992; Kaipainen *et al.*, 1994). For example, Fujikawa et al showed that endothelial cells re-populating the denuded surface of carotid arteries express Tie1 (Fujikawa *et al.*, 1999). Interestingly, expression of Tie1 is increased in a number of tumors such as breast, colon, gastric and thyroid (Cance *et al.*, 1993; Lin *et al.*, 1999; Tseng *et al.*, 2001; Yang *et al.*, 2003; Ito *et al.*, 2004; Uruno *et al.*, 2004), and it has also been found to correlate inversely with survival of gastric cancer patients (Lin *et al.*, 1999).

Tie1 expression is driven by an octamer element (Boutet *et al.*, 2001) that binds the transcription factor Oct1 and another unidentified co-factor, thereby functioning as a positive regulator. In this chapter, our studies in Tie1 expression utilize a genetically modified mouse generated by the insertion of a LacZ reporter gene downstream of this promoter element.

Expression of Tie1 has also recently been associated with regions of the vasculature experiencing disturbed flow with low shear stress (Porat *et al.*, 2004). Tie1 promoter driven LacZ expression was found to be upregulated at regions of disturbed flow such as

vessel bifurcations and the aortic sinuses. Mirroring these *in vivo* findings, disturbed flow conditions *in vitro* also upregulated Tie1 promoter activity (Chen-Konak *et al.*, 2003; Porat *et al.*, 2004) whereas laminar flow with high shear stress *in vitro* decreased levels of Tie1 (Chen-Konak *et al.*, 2003). A putative negative shear stress response element has been found to be involved in shear stress mediated suppression of Tie1 expression. However, the pattern of Tie1 promoter activity in adult vasculature has not been extensively studied.

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^{o}C. Mice were genotyped by polymerase chain reaction with REDExtract-N-Amp PCR Reaction Mix (Sigma) using the following primers,

tie1-lacZ: 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';

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'.

Animal Breeding and Tamoxifen Injections

The Tie1$^{lz/+}$ mice (Puri *et al.*, 1995) was a kind gift from Dr. Puri. Tie1$^{lz/+}$:ApoE$^{-/-}$ and Tie1$^{+/-}$: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.

ß-Galactosidase Detection

Whole tissue were collected in Hank's Buffered Salt Solution, fixed in 0.2% glutaraldehyde, 2mM $MgCl_2$, 5mM EGTA solution, and stained in 1mg/ml X-gal, 2mM $MgCl_2$, 5mM EGTA, 0.01% deoxycholate, 0.02% NP-40, 0.1M phosphate buffer (pH 7.3) solution overnight at 30°C. The stained tissues were post fixed in 4% paraformaldehyde overnight, cleared in a glycerol gradient and photographed in 100% glycerol with a dissecting photomicroscope. Serial cryosections of aortic sinus adjacent to oil red O-stained sections were stained as described above, post-fixed with 4% paraformaldehyde for 10 minutes, dehydrated in ethanol, cleared in xylene, counterstained with Eosin and mounted in Permount.

Shear Stress Modifying Cast

Casts were made with medical grade polyetherketone (Invibio Inc.) using single lip micro cutters (manufactured by and kindly gifted from Alignment Tools Pte Ltd, Singapore) and manufactured at the Vanderbilt Physics Machine Shop. Casts with non-constricting bores were used as controls. As described by Cheng et al, the tapered casts induce lowered shear stress ($10N/m^2$) immediately upstream, increased shear stress inside the cast and oscillatory shear stress with vortex flow immediately downstream(Cheng *et*

al., 2005; Cheng *et al.*, 2006). The tapered region increases shear stress from 10 N/m^2 to 25 N/ m^2.

Eight mice 12 weeks of age were assigned randomly to two groups. Shear stress in the right common carotid artery of was altered by cast placement. Each animal was anesthetized with isoflurane and the anterior cervical triangle was accessed by a sagittal anterior neck incision. Both halves of the cast were placed around the right common carotid artery and fixed with a suture. Wounds were closed and the animals were allowed to recover. Animals with control and tapered casts were sacrificed 7 days post-surgery.

Results

Tie1 is Expressed Exclusively at Regions of Atherogenic Disturbed Flow but not at Areas of Atheroprotective Laminar Flow

Previous studies have shown that Tie1 is ubiquitously expressed in various organ endothelia at birth (Taichman *et al.*, 2003). To map the expression pattern of Tie1 in adult macrovasculature we utilized a transgenic mouse with Tie1 promoter driven LacZ expression (Puri *et al.*, 1995). In this model, LacZ is "knocked into" the Tie1 locus ($Tie1^{lz/+}$) replacing one Tie1 allele and the LacZ gene is placed under the control of endogenous Tie1 regulatory elements. As we expected, Tie1-LacZ activity was ubiquitously expressed throughout the aorta and its branches of 4 week-old animals (Figure 2.1A), consistent with the role of an activated endothelium. Interestingly, in the 12 week-old adult mouse aorta this expression was restricted to specific locales with disturbed flow. We observed that whereas expression in the thoracic and abdominal aorta

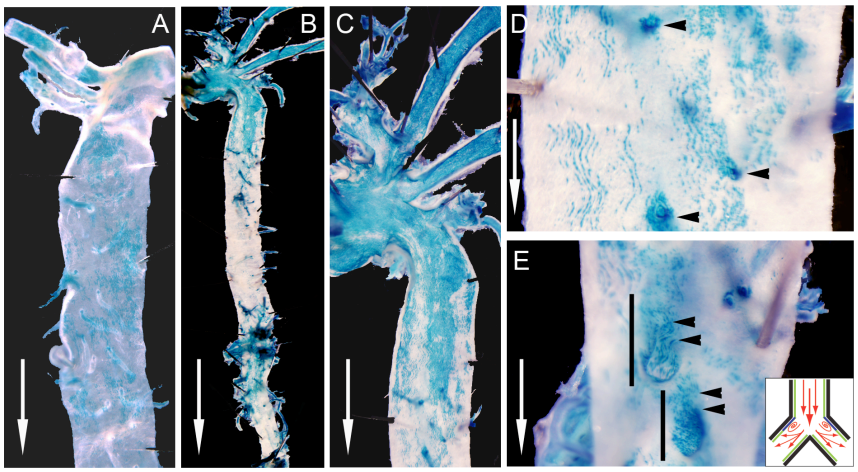

Figure 2.1 ***Tie1 Expression is Restricted to Shear Stress Specific Sites in the Adult Aorta*** Representative en-face X-gal stained, wholemount distal aorta of 4 week-old (**A**) and 12 week-old (**B-E**) Tie1-LacZ mice. (**A**) Pervasive endothelial Tie1-LacZ expression in the aortic arch, branch arteries and descending aorta wall of immature 4 week-old mouse (20x magnification). (**B**) Low magnification (10x) of aorta (aortic arch, thoracic and abdominal aorta) and (**C**) higher magnification (20x) depicting endothelial Tie1-LacZ expression in the aortic arch diminishing in the thoracic aorta as laminar flow is restored. (**D**) High magnification (40x) image of thoracic aorta wall illustrating endothelial Tie1-LacZ expression at ostia of vertebral artery branches (arrowheads). (**E**) Close-up (40x magnification) view of abdominal aorta wall showing distinct X-gal staining at the outer walls of renal artery bifurcations (arrowheads) and (inset) illustration of proatherogenic, disturbed flow as experienced at aortic branch points. (White arrows indicate direction of blood flow)

was diminished, X-gal staining persisted in the aortic arch, its branch arteries (Figures 2.1B, C), and branches of the descending aorta including vertebral (Figure 2.1D) and renal arteries (Figure 2.1E). We also found distinct staining at the outer walls of these vascular bifurcations where conditions of disturbed flow and low shear stress prevail (Figure 2.1E, inset).

LacZ expression was also detected in the endothelium of the aortic sinus and the aortic valves, areas characterized by disturbed flow although X-gal staining was more widespread on the aortic valve endothelium (Figure 2.2A, B). We noted more robust LacZ expression on the fibrosa surface, which experiences disturbed flow, as compared to the ventricularis, which undergoes laminar flow (Peacock, 1990; Simmons *et al.*, 2005). Additionally, X-gal staining was minimal in the ascending aorta, where laminar flow prevails (Figure 2.2G). Since the aortic valve region is prone to atherosclerosis, we studied the effect of hypercholesterolemia due to ApoE deletion on the expression pattern of Tie1. In young 4 week-old $Tie1^{lz/+}$:$ApoE^{-/-}$ mice, prior to the appearance of quantifiable atherosclerotic lesions, we noted a similar Tie1-LacZ expression pattern at the aortic sinus (Figures 2.2C, D) as seen in non-atherosclerotic littermates. This expression pattern persisted in 24 week-old $Tie1^{lz/+}$:$ApoE^{-/-}$ mice with advanced atherosclerotic plaque burden (Figures 2.2E, F). These results suggest a specificity of Tie1 expression in regions that are characterized by disturbed flow, but absent in areas experiencing laminar flow.

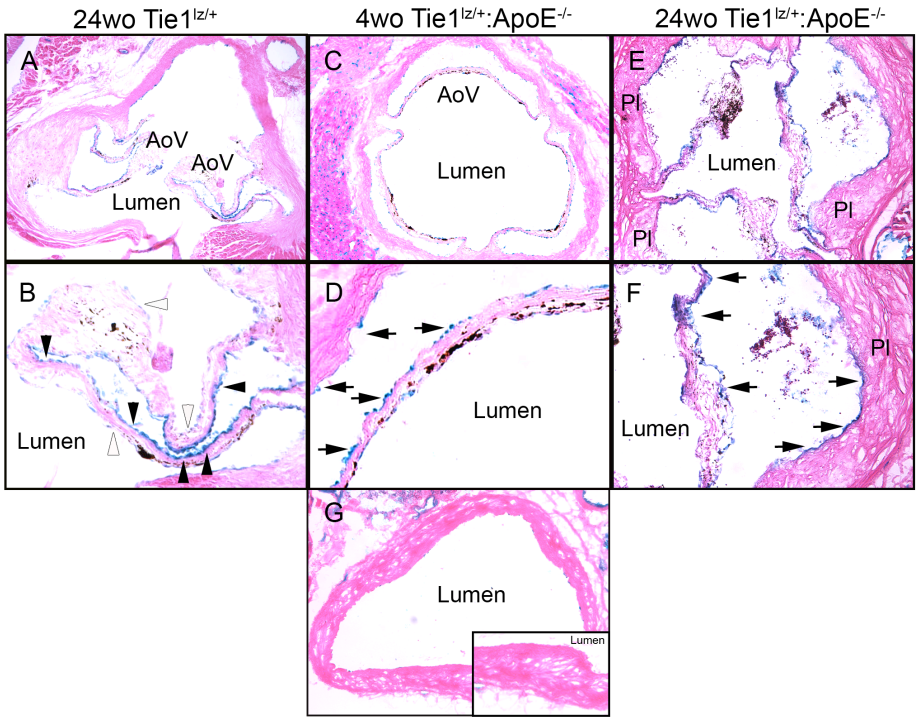

Figure 2.2 ***Tie1 is Expressed in Regions of Disturbed Flow*** (**A-G**) Cryosections of aortic valves from Tie1-LacZ mice, X-gal stained and H&E counterstained. (**A**) 24 week-old Tie1$^{lz/+}$ mouse aortic valves shown in high magnification (**B**), illustrating specific X-gal staining on fibrosa surface of aortic valve leaflet (black arrowheads) but not on ventricularis surface (white arrowheads). Tie1-LacZ expression (black arrows) persists on the fibrosa surface of the aortic valve leaflet and aortic wall of Tie1$^{lz/+}$:ApoE$^{-/-}$ mouse, prior to atherosclerotic lesion formation at (**C**) 4 weeks, (**D**) high magnification and during advanced disease progression at (**E**) 24 weeks, (**F**) high magnification. (**G**) Cryosection of ascending aorta (immediately downstream of aortic valves) depicting absence of endothelial LacZ staining where laminar flow is predominant (inset, higher magnification of aorta wall and endothelium). (**A,C,E,G** – 50x magnification; **B,D,F** – 100x magnification; AoV, aortic valve leaflets; Pl, atherosclerotic plaques).

In vivo Tie1 expression is Dynamically Reduced by Laminar Flow with High Shear Stress

To study whether shear stress modification *in vivo* would affect Tie1 expression, we utilized shear stress modifying casts (Figures 2.3A, B). These casts induce lowered shear stress immediately upstream, increased shear stress inside the cast and oscillatory shear stress with vortex flow immediately downstream (Cheng *et al.*, 2005; Cheng *et al.*, 2006). Cheng et al previously described increased eNOS activity in the tapered region of the cast where laminar flow with increased shear stress is found (Cheng *et al.*, 2005). Also, atherosclerotic lesions formed in the adjacent upstream region experiencing laminar flow with low shear stress and in the immediate downstream area undergoing oscillatory flow. We implanted casts around the right common carotid artery of Tie1-LacZ mice for 7 days. Right common carotid arteries implanted with control non-tapered casts (n=4) expressed LacZ uniformly along the vessel (Figure 2.3D) similar to non-implanted left subclavian and left carotid arteries. In contrast, X-gal staining was attenuated in the tapered region of the cast where laminar flow with increased shear stress is induced (n=4) (Figure 2.3C). Comparable to controls, LacZ activity persisted in the aforementioned regions adjacent to the cast. These results suggest that Tie1 expression is attenuated by conditions of laminar flow with high shear stress. Further, these results demonstrate a dynamic repression of Tie1 promoter activity in the short term (7 days).

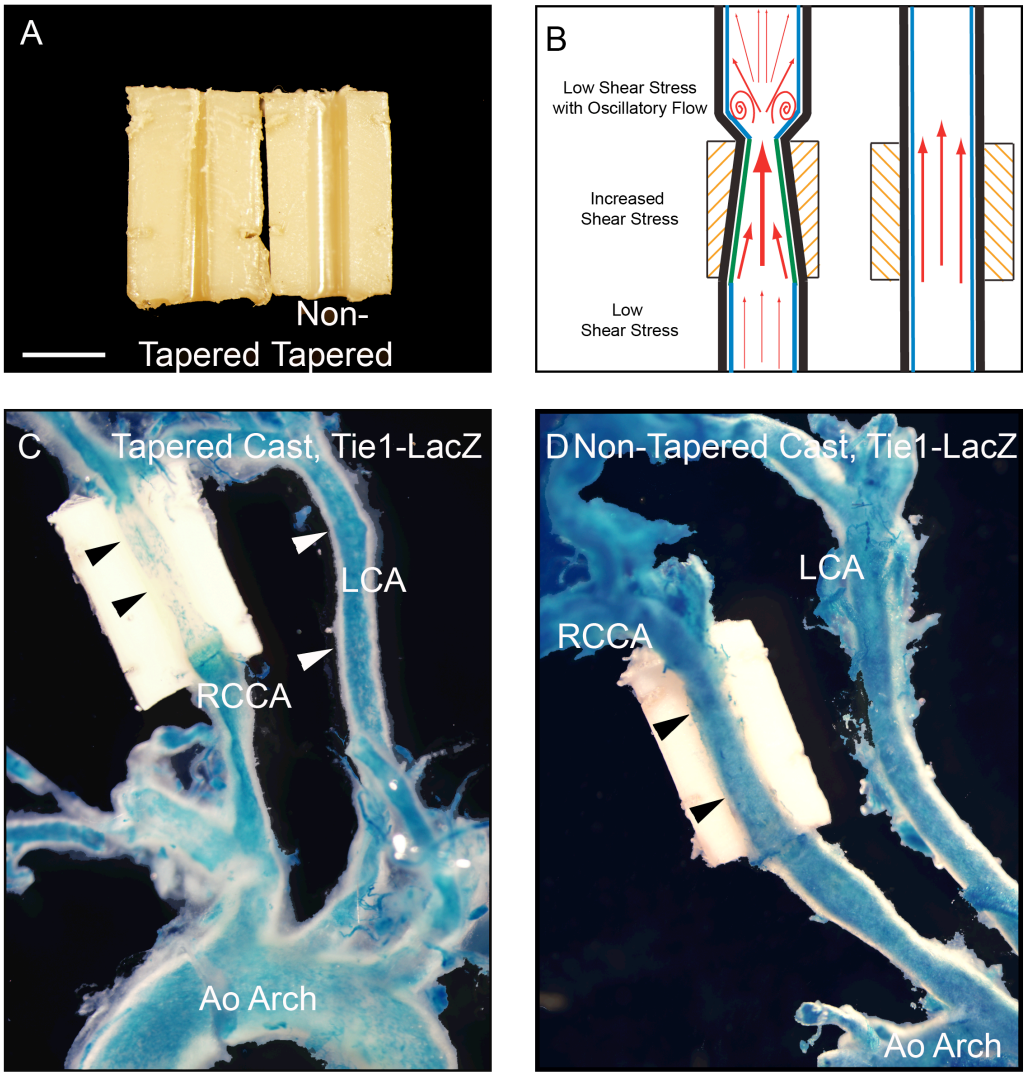

Figure 2.3 ***Tie1 Promoter Activity is Downregulated by High Shear Laminar Flow***
(**A**) Photograph of tapered (left) and non-tapered control (right) casts that were surgically implanted in mouse carotid arteries. (White bar indicates 1mm scale) (**B**) Illustration of tapered casts manipulating *in vivo* shear stress inducing lowered shear stress upstream, increased shear stress inside and oscillatory flow with shear stress immediately downstream of the cast. Diagram also shows control non-tapered casts do not interfere with *in vivo* shear stress. (**C, D**) Representative wholemount X-gal stained carotid arteries photographed with shear stress modifying casts 7 days post implantation (50x magnification). (**C**) Tie1-LacZ mouse carotid artery pictured with tapered cast showing diminished X-gal staining along the region of increased shear stress inside the cast. (**D**) Control non-tapered casts implanted in Tie1-LacZ mouse carotid artery illustrating persistent uniform X-gal staining. (RCCA, right common carotid artery; LCA, left carotid artery; Ao arch, aortic arch)

Discussion

Previous *in vivo* studies have focused on the role of Tie1 in early stages of embryonic development. Puri et al documented a heterogeneous requirement for Tie1 in the maintenance of various adult vascular endothelial cell populations (Puri *et al.*, 1995). In addition, Porat et al showed a predilection of Tie1 expression in bifurcations of microvasculature in early post-natal mice (Porat *et al.*, 2004). Here, we show that the expression pattern of Tie1 in the 12 week-old adult macrovasculature is different from that of the immature 4 week-old mouse. In the aorta of a 4 week-old mouse, we observed pervasive endothelial Tie1 expression consistent with aortic immaturity. However, in the 12 week-old adult mouse, Tie1 expression was decreased specifically in the descending aorta, an area characterized by atheroprotective laminar flow with high shear stress. We noted strong X-gal staining at established locales subjected to atherogenic disturbed flow (Suo *et al.*, 2007), specifically at the aortic arch and its branch vessels, and bifurcations of the vertebral and renal arteries. LacZ expression was also observed at the aortic sinus and the aortic valves, areas characterized by disturbed flow.

The direct effect of altered shear stress on Tie1 expression was demonstrated *in vivo* by the use of vascular casts. Cheng et al previously demonstrated use of these *in vivo* shear stress modifying casts to alter the shear forces in the carotid arteries of ApoE null mice (Cheng *et al.*, 2006). Within the boundaries of high shear laminar flow in the tapered cast, eNOS expression was increased and no atherosclerotic lesions were detected (Cheng *et al.*, 2005). They also found that lowered shear stress upstream of the cast induced formation of unstable plaques and the immediate downstream region of the cast formed stable plaques in the presence of oscillatory shear. In our Tie1-LacZ reporter

mice, we found persistence of Tie1 promoter driven LacZ expression also in these immediate vicinities around the cast. We observed LacZ activity in the upstream region experiencing low shear stress, laminar flow (10 N/m^2), and in the downstream oscillatory shear stress areas that experience wide variations of force (10 to 60 N/m^2). This Tie1 expression was similarly observed in unimplanted carotid arteries and in controls implanted with non-tapered casts (15 N/m^2). Interestingly, we found a dynamic and dramatic reduction in Tie1 promoter activity only after seven days of implanting tapered casts in Tie1-LacZ mice. Attenuation of LacZ expression was distinctly confined to the boundaries of the tapered cast where shear stress increases from 10 to 25 N/m^2. Hence, the attenuation of Tie1 expression in the region of laminar shear increasing from 10 to 25 N/m^2 is likely due, at least in part, to both the higher shear forces and laminar flow conditions.

Thus, while Tie1 expression is accentuated during both embryonic development and post-natal growth, Tie1 activity is also regulated in the adult by shear stress. Whether this is a negative effect of promoter suppression by atheroprotective laminar shear or activation by atherogenic shear is not known. Chen-Konak et al previously reported a octameric negative shear stress response element (nSSRE) downregulating Tie1 expression (Chen-Konak *et al.*, 2003). Shear stress response elements are targets of nf-κB transcription factor, which in turn can be regulated by shear stress(Gimbrone *et al.*, 1999). In light of the fact that SSREs have been found in MCP-1, VCAM-1, PDGF and TGF-β, we speculate that Tie1 expression may also be regulated by nf-κB.

In summary, we showed that post-natal Tie1 expression is pervasive throughout the aorta, but in the adult mouse this expression is limited to regions exposed to disturbed

flow with low shear stress. Additionally, we also showed that modified exposure to laminar flow with high shear stress dynamically downregulates Tie1 promoter activity. Hence, the expression of Tie1 is upregulated by shear stress profiles that promote atherosclerosis and it is decreased by anti-atherogenic flow patterns.