Regulation of angiogenesis and vascular remodeling by angiogenic factors

Taren Michelle Grass
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REGULATION OF ANGIOGENESIS AND VASCULAR REMODELING BY ANGIOGENIC FACTORS

by

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ABSTRACT

Coronary angiogenesis is a natural response to the hypoxia associated with cardiovascular disease. Neovascularization may increase blood flow to ischemic tissue thereby improving cardiac function. However, in order for new vessel growth to become permanent and perfuse the cardiac muscle, these capillaries must mature and be stabilized by attachment of smooth muscle cells or pericytes during vascular remodeling. Therefore, a net increase in perfusion of the cardiac tissue following vascular remodeling is the essential goal of therapeutic coronary angiogenesis.

Therapeutic angiogenesis strategies have primarily focused on angiogenic regulatory factors, including members of the VEGF and angiopoietin (ANG) families. However, temporal expression of these factors in relationship to the growth and remodeling phases of angiogenesis has not been established. The current study was designed to characterize the transition between angiogenesis and vascular remodeling during tissue remodeling in the ischemic murine heart and establish temporal expression patterns of ANG-1, ANG-2, Tie2 receptor, VEGF, and the phosphorylated Tie2 receptor within the context of a model of vascular growth and remodeling.

Microcauterizer-treatment of the Tie2LacZ transgenic mouse heart to generate a myocardial infarction provides a model for histologically defining angiogenic growth and remodeling. Whole mount staining of infarcted heart tissue in combination with Gomori trichrome staining, PECAM labeling, and smooth muscle α-actin labeling of tissue sections showed an immediate angiogenic response with a transition to vascular remodeling evident at 10d post-treatment. ANG-1, ANG-2, Tie2, and VEGF mRNA levels increased at 10d post-infarction in all targets suggesting a coordinate role for the ANG and VEGF systems in regulating the remodeling process. However, no temporal changes in protein expression were seen in ANG-1, ANG-2, Tie2, phospho-Tie2, and VEGF. In addition, immunostaining for ANG-2 demonstrated no difference in expression among the time-points. In contrast, immunostaining for phospho-Tie2 showed different patterns of expression at each of the time-points studied. The results of ANG/Tie2 and VEGF expression studies emphasize the potential complexity of determining the specific roles of these factors in the adult cardiac vasculature. Furthermore, the data suggest that future therapeutic angiogenesis strategies in the heart need to focus on both the growth and remodeling phases as critical components of effective therapies.
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INTRODUCTION

Cardiovascular Disease

Cardiovascular disease is the leading cause of death in the industrialized world, resulting in almost 1 million deaths in the United States alone in 2001\(^1\). The majority of deaths result from coronary artery disease, which is characterized by damage to the heart tissue due to loss of blood supply. Coronary artery occlusion causes loss of oxygen to the myocardium due to reduced blood supply often resulting in myocardial infarction. A large portion of the damage to the cardiac tissue is irreversible due to cardiomyocyte death. However, reoxygenation of the remaining tissue can prevent further damage. Current therapies are designed to either revascularize the cardiac tissue or clear the existing blocked vessels in order to restore oxygen and nutrient delivery to the peri-infarct region and prevent disruption of flow to the undamaged tissue.

Therapeutic Angiogenesis

Treatment of individuals suffering from coronary artery disease typically consists of bypass surgery or angioplasty to clear the obstructed vessels. However, for a proportion of patients the traditional therapeutic options are either not viable, due to diffuse coronary artery disease or impaired microcirculation, or ineffective, resulting in restenosis of the artery. For these individuals, additional treatment strategies are needed. One such option is therapeutic angiogenesis. Development of collateral circulation is an inherent response to myocardial hypoxia or ischemia associated with coronary artery disease \(^1\). Neovascularization, by way of angiogenesis, may aid restoration of cardiac

function by increasing perfusion of the affected area [2], thereby providing oxygen and nutrients necessary for survival of the tissue. However, the natural angiogenic response is often insufficient to effectively perfuse the damaged myocardium. Supplementing the innate response through gene or protein therapy is a desirable treatment strategy that has gained much focus. Nonetheless, efforts to facilitate or exploit coronary angiogenesis have been hindered by insufficient knowledge of the mechanisms by which the body stimulates the formation and maturation of new blood vessels.

*Angiogenesis*

Angiogenesis, the development of new capillaries from the existing vasculature [3], is a physiological process that occurs naturally in the female reproductive system and in the wound healing response. Pathological angiogenesis is evidenced either as excessive vascular formation, as seen in tumor formation and progression, atherosclerosis, chronic inflammation, and diabetic retinopathy, or inadequate neovascularization characteristic of cardiovascular disease and ischemic limb disease.

Angiogenesis is a multi-step process comprised essentially of growth and remodeling phases. Growth of neovasculature occurs by sprouting of a new vessel from an existing vessel. Formation of new vessels necessitates destabilization of the current vascular structure, thereby allowing the vascular components to become receptive to additional angiogenic signals. Angiogenesis is often stimulated by hypoxia, growth factors or other biochemical signals produced by the surrounding tissue or vascular wall cells, and hemodynamic factors, such as shear stress and pressure [4]. The initial stimulus triggers degradation of the basement membrane by matrix metalloproteinases 2.
thereby enabling endothelial cell (EC) migration and proliferation. In addition, enhanced vascular permeability allows release of plasma proteins, which can form a provisional matrix for migration of ECs [5, 6]. The cells assemble into simple chord-like structures and acquire a lumen. An alternative method of vessel formation in angiogenesis is intussusception; neovascularization occurs by division of an existing vessel by inward growth of ECs (for a review see [7]).

In order for the neovasculature to enhance circulation, these newly formed capillaries must first undergo remodeling into a branched network of mature, stable vessels. Remodeling is distinguished by reorganization of vascular structures in response to mechanical and biochemical stimuli [4]. Stabilization and maturation of the vascular network occurs by recruitment of pericytes and smooth muscle cells to the endothelial cells [8, 9] as well as formation of the new basement membrane. Binding of periendothelial cells to the newly formed vessel prevents endothelial cell proliferation and migration in addition to making vessels less susceptible to regression [10].

Vascular regression may occur via endothelial cell apoptosis [7, 11] and is essentially pruning of the vasculature resulting in the elimination of excess vessels and, therefore, a more efficient vascular network. While the signaling mechanisms resulting in regression are not understood, physiological factors including perfusion, oxygenation state, and shear stress appear to affect vascular regression. Non-perfused vessels and those exposed to hyperoxia regress as do those vessels experiencing reduced or absent levels of shear stress [10].
Pericyte/Smooth Muscle Cell and Endothelial Cell Interactions

A key aspect of the remodeling process is the stabilization of vessels of all sizes by periendothelial (mural) cells. Pericytes are characterized by a cell body with long processes that enable them to embed within the basement membrane and contact ECs directly [9]. Detection of pericytes within tissues has proved challenging as a single surface marker does not usually detect all such cells. In addition, markers, such as platelet derived growth factor (PDGF)-β and high molecular weight-melanoma-associated antigen, that characteristically detect activated pericytes do not detect the cells in quiescent tissues [12]. Electron microscopy provides the most reliable means of detecting pericytes embedded within the basement membrane [13].

Currently, mural cells are believed to play a role in inhibiting EC proliferation [14, 15] and migration as well as production of extracellular matrix components to stabilize vessels. Vessels lacking periendothelial coverage are more prone to regression [16]. Furthermore, the number and structure of pericytes varies among tissues and vessels suggesting periendothelial cells may assist ECs in acquiring distinct functions depending on the needs of the vascular bed [9]. In addition to their structural role, pericytes and smooth muscle cells provide molecular signals via growth factors and cytokines that influence function of the vascular network.

Regulation of Angiogenesis & Vascular Remodeling

Regulation of angiogenesis and vascular remodeling is known to be a multifactorial process involving a precise balance between positive and negative endogenous regulators. Each step in the process must be tightly controlled to prevent excessive
vascular growth. For example, activating signals stimulate the proliferation and migration of ECs; these actions are counter-balanced by inhibitory signals. In fact, some naturally occurring inhibitors of angiogenesis induce EC apoptosis [17]. In an unperturbed system, proper regulation results in quiescent vessels.

Angiogenic factors are believed to be the primary regulators of the angiogenic process. Among the most prominent are vascular endothelial growth factor (VEGF) and angiopoietin (ANG)-1 and -2. VEGF and fibroblast growth factor (FGF)-2 have generated a great deal of interest due to their ability to regulate a number of distinct angiogenic events. A number of additional factors, which may play a role in the angiogenic process, have also been identified suggesting the signaling pathways for angiogenesis are likely to be many and complex. Furthermore, these factors are proposed to be tightly regulated temporally and spatially resulting in a functional vascular network [18].

Vascular Endothelial Growth Factor

VEGF was first described as both a potent endothelial cell mitogen [19, 20] and a vascular permeability factor [21, 22]. Since its initial characterization a total of five VEGF family members have been isolated, several of which consist of multiple isoforms. VEGF-A (VEGF) is the most well characterized and highly studied member of the family.

VEGF is a 34-46 kDa homodimeric glycoprotein [21]. Alternative splicing produces four isoforms of murine VEGF: VEGF-120, -144, -164, and -188. Exons 6 and 7, lacking in VEGF-120, encode heparin and heparan proteoglycan binding domains.
VEGF-120 is a freely soluble form of the factor, whereas the remaining isoforms may bind to the cell surface or extracellular matrix thus restricting their bioavailability. VEGF-164 is the predominant isoform measured in many tissues and cells; however VEGF-120 and VEGF-188 are also commonly detected [23].

A number of different cell types, including endothelial cells, fibroblasts, smooth muscle cells, and macrophages, produce VEGF leading to a primarily paracrine mode of action. VEGF binds to endothelial tyrosine kinase receptors, VEGF receptor-1 (Flt-1) and VEGF receptor-2 (Flk-1/KDR). Activation of either receptor by the VEGF ligand results in homo-dimerization and autophosphorylation on tyrosine residues. Binding of VEGF to Flk-1 stimulates EC proliferation in addition to mediating increased vascular permeability. In addition, VEGF promotes endothelial survival by inhibiting apoptosis through up-regulation of Bcl-2 and survivin [24, 25]. Generation of VEGF null mice has established VEGF as a critical mediator of the early stages of vascular development. In addition to impaired blood vessel development in VEGF null mice, embryos heterozygous for the VEGF allele exhibit a lethal phenotype, emphasizing a critical dose-dependence on the factor during embryonic vessel development [26, 27].

Hypoxia has been shown to mediate VEGF expression by either stimulating transcription [28] or increasing stability of the message [29, 30]. The transcriptional regulator hypoxia inducible factor (HIF)-1 has been shown to bind and stimulate transcription of VEGF [31]. In addition, TGF-β, interleukin-1 and interleukin-6 have been shown to up-regulate VEGF in vitro [32-34].
Tie2

Tie2 is a transmembrane tyrosine kinase receptor expressed predominantly in the vascular endothelium and on some hematopoietic cells [35, 36]. Tie2 was first isolated by RT-PCR and identified as a novel receptor tyrosine kinase (RTK) based on its amino acid sequence [35] and structural homology with the RTK gene family [37]. Enzymatic characterization of human Tie2 and phosphorylated (phospho-) Tie2 demonstrated that autophosphorylation occurs first at Y992 of the activation loop; subsequent autophosphorylation of the C-terminal tail occurs at Y1108 [38]. Additional evidence suggests that Y1101 of Tie2 may be important to the biological role of the receptor. Decreased tyrosine phosphorylation has been demonstrated in the tyrosine-to-phenylalanine mutant Y1101F [39]. In addition, intact Y1101 is required for phosphorylation and activation of Akt and activation of PI3-kinase [39], which has been shown to regulate EC survival [40]. A study mapping the interactions of putative Tie2 phosphorylation sites with known RTK targets demonstrated interactions with murine Y1100, Y1106, and Y1111 (corresponding to human Y1101, Y1108, and Y1112) [41]. Furthermore, a potential role for murine Y897 in the negative regulation of Tie2 has been suggested [37].

While the downstream signaling pathways responsible for the vascular effects of Tie2 are not well understood, ANG-1 and ANG-2 have been identified as ligands for the receptor. Binding of ANG-1 to Tie2 induces dimerization and autophosphorylation of the receptor [42]. Expression of phosphorylated Tie2 has been observed in both active and quiescent ECs in numerous adult tissues, suggesting constitutive activation, and therefore a possible role in vascular maintenance [43]. Furthermore, investigation of
Tie2 null mice has suggested that the receptor is critical for proper development of mature vessels. Tie2 knockout mice die at embryonic day 9.5-10.5 with deficits in remodeling of the initial capillary plexus into a more diverse and complex vascular network [44].

Evidence for regulation of Tie2 by hypoxia is contradictory. Prolonged exposure (48 h) of rats to hypoxic air resulted in a decrease in both mRNA and protein levels in the heart [45]. However, Tie2 expression increased in human ECs in vitro [46] and remained stable in cultured bovine microvascular ECs (BMECs) [47] following hypoxic treatment; in addition, Tie2 expression was unaffected by VEGF treatment in BMECs [47]. Up- or down-regulation of Tie2 by ANG-1 or ANG-2 has not been clearly demonstrated.

**Angiopoietin-1**

Angiopoietin-1 was the first of a family of four angiopoietins to be isolated as a ligand for Tie2. ANG-1 is an ~70 kDa, secreted glycoprotein that organizes into higher-order multimers. Expression of ANG-1 has been localized to pericytes, including those expressing markers of activation, and smooth muscle cells [12], thus suggesting a paracrine mechanism of action. Similar to Tie2, expression of ANG-1 mRNA has been noted in numerous adult tissues including those involved in vascular remodeling in a healthy adult, such as uterus and ovary, and those considered quiescent, such as heart and brain [48].

Initial characterization of ANG-1 showed it to be non-mitogenic, unlike previously identified angiogenic factors, such as VEGF [42]. However, additional in vitro studies have suggested several potential roles for ANG-1 in the vasculature.
Treatment of cultured ECs with ANG-1 results in a chemotactic response of the cells toward ANG-1 [49, 50] that appears to be mediated via the PI3-kinase pathway [50] and in tubule formation [51]. Furthermore, a role has been established for ANG-1 in the promotion of EC survival [40, 49-52]. Stimulation of ECs by ANG-1 results in an elevation of the anti-apoptosis factor, Survivin, via a PI3-kinase/Akt-dependent mechanism [52].

In vivo, the phenotype of the ANG-1 null mouse first led to the suggestion that ANG-1 may have a role in the remodeling process. Embryos lacking ANG-1, or Tie2, develop a seemingly conventional primary vasculature, which then fails to undergo further remodeling, suggesting ANG-1 is involved in later stages of vascular development [53]. In addition, ANG-1 -/- embryos exhibit poor endothelial cell/periendothelial support cell interactions when compared to wild-type embryos. A postnatal cornea micropocket assay studying the effect of ANG-1 in combination with VEGF showed abundant periendothelial cells when ANG-1 was administered with VEGF but few such cells when VEGF was given alone [54]; the cornea micropocket assay allows assessment of neovascularization directly stimulated by cytokine-containing pellets implanted beneath the cornea. This evidence supports the view that activation of Tie2 by ANG-1 may initiate the release of factors that recruit perivascular support cells to the remodeling vasculature [44, 53].

While the downstream signaling pathways are not yet known, ANG-1 also appears to play a key role in inhibiting the inflammatory response and mediating the reduction of vascular permeability in vitro and in vivo [55-57]. Over-expression of ANG-1 reduces inflammation associated with VEGF-induced neovascularization [56, 57].
Additionally, ANG-1 is believed to act as an anti-permeability factor, reciprocal to VEGF. Transgenic over-expression of VEGF has been shown to result in leaky vessels, which can be stabilized by simultaneous over-expression of ANG-1 [57]. *In vitro* examination of human umbilical vein ECs treated with ANG-1 suggests that the anti-permeability characteristics of the factor are likely to be mediated by regulation of endothelial cell junctional complexes [55].

Additional evidence strongly suggests that the role of ANG-1 in vascular remodeling is due to binding and activation of Tie2 by ANG-1. Initial characterization of ANG-1 showed that tyrosine phosphorylation of Tie2 induced by the ligand could be inhibited by addition of soluble Tie2 (sTie2) [42]. In addition, *in vitro* treatment of endothelial cells with sTie2 blocks migration, tubule formation, and the anti-apoptotic effect induced by ANG-1 [40, 49, 51]. In the cornea micropocket assay, modulation of VEGF-induced neovascularization by ANG-1 is abolished by the addition of sTie2 [54].

Little is known about the regulation of ANG-1. Hypoxia has been shown to down-regulate ANG-1 in rat glioma cells *in vitro* [58] and in rat heart tissue after a 48 h hypoxic exposure [45] but have no effect on ANG-1 expression in cultured bovine microvascular endothelial cells (BMECs) [47]. Furthermore, ANG-1 expression remains stable after VEGF exposure in BMECs [47].

*Angiopoietin-2*

ANG-2 is a ligand for Tie2 as well, acting as a natural antagonist to ANG-1 in the vascular system. Similar to ANG-1, ANG-2 is a non-mitogenic, secreted glycoprotein, which forms homodimers. *In vitro*, ANG-2 expression has been identified in numerous
endothelial cell lines [12, 49, 59]. However, contrary in vivo evidence suggests that cellular localization of ANG-2 may vary by tissue or experimental system; ANG-2 has been identified in endothelial cells of glioma tumors [60] but also in pericytes and smooth muscle cells of the developing aortic branches in the mouse [48, 61]. Examination of murine tissues by in situ hybridization and Northern blot analyses has shown ANG-2 expression at sites of vascular remodeling, including ovary, placenta, uterus, and corpus luteum [48].

ANG-2 and ANG-1 have demonstrated comparable binding affinity for the Tie2 receptor [42, 48]. However, a fourfold to eightfold molar excess of ANG-2 was shown to considerably block ANG-1 mediated activation of the Tie2 receptor in a human endothelial hybrid cell line [48] thus resulting in the conclusion that ANG-2 serves as an antagonist for the receptor. In contrast to ANG-1, ANG-2 does not elicit a chemotactic response in ECs [49]. Furthermore, activation of Tie2 is not evidenced when ECs are treated with ANG-2 for short duration or at concentrations comparable to those at which ANG-1 is capable of inducing Tie2 phosphorylation. However, additional in vitro data have shown that prolonged EC exposure to ANG-2 [62], administration of ANG-2 at high concentration [63], and treatment of non-endothelial cells ectopically expressing Tie2 [48] results in phosphorylation of the Tie2 receptor suggesting a potentially complex role for ANG-2 in the vascular system.

The phenotype of the ANG-2 transgenic mouse is reminiscent of that of the ANG-1 and Tie2 knockout mice providing additional support for the proposal that ANG-2 serves as an antagonist in the vascular system [44, 48, 53]. Generation of the ANG-2 null mouse however, provided further evidence that the role of ANG-2 may be system and
context dependent. The ANG-2 knockout proceeds through vascular development normally but undergoes impaired vascular remodeling of the neonatal eye. In addition, evidence suggests that ANG-2 acts as an agonist in the lymphatic system with the ANG-2 knockout exhibiting abnormal organization and function of the lymphatic vasculature, which can be recovered by treatment with ANG-1 [61].

In contrast to ANG-1, ANG-2 is thought to act in concert with VEGF, promoting angiogenesis by destabilizing vessels through disruption of endothelial cell – pericyte interactions [64]. Coexpression of ANG-2 and VEGF has been noted at sites of angiogenesis, including the corpus luteum and vascularized tumors [48, 65]. Furthermore, in pupillary membrane and corneal micropocket models, ANG-2 has been shown to have a proangiogenic effect when administered with VEGF [66, 67].

Up-regulation of ANG-2 mRNA is stimulated by a variety of stimuli including hypoxia, VEGF, basic fibroblast growth factor, and tumor necrosis factor-α [47, 59, 68]. VEGF treatment in vitro increases the rate of ANG-2 transcription with an accompanying increase in protein synthesis [47]. Suggesting a feedback mechanism, ANG-2 is down-regulated by ANG-1 and ANG-2 itself [59]. A decrease in ANG-2 mRNA also results from exposure to TGF-β [59]. Furthermore, hypoxic regulation of ANG-2 in vivo has been shown to vary by organ with a decrease in ANG-2 mRNA in the heart following prolonged hypoxia [45].

*Gene and Protein Angiogenic Therapy*

Existing therapeutic angiogenesis strategies have focused primarily on the VEGF and fibroblast growth factor (FGF) families, due largely to their demonstrated ability to
stimulate angiogenesis in animal models and \textit{in vitro}. Currently, greater than twenty clinical trials utilizing a member of the VEGF or FGF families are underway. Use of a single factor is based on the assumption that administration of the gene or protein will trigger all downstream signaling necessary for growth and establishment of the neovasculature. The results of many early trials have been promising, demonstrating vascular formation, increases in perfusion, and improvements in objective measures of function. However, side effects such as edema [69] or hypotension [70] as well as the question of whether these factors are capable of stimulating long-term neovascularization have led investigators to hypothesize that a single angiogenic factor may be insufficient to induce a stable, functional vasculature. Additional molecules, which have been recently considered, are HIF-1$\alpha$ and ANG-1. HIF-1$\alpha$ is a transcriptional activator known to stimulate VEGF expression. Generation of HIF-1$\alpha$ transgenic mice, using an altered form of the gene, demonstrated vascular formation without the edema and inflammation associated with the VEGF transgenic mouse [71]. In addition, in a hindlimb ischemia model, induced by dissection and removal of the femoral artery, sequential administration of ANG-1 naked DNA followed by VEGF resulted in improved vascularity, including an increase in the number of vessels positive for a smooth muscle marker, without VEGF-mediated edema [72]. This evidence suggests an upstream molecule capable of initiating multiple angiogenic factors or sequential administration of unique angiogenic factors may stimulate a more complete vascular response.

\textit{Limitations of Existing Models for the Study of Angiogenesis and Vascular Remodeling}

Development of effective angiogenic therapies will require understanding of the
complex mechanisms regulating the entire angiogenic process from stimulation of growth through stabilization and maturation of a functional vasculature. Existing experimental models attempt to establish various growth factors as regulators of the angiogenic process but often fail to place these angiogenic factors within the context of a defined model for vascular growth and remodeling. A recent study in which hypoxia was induced in the rat heart for various lengths of time followed by 24 h of reoxygenation demonstrated differential expression of VEGF, the VEGF receptors, the angiopoietins, and Tie receptors when compared to normoxic control [73]. However, references to the “initial” and “later stages of the early angiogenic process” were not defined temporally or histologically, thereby weakening the conclusions to be drawn from the protein data. Determination of temporal expression patterns of angiogenic factors in a cardiac model in which the morphological characteristics associated with vascular growth and remodeling have been described will facilitate the refinement of therapeutic strategies for cardiovascular disease.

Tie2LacZ Mouse Model

A key factor in determining the regulatory mechanisms of angiogenesis and vascular remodeling is the ability to assess these phases within an in vivo system. Tie2LacZ transgenic mice provide a model for histologically defining angiogenic growth and remodeling. Specific expression of the LacZ gene in the vascular endothelium via the Tie2 promoter [74] allows qualitative assessment of morphological changes in the vasculature by whole mount β-galactosidase staining of the tissue. Microcauterizer-treatment of the heart to generate an infarct serves as a means of stimulating a complete
angiogenic response comprised of both growth and remodeling phases. In a prior study, assessment of microcauterizer-wounded tissue at 7 days post-treatment revealed an apparently avascular area when perfused with fluorescein-dextran [75]. However, the Tie2LacZ model allows detection of an initial capillary plexus around the wound as early as 1 day post-treatment. In addition, tissue remodeling associated with the microcauterizer-generated infarct is consistent with that evidenced in a murine model of myocardial infarction induced by permanent left anterior descending coronary artery occlusion [76]. Microcauterizer stimulation of angiogenesis in the Tie2LacZ transgenic mouse provides a model for the study of vascular growth and remodeling in addition to the characterization of the regulatory pathways mediating these phases.

Proposed Model for Regulation of Angiogenesis and Vascular Remodeling

The ANG and VEGF systems are strong candidates for distinct roles in the regulation of vascular growth and remodeling. The model shown in Fig. 1 summarizes the proposed roles for the ANG/Tie2 and VEGF systems in coordinately regulating the growth and later stabilization of the vasculature in response to myocardial ischemia; these roles are described in detail below.
Co-expression of ANG-2 and VEGF has been documented at sites of vascular invasion, including the corpus luteum and vascularized tumors, whereas the presence of ANG-2 in the absence of VEGF was associated with vascular regression [48, 64]. These observations have led to the hypothesis that ANG-2 and VEGF together may be involved in destabilizing the existing vasculature. ANG-2 is believed to bind Tie2 inhibiting the stabilizing influence of ANG-1 and thereby facilitating sprouting of new vessels in the presence of VEGF. Additional support for this role was provided by the study of these factors in an in vivo corneal micropocket assay. Administration of ANG-2 in combination with VEGF was pro-angiogenic, causing an increase in the length and circumference of newly formed vessels when compared to VEGF alone [54]. Furthermore, injection of ANG-2 in an in vivo pupillary membrane model stimulated a vascular response, which included increased capillary diameter, EC migration, and sprouting; however, co-injection of soluble VEGF receptor-1 and ANG-2 resulted in
increased endothelial apoptosis suggesting the pro-angiogenic effect of ANG-2 was
dependent on endogenous VEGF [66]. Animal models of myocardial ischemia have also
demonstrated coincident increases in ANG-2 and VEGF following either repetitive [77]
or permanent [78] occlusion of the left anterior descending artery.

Numerous in vitro and in vivo studies have documented the roles of VEGF in
vascular formation, especially endothelial cell proliferation and migration. In addition,
the importance of VEGF in neovascularization of ischemic tissue is suggested by analysis
of ischemic limb [79] and ischemic cardiac tissue [77, 78, 80], which have demonstrated
elevated levels of VEGF, and its receptor Flk-1, when compared to non-ischemic regions.
Vascular formation has also been observed as an outcome in gene and protein therapies
utilizing VEGF [69, 72, 81-85].

While ANG-2 and VEGF are thought to initiate destabilization of the existing
vasculature and formation of new vessels, the Tie2 agonist, ANG-1, is believed to
promote maturation and stabilization of the neovasculature. The stabilizing effect of
ANG-1 via activation of the Tie2 receptor was first suggested by the phenotypes of the
ANG-1 and Tie2 mice; embryos null for either molecule exhibited poor perivascular
support cell interactions when compared to their wildtype counterparts [44, 53].
In addition, co-administration of ANG-1 and VEGF in the postnatal cornea micropocket
assay showed abundant periendothelial cells when ANG-1 was administered with VEGF
but few such cells when VEGF was given alone [54].

While in vitro data have suggested roles for ANG-1 in EC migration and tube
formation [49-51], in vivo support for those functions has been limited to the stimulation
of neovascularization in hindlimb ischemia and corneal micropocket models upon co-

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expression of ANG-1 and VEGF [54, 72, 86]; the in vivo or ex vivo effects of ANG-1 on EC migration and/or tube formation have not been studied. In addition, studies have shown constitutive expression of ANG-1, similar to Tie2, in the heart and brain with no change in expression following myocardial infarction or middle cerebral artery occlusion [77, 87].

The most dramatic role for ANG-1 demonstrated in postnatal in vivo models is that of an anti-permeability factor. Transgenic over-expression of ANG-1 in the skin and heart has resulted in suppression of vascular leakage induced by simultaneous over-expression of VEGF [57, 88]. Furthermore, administration of exogenous ANG-1 has also been shown to counter vascular permeability resulting from blockage of platelet derived growth factor (PDGF) signaling [89]. In vitro data suggests that ANG-1 supports localization of cell adhesion molecules into endothelial cell junctions and stabilizes junctions thereby decreasing permeability [55]. This role is believed to be facilitated by stabilization from perivascular cells. In addition, previous models have suggested that binding of ANG-1 to Tie2 may stimulate the release of factors responsible for the recruitment of mural cells to the immature vasculature [44, 53] thereby stabilizing the vessels.

Understanding the regulation of angiogenesis and vascular remodeling in the adult heart will facilitate development of effective therapeutic angiogenic strategies. Based on the evidence described, the ANG and VEGF systems are candidates for critical roles in the regulatory process. Furthermore, the putative stabilizing effects of ANG-1 are likely to be mediated via phosphorylation of the Tie2 receptor. Assessment of the phosphorylation state of Tie2 in microcauterizer-treated murine heart tissue will allow

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further understanding of the role of the ANG-Tie2 system in the vascular growth and remodeling processes. The goal of this project is to advance development of gene therapies by further establishing the roles of these factors within the context of a morphologically characterized model of vascular growth and remodeling. The research described will test the hypothesis that VEGF and ANG-2 will be up-regulated during the growth phase of angiogenesis while elevation of ANG-1 and Tie2 and an increase in phosphorylated Tie2 receptor will occur later, coincident with remodeling.
The current study addressed the following Specific Aims:

**Specific Aim I.** Establish the Tie2LacZ transgenic mouse as a model for *in vivo* study of angiogenic growth and remodeling in the ischemic murine heart.

**Specific Aim II.** Characterize the temporal expression patterns of known angiogenic regulatory proteins as associated with the growth and remodeling phases of coronary angiogenesis.

**Specific Aim III.** Demonstrate altered phosphorylation of Tie2 receptor in heart tissue actively undergoing vascular growth and remodeling that occurs following microcauterizer wounding.
MATERIALS and METHODS

Animals and Treatment Groups

This study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Tie2LacZ transgenic mice on a FVB/N background were initially obtained from Jackson Laboratories (Bar Harbor, ME, USA). They were raised and maintained at The University of Montana according to Institutional Animal Care and Use (IACUC protocol #ACC-005-02) and Public Health Service standards. The mice were housed in microisolation cages under specific pathogen free conditions with constant temperature and humidity. They received food and water ad libidum and had a controlled 12 h light/dark cycle. Animals were genotyped by polymerase chain reaction for the Tie2LacZ transgene. All mice positive for the transgene were hemizygous based on parental genotypes. The mice were divided into three treatment groups: non-surgical control (NSC), surgical control (SC) and microcauterizer-infarcted (MC). Each group was composed of Tie2LacZ transgenic mice at 10-12 weeks of age. Within the SC and MC groups, animals were divided into five sub-groups: 1, 4, 7, 10, and 14d post-treatment. The ‘n’ number and gender of mice assigned as well as the time-points assessed for each experimental study is shown in Table 1. A total (including the NSC group) of 11 sub-groups was analyzed.

Microcauterizer Treatment

Surgery was carried out similar to Chiotti et al [75]. Mice were anesthetized with an

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2 The Jackson Laboratory (http://www.jax.org)
Table 1. Summary of experimental groups. Non-surgical control mice were studied in all experiments, as were surgical control and microcauterizer-treated mice at each of the time-points listed.

<table>
<thead>
<tr>
<th>Experimental Technique</th>
<th>n</th>
<th>Gender</th>
<th>Time-points</th>
</tr>
</thead>
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<td>Whole mount histology</td>
<td>3-5</td>
<td>Mixed</td>
<td>1d, 4d, 7d, 10d, 14d</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>3-5</td>
<td>Mixed</td>
<td>4d, 7d, 10d, 14d</td>
</tr>
<tr>
<td>Real-time RT-PCR</td>
<td>8</td>
<td>Female</td>
<td>1d, 4d, 7d, 10d, 14d</td>
</tr>
<tr>
<td>Western Blot &amp; ELISA</td>
<td>8</td>
<td>Male</td>
<td>1d, 4d, 7d, 10d, 14d</td>
</tr>
</tbody>
</table>

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intraperitoneal injection of avertin [90] (0.015 cc/g body weight), intubated orally using a 22 gauge cannula and ventilated (Harvard Instruments, Boston, MA) with 25 cc/min oxygen-supplemented air. Subsequently, the thorax was shaved and swabbed with betadine and a mediolateral incision made between the fifth and sixth ribs to expose the heart. For MC treatment a microcauterizer was used to make a small infarct approximately 1.5-2 mm in diameter on the left ventricle near the apex of the heart. The chest wall was sealed with one to two sutures and the pneumothorax was relieved with a 22 gauge cannula and 5 cc syringe. Wound clips closed the skin incisions and the mice were allowed to recover on a warming pad. Surgical control animals received thoracotomy with a touch to the heart with the unheated microcauterizer. Survival rate in both groups was greater than 90%. Following treatment, mice recovered for 1, 4, 7, 10, or 14 d after which they were sacrificed and the heart harvested. Animals in the NSC group received no anesthesia and underwent no surgery or treatment.

**Whole Mount Histology**

Mice were anesthetized with avertin and perfused through the left ventricle with 0.1 M phosphate buffered saline pH 7.4 (PBS) followed by PLP fixative (4% paraformaldehyde, 0.12 M L-lysine hydrochloride, and 15.6 mM Na-m-periodate); care was taken to avoid the infarct when inserting the perfusion needle. Following perfusion, the heart was removed and transferred to Bluo-Gal stain solution (4.9 mg Bluo-Gal (5-bromo-3-indolyl-β-D-galactopyranoside; Diagnostic Chemicals Limited, Prince Edward Island, Canada), 0.1 ml N,N-dimethylformamide, 10 ml PBS, 1 mM MgCl$_2$, 3 mM K$_4$Fe(CN)$_6$). Hearts were stained overnight at 37°C with gentle agitation and minimal light exposure.

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After satisfactory staining was achieved (8-12 h), hearts were rinsed in PBS twice for 5 min each. The tissue was examined and photographed as a whole mount with an Olympus SZ-STS Zoom Stereo Microscope (Olympus America, Melville, NY).

Fixation and Processing of Tissue for Histology

Mice were anesthetized with avertin and perfused for 10 min through the left ventricle with 0.1 M Tris buffer, pH 7.4. Following perfusion, hearts were removed, cut into three equal thickness cross sections from apex to base and post-fixed in Tris-buffered zinc fixative (2.8 mM calcium acetate, 22.8 mM zinc acetate and 36.7 mM zinc chloride in 0.1 M Tris buffer, pH 7.4) [91] for 12-24 h at room temperature (RT). Tissue was then processed through a graded alcohol series, methyl salicylates, paraffin-embedded, and sectioned at 5 μm. Tissue was mounted as 1 in 10 series onto Superfrost® Plus slides (VWR Scientific, West Chester, PA). Slide-mounted sections were deparaffinized and rehydrated prior to immunohistochemistry and trichrome staining.

PECAM/Smooth Muscle α-Actin Immunostaining and Gomori Trichrome Stain

Following rehydration, slides were rinsed in Tris buffer pH 7.4 (Tris) and blocked for 20 min with 4% normal goat serum in 0.1 M Tris buffer pH 7.4 with 1% bovine serum albumin and 0.1% sodium azide (TAB). All antibodies were diluted in TAB and rinses carried out in Tris. Slides were incubated overnight at 4°C with biotinylated anti-PECAM (1:50; Pharmingen, San Diego, CA; 553371). Slides were rinsed and incubated for 1 h at RT with streptavidin 488 (1:500; Molecular Probes, Eugene, OR; S11223). Slides were then rinsed and incubated 1 h at RT in the dark with anti-smooth muscle

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alpha actin (SMA; 1:150; Dako, Carpinteria, CA; M0851). Following a Tris rinse, slides were incubated for 1 h at RT with goat anti-mouse 546 (1:1500; Molecular Probes; A11034), rinsed sequentially in Tris, ddH$_2$O, and PBS followed by coverslipping with PBS. Using an automated stainer (ThermoShandon, Pittsburgh, PA) an alternate series of serial sections was rinsed with dH$_2$O, fixed in 10% formalin, rinsed with dH$_2$O, and stained with Gomori trichrome following the ThermoShandon protocol to delineate the infarct region in MC-treated tissue.

Angiopoietin-2 and phospho-Tie2 Immunostaining

Immunostaining was performed on alternate series of serial sections previously mounted. All antibodies were diluted in TAB and rinses carried out in Tris buffer pH 7.4 or Tris with 1% bovine serum albumin (Tris/BSA). Following rehydration, endogenous peroxidase was blocked by 0.3% H$_2$O$_2$/40% MeOH in PBS overnight at RT. Slides were rinsed and blocked with 4% normal goat serum in TAB for 20 min. Slides were incubated overnight at 4°C with anti-Ang2 (1:500; Chemicon, Temecula, CA; AB3121) or anti-phospho-Tie2 (1:8000; Oncogene Research Products, San Diego, CA; PC449). Slides were rinsed in Tris followed by Tris/BSA and incubated for 1 h at RT with biotinylated goat anti-rabbit secondary (1:400; Vector Laboratories, Burlingame, CA). Slides were rinsed and incubated for 1 h at RT with Vector Elite ABC avidin-biotin complex (Vector Laboratories) followed by visualization with 3-3’ diaminobenzidine (DAB; Sigma). Slides were dehydrated and coverslipped using DPX mounting medium (BDH Limited, Poole U.K.).
**Preparation of Tissue for ELISA and Western Blot Analysis**

Mice were sacrificed by cervical dislocation. Following removal and bisection of the heart, a 3 mm punch of tissue including the wound site was isolated and snap-frozen in liquid nitrogen; tissue from a similar region of the heart was harvested from NSC and SC mice. Tissue punches were homogenized in RIPA lysis buffer [92] containing 20 mM EDTA and 1 Complete Protease Inhibitor tablet (Roche, Indianapolis, IN; 1697498) per 50 ml 1X buffer. BCA protein assays (Pierce, Rockford, Illinois, USA) were performed on individual samples to determine total protein (TP). Within each sub-group (including NSC), two tissue samples were pooled by equivalent amounts of protein. A second protein assay was run on the pooled supernatant to quantitate TP for use in ELISA and Western blot analysis. The experiment was performed in quadruplicate for a total of eight animals per sub-group. For the NSC sub-group, a second set of pooled protein comprised of equivalent amounts of TP from all eight animals was prepared for use in Western Blotting.

**ELISA**

VEGF ELISA (R&D Systems, Minneapolis, MN; MMV00) was performed according to the manufacturer’s instructions using 100 µg TP. Each two-sample pool (including NSC) was run in duplicate. Plates were analyzed and VEGF protein (pg/ml) results obtained using SOFTmax® PRO software (Molecular Devices, Sunnyvale, CA). The experiment was carried out in duplicate.
Western Blot and Densitometry

Protein samples were boiled in Laemmlı Sample Buffer (BioRad, Hercules, CA; 1610737) for 5 min, and 30 µg TP from each two-sample pool was run on reducing 10% SDS-PAGE gels (Cambrex Bio Science, Walkersville, MD; 58502). The NSC 8-sample pool was run on every gel for purposes of gel-to-gel standardization. Biotinylated protein standards (Cell Signaling, Beverly, MA) and prestained protein standards (BioRad) were run on each gel. Separated proteins were transferred to Hybond-P PVDF membrane (Amersham Biosciences, Piscataway, NJ) in transfer buffer (BioRad; 1610771) with 0.05% SDS (ANG-2, Tie2) or without SDS (ANG-1, phospho-Tie2) for 5 h at 100V. ANG-1, ANG-2, and Tie2 blots were blocked in 5% dry milk/TBST (10 mM Tris base, 150 mM NaCl, 0.2% Tween-20 pH 8.0) for 1.5 h at RT while phospho-Tie2 blots were blocked in 10% bovine serum albumin (BSA)/TBST. Blots were rinsed in TBST once for 15 min and two times at 5 min each. Primary antibodies against ANG-1 (1:1000; R&D Systems; AF923), ANG-2 (1:250; Chemicon), and Tie2 (1:250; Pharmingen, San Diego, CA; 557039) were diluted in 5% dry milk/TBST while phospho-Tie2 (1:1000; Oncogene) was diluted in 10% BSA/TBST. Blots were incubated with their respective primary antibody overnight at RT. Following TBST rinses, ANG-2 and phospho-Tie2 immunoblots were incubated with peroxidase-conjugated anti-rabbit secondary antibody (1:5000; Vector Laboratories) and Tie2 immunoblots with peroxidase-conjugated antimouse secondary antibody (1:5000; Vector Laboratories) for 1 h at RT. ANG-1 immunoblots were incubated with biotinylated anti-goat secondary (1:5000; Vector Laboratories) for 1 h followed by incubation with horseradish-peroxidase streptavidin (1:2000; Vector Laboratories) for 1 h. Proteins were detected with enhanced
chemiluminescent detection (ECL) reagents (Amersham) and images captured using a VersaDoc model 3000 system (BioRad). Bands were quantitated with local background subtraction using Quantity One software version 4.3.0 (BioRad). Quantitative data was normalized to NSC prior to data analysis.

*Real-time RT-PCR*

Mice were sacrificed and tissue harvested and snap-frozen as for protein analysis. Total RNA was isolated from each tissue punch using the Qiagen RNeasy® Mini Kit (Qiagen, Valencia, CA; 74104) in the method described by the manufacturer. Real-time RT-PCR was used to quantitate relative levels of ANG-1, ANG-2, Tie-2, and VEGF mRNA. Real-time RT-PCR was carried out using the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Murine growth factor sequences were obtained from NCBI, and PCR primers and dual-labeled probes (Table 2) designed using Primer Express® Version 1.5 (Applied Biosystems). HPRT served as endogenous control and was assayed separately from target amplicons. An assay specific synthetic oligonucleotide encompassing the entire length of the amplicon was diluted in 100ng/μl tRNA/DEPC-H2O and used in the creation of a standard curve for each transcript. All samples were diluted and DNase-treated prior to reverse transcription. Each sample was assayed in triplicate plus one no amplification control without reverse transcriptase. For each replicate, the reverse transcription reaction mixture (10 μl) contained 1x reverse transcriptase buffer, 500 μM deoxynucleotides, 400 nM assay-specific reverse primer, 10U reverse transcriptase, and 4 μl DNase-treated sample RNA (12.5 ng/μl). Cycling conditions were as follows: 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. Real-
Table 2. Real-time RT-PCR Primer and Probe Sequences

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward Primer, Dual-labeled Probe, Reverse Primer</th>
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<td>ANG-1</td>
<td>Fwd: 5'-GCTTGATCTTTACACGAGTGCTG</td>
</tr>
<tr>
<td></td>
<td>Probe: 5'-FAM-TCAGCACAAGGATGCTGATAACGACA-BHQ2</td>
</tr>
<tr>
<td></td>
<td>Rev: 5'-AGAGCGCATTTGCACATACAG</td>
</tr>
<tr>
<td>ANG-2</td>
<td>Fwd: 5'-GCAAGTGTTCCCAGATGCTCT</td>
</tr>
<tr>
<td></td>
<td>Probe: 5'-FAM-AGGAGGCTGGTGTTTGCACGATGT-BHQ2</td>
</tr>
<tr>
<td></td>
<td>Rev: 5'-TGTTGGTAGTACTGTCATCCATTCAAGTT</td>
</tr>
<tr>
<td>HPRT</td>
<td>Fwd: 5'-GCTCGAGATGTCAAGGAGGA</td>
</tr>
<tr>
<td></td>
<td>Probe: 5'-HEX-CATCACATTGTCGGCCTCTGTGTGCT-BHQ2</td>
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<tr>
<td></td>
<td>Rev: 5'-AGGTCAGCAAGAAGAATTTATAGGC</td>
</tr>
<tr>
<td>Tie2</td>
<td>Fwd: 5'-ATCACCGTGCTGGTTGGC</td>
</tr>
<tr>
<td></td>
<td>Probe: 5'-FAM-CAACTGAAGAGGCAATGTCCAAGGAGA-BHQ2</td>
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<tr>
<td></td>
<td>Rev: 5'-CACGTTCTGGGAATGCGCTGAG</td>
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<tr>
<td>VEGF</td>
<td>Fwd: 5'-CCCTGGCTTTACTGCTGTACC</td>
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<td></td>
<td>Probe: 5'-FAM-ACCATGCAAGTGGTCCAGGC-BHQ1</td>
</tr>
<tr>
<td></td>
<td>Rev: 5'-CATGGGACTTCGTCTCCTT</td>
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time PCR was carried out using the above cDNA as template. The reaction mixture (50 μl) contained 1x PCR buffer, 5 mM MgCl₂, 200 μM dNTPs, 1x ROX dye, 400 nM assay-specific forward primer, 400 nM assay-specific reverse primer, 100 nM assay-specific dual-labeled probe, and 1.25U Taq polymerase. Thermal cycling conditions for the ABI PRISM® 7700 Sequence Detector were as follows: 95°C for 1 min and 40 cycles of 95°C for 12 s and 60°C for 30 s. Analysis of raw data was accomplished with the SDS software version 1.9 (Applied Biosystems). The data was expressed as a ratio of the mean triplicate value of target amplicon (i.e. ANG-1) to the mean value of HPRT.

Data Analysis

Data were expressed as mean +/- standard error of the mean (SEM). Prior to statistical analysis, a log10 transformation was performed on ANG-1 and ANG-2 real-time RT-PCR data resulting in a lognormal distribution. For real-time RT-PCR and ELISA data, comparisons to NSC were carried out using one-way ANOVA followed by the Dunnett post hoc test for multiple comparisons. Two-way ANOVA was run on all data sets to obtain the mean square error for post hoc analysis. The mean square error was used to calculate the Tukey test for all possible pairwise comparisons between surgical control and microcauterizer groups at a given time-point or within microcauterizer groups. Data were represented graphically as percentage of NSC; statistical analyses for real-time RT-PCR and ELISA were carried out on raw data prior to normalization to NSC. Statistical analyses were carried out using SPSS software version 11.0 (SPSS, Chicago, IL), and statistical differences were considered significant at p< 0.05.
MORPHOLOGICAL RESULTS

β-galactosidase staining of whole mount infarcted tissue provides a global view of angiogenic growth and remodeling.

Microscopy of Bluo-Gal stained, MC-treated hearts (Fig. 2) revealed an infarct of approximately 1.5-2 mm in diameter at all time-points studied. Initial formation of a capillary plexus surrounding the infarct area was visible as early as 1d post-treatment (PT; Fig. 2B). A similar response was observed at 4d PT (Fig. 2C); however, expansion of the zone of growth surrounding the infarct had begun to occur. The extent and density of the capillary ring progressively increased through 7d PT (Fig. 2D), and vessels of increased diameter were also noted in the vascular network. By 10d PT (Fig. 2E), the infarct was completely covered by neovasculature and both fine capillary vessels and larger vessels were evident. At 14d post-infarction (Fig. 2F), a shift in the appearance of the vasculature was evident from the presence of fine microvessels visible at previous time-points to exclusively larger vessels. SC tissue (not shown) showed no difference from NSC (Fig. 2A); neither control group demonstrated a vascular response such as that seen in the MC-treated groups.

Gomori trichrome staining of heart tissue reveals tissue remodeling in the infarct.

A Gomori trichrome stain of zinc-fixed hearts harvested 4, 7 (Fig. 3B-C), 10, and 14d (Fig. 4A-B) post-treatment revealed the infarcted area as demonstrated by collagen-staining throughout the infarct and distinct staining of intact cardiac muscle outside of the wound site. At 4d PT (Fig. 3B) cellular debris, including damaged cardiac muscle and red blood cells, remained within the infarcted area. Also noticeable was a greater density...
Figure 2. Whole mount β-galactosidase staining of infarcted Tie2LacZ murine heart tissue (B-F). Capillary plexus formation around the infarct was evident as early as 1d PT (B) with broadening of the growth area at 4d PT (C). At 7d PT (D), the capillary ring had increased in density and larger vessels had begun to appear. By 10d PT (E), the infarcted area was covered by neovasculature and both fine capillary vessels and larger, remodeling vessels were evident. At 14d PT (F), the infarct was traversed almost exclusively by larger vessels. Surgical control hearts (not shown) showed no difference in appearance when compared to non-surgical control (NSC; A). Arrowheads indicate representative areas of angiogenic growth. Arrows indicate representative areas of remodeling. Bar = 0.5mm
of cells, as evidenced by nuclear staining, in the periphery of the infarct when compared to surrounding uninjured myocardium. Although collagen had been deposited, the infarct maintained an unorganized appearance. By 7d PT (Fig. 3C) clearance of cellular debris from the infarcted area was essentially complete and the site was characterized by loose connective tissue. At 10d post-infarction (Fig. 4A), the tissue was characterized by an increase in larger caliber vascular structures including a medial layer. In addition, the cellular density within the infarcted region was comparable to surrounding uninjured tissue. By 14d PT (Fig. 4B), a well-established connective tissue layer characterized by dense collagen was visible in the infarct area. Additionally, the site was filled with numerous large vessels as evidenced by well-formed medial layers surrounding the intima. In 10d and 14d PT tissue, both arterial and venous vessels had formed as evidenced by the thickness of medial and adventitial layers surrounding the intima. Control vessels in an area of uninjured tissue (Fig. 3A) showed well-formed medial layers.

*Transition from vascular chord formation to remodeling is demonstrated by PECAM and smooth muscle α-actin double-label immunohistochemistry.*

Platelet endothelial cell adhesion molecule (PECAM) and smooth muscle α-actin (SMA) immunohistochemistry (Fig. 3D-F; 4C-D) revealed distinct labeling of endothelial cells by PECAM and both pericytes and smooth muscle cells by SMA. At 4d PT (Fig. 3E), the central area of the infarct was essentially void of PECAM labeling; however, numerous PECAM-labeled vascular chords were visible in the periphery of the infarcted region. In addition, diffuse SMA-staining in the central area of the infarct may be
Figure 3. Evidence of vascular growth in response to microcauterizer-treatment at 4d and 7d PT. Gomori trichrome stain of infarcted Tie2LacZ mouse heart tissue (B-C) delineates the infarct with collagen in the infarcted area staining light blue and uninjured muscle staining dark red; nuclei are dark purple. Double-label immunohistochemistry for smooth muscle α-actin (SMA; red) and PECAM (green) labels smooth muscle cells/pericytes and endothelial cells, respectively (D-F). Representative control vessels in non-infarcted tissue show strong labeling with both PECAM and SMA (D); the corresponding trichrome-stained section is shown (A). Vascular chord formation is evident in the periphery at 4d PT (E) with expansion of the vascular response into the center of the infarcted area by 7d PT (F). Arrowheads indicate vascular chords and microvessels characteristic of the growth response. Arrows designate vessels with intimal and medial layers labeled with PECAM and SMA, respectively. Asterisk (*) indicates reference vessel for comparison to ANG-2 and phospho-Tie2 immunostaining. Representative images are shown. Bar = 50μm
myofibroblasts. By 7d PT (Fig. 3F) expansion of PECAM-labeled vascular chords and/or small caliber blood vessels into the central area of the infarct was evident. At 10d post-infarction (Fig. 4C), large vessels were common throughout the area with stabilization of the intima by a SMA-labeled medial layer that varied in thickness and intensity of staining. A small number of PECAM-labeled microvessels remained in the periphery of the infarct and had assumed a rounded appearance as compared to the vascular chords seen at 4d PT. The infarct tissue harvested 14d PT (Fig. 4D) was predominated by large caliber vessels. A well-defined intimal layer stained with PECAM and a developing medial layer consisting of tightly packed cells arranged radially was evident. However, SMA staining of the medial layer was diffuse compared to mature control vessels (Fig. 3D). A majority of large vessels seen at both 10d and 14d PT exhibited an irregular border demonstrating punctate SMA-labeling as well as weakened PECAM-labeling when compared to peripheral microvessels and a small number of large vessels in the same infarct.
Figure 4. Transition to vascular remodeling by 10d and 14d post microcauterizer-treatment. The infarct is delineated by Gomori trichrome stain of murine heart tissue (A-B). Collagen in the site stains light blue, nuclei dark purple, and muscle dark red. Double-label immunohistochemistry for smooth muscle α-actin (red) and PECAM (green) labels smooth muscle cells/pericytes and endothelial cells, respectively (C-D). Large caliber vessels are evident throughout the infarcted area at both time points. PECAM-staining defines the intima whereas medial layers not yet fully established are characterized by SMA-labeling of varying intensity. Arrows indicate vessels with a well-formed intimal layer and a medial layer demonstrating SMA staining. Arrowheads designate vessels showing weakened PECAM labeling as well as irregular borders suggestive of vascular regression. Representative images are shown. Bar = 50μm.
mRNA AND PROTEIN EXPRESSION RESULTS

**ANG-1, ANG-2, Tie2, and VEGF mRNA expression**

In order to better understand the molecular regulation of the morphological changes associated with vascular growth and remodeling, mRNA analysis was performed. The results of real-time RT-PCR analyses for ANG-1, ANG-2, Tie2, and VEGF expression are shown in Fig. 5 (ANG-2, VEGF) and Fig. 6 (ANG-1, Tie2). Comparisons between non-surgical control (NSC) and microcauterized (MC) groups were carried out using one-way ANOVA followed by the Dunnett *post hoc* test. Two-way ANOVA followed by the Tukey test for multiple comparisons was used to determine statistical differences between surgical control and microcauterizer groups at a given time-point or within the microcauterizer group. A summary of statistically significant differences (p<0.05) resulting from pairwise comparisons is listed in Table 3.

No statistically significant differences were seen when comparing surgical control (SC) to infarcted (MC) tissue at any time point for any of the targets measured. A biphasic pattern was evident in ANG-2 expression of the MC groups with increases in message at 4d and 10d post-treatment (PT) when compared to NSC and 1d PT tissue. Only the 4d group demonstrated a significant increase when compared to the 7d and 14d PT groups. VEGF mRNA levels differed from NSC as well as 1d MC at the 4d and 10d post-MC time points. ANG-1 mRNA levels were significantly different in the MC group with an increase at 1d and a decrease at 14d when compared to NSC. In addition, ANG-1 displayed a similarly biphasic pattern of expression in the MC group with significant differences in mRNA at 1d, 4d, and 10d PT when compared to 7d and 14d time points.
An increase in Tie2 message was measurable at 10d post-MC when compared to NSC as well as 7d and 14d MC groups.

**Western Blot Method Development**

In an attempt to characterize the regulatory mechanisms of angiogenesis and vascular remodeling at the protein level, Western blot analyses and ELISA (for VEGF only) were carried out. A standard Western Blot protocol previously used in the Coffin laboratory was followed for initial testing of antibodies against ANG-1, ANG-2, Tie2, and phospho-Tie2; antibodies from two different manufacturers were tested for ANG-2 and Tie2. The results demonstrated specific detection of ANG-1, ANG-2, and phospho-Tie2; however, non-specific binding was high on both ANG-2 and phospho-Tie2 blots. In addition, Tie2 was not detected. Consultation with technical support specialists from the manufacturers of the ANG-2, Tie2, and phospho-Tie2 antibodies resulted in the following recommendations:

1) Dilute antibodies in Tris-buffered saline (TBS) only without dry milk or Tween-20 because the detergent may cause the formation of micelles, which may interfere with antibody binding

2) Consider testing a different secondary antibody for one of the Tie2 antibodies

3) Consider increasing the concentration of dry milk for blocking and antibody dilution

Each of these recommendations was tested individually. Dilution of the antibodies in either TBS or TBST without milk resulted in significantly higher background with no improvement in specific signal. Similarly, a higher concentration of milk/TBST resulted
Figure 5. Real-time RT-PCR analyses of ANG-2 and VEGF mRNA expression demonstrated significant (p<0.05) increases in both ANG-2 and VEGF at 4d and 10d PT compared with NSC (indicated by *). Additional statistically significant differences (p<0.05) were seen when comparisons were made within the MC group (Table 3). Raw values were normalized to HPRT and expressed as percentage of NSC +/- SEM (n = 8-9 per sub-group). Hyphenated (----) line indicates NSC (baseline) expression. MC = microcauterizer-treated, SC = surgical control, NSC = non-surgical control, d = days post-treatment
Figure 6. Real-time RT-PCR analyses of ANG-1 and Tie2 mRNA expression relative to NSC demonstrated significant (p<0.05) increases in ANG-1 and Tie2 at 1d and 10d PT respectively and a decrease in ANG-1 at 14d PT (indicated by *). Significant differences in ANG-1 or Tie2 message were also noted when comparisons were made within the MC group (Table 3). Raw values were normalized to HPRT and expressed as percentage of NSC +/- SEM (n = 8-9 per sub-group). Hyphenated (—) line indicates NSC (baseline) expression. MC = microcauterizer-treated, SC = surgical control, NSC = non-surgical control, d = days post-treatment
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Table 3. Summary of pairwise comparisons carried out for determination of statistical significance. Comparisons between non-surgical control and microcauterizer-treated subgroups were made by one-way ANOVA followed by the Dunnett post hoc test. Two-way ANOVA followed by the Tukey test for multiple comparisons was carried out to determine differences between surgical control and microcauterizer-treated groups at a given time-point or between the microcauterizer-treated sub-groups. An asterisk (*) indicates a statistically significant (p<0.05) difference when comparing sub-group A and sub-group B.
in slightly higher background than the standard concentration of 5% milk/TBST. In
addition, Tie2 remained undetectable with any of the secondary antibodies tested.

Upon further consultation, Pharmingen (maker of one of the Tie2 antibodies)
technical support suggested that the transfer step may be inadequate; Pharmingen
recommended an equivalent of 1 Amp for 90 min with a transfer buffer containing 0.05%
SDS. The extended transfer time (compared to the standard protocol originally used) and
addition of 0.05% SDS to the transfer buffer were tested independently. Side-by-side
comparison of blots transferred at 100V for 5 h to blots transferred at the standard 100V
for 2 h demonstrated a specific band corresponding to the Tie2 protein without non-
specific binding only with the 5 h transfer. In addition, the increased transfer time
combined with further dilution of the antibodies resulted in elimination or improvement
of non-specific binding for ANG-2 and phospho-Tie2, respectively. Comparison of the
extended transfer to a transfer at 25V overnight in the cold showed no difference.
Addition of 0.05% SDS to the transfer buffer (which can facilitate detection of high
molecular weight proteins) resulted in improvement of ANG-2 and Tie2 signals.
Furthermore, Coomassie stain of protein gels post-transfer showed numerous proteins
remaining in the gel transferred without SDS and virtually no protein in the gel
transferred with SDS. However, the addition of SDS was detrimental to the quality of the
ANG-1 and phospho-Tie2 signals. Therefore, Western blotting for all proteins utilized a
5 h 100V transfer with the addition of 0.05% SDS to the ANG-2 and Tie2 transfer buffer
and no SDS in the ANG-1 and phospho-Tie2 transfer buffer.

In an effort to further reduce non-specific signal detected by the phospho-Tie2
antibody, blocking of the membrane and dilution of the antibodies was carried out in 10%
BSA/TBST rather than 5% milk/TBST. The modification resulted in improved signal intensity with a relatively minor increase in non-specific binding. Use of 10% BSA/TBST for ANG-2 and Tie2 blots resulted in loss of specific signal and an increase in background without a significant increase in specific binding, respectively. Therefore, membrane blocking and dilution of antibodies was carried out in 10% BSA/TBST for phospho-Tie2 blots and 5% milk/TBST for ANG-1, ANG-2, and Tie2 blots.

Following final optimization of antibody dilutions using the transfer and blocking/dilution conditions described above, an initial set of ANG-1 and phospho-Tie2 blots was run on two sets of pooled tissue. In addition to ANG-1 and phospho-Tie2, the blots were probed with anti-actin to establish equal loading. Densitometric analysis of the actin bands demonstrated appreciable changes between the sub-groups. Later investigation showed that the anti-actin antibody that had been used detected all forms of actin rather than β-actin alone. The other housekeeping gene commonly used as a loading control, GAPDH, was eliminated as a candidate due to its regulation by hypoxia inducible factor (HIF)-1. Upon further consultation, β-tubulin was selected for testing. At a very high antibody concentration, only a faint band corresponding to β-tubulin was detected, therefore use of the protein as a loading control was not feasible.

The next alternative considered was Ponceau staining for total transferred protein; Ponceau staining of the membrane is the equivalent of Coomassie staining of a gel. Ponceau staining was attempted on several test blots but proved ineffective because wetting the blot to obtain an image for quantification caused rapid fading of the protein bands. The final method tested for assessment of equal loading of total protein was MemCode™ Reversible Protein Stain commercially available from Pierce. MemCode™

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is a total protein stain similar to Ponceau but is considered more sensitive and does not exhibit the problematic fading associated with Ponceau stain. In practice, bands designating proteins detected by the MemCode stain faded but did so less rapidly than Ponceau stain allowing sufficient time to capture an image. However, numerous methods of densitometric analysis resulted in highly variable results suggesting that the utility of MemCode™ as a loading control is questionable. Because of the inability to identify a reliable loading control for the current experiments, the data were considered to be more representative without normalization to a loading control. Repeated protein assays on a proportion of the samples to confirm protein concentrations, as well as previously established accuracy of pipetting technique, facilitated establishment of equal loading without the use of a true loading control.

**Analysis of ANG-1, ANG-2, Tie2, phospho-Tie2, and VEGF protein levels**

VEGF ELISA results and Western blot analyses for ANG-1, ANG-2, Tie2, and phospho-Tie2 are depicted in Fig. 7 (ANG-1, ANG-2), Fig. 8 (Tie2, phospho-Tie2), and Fig. 9 (VEGF). No statistically significant differences were seen when comparing SC to MC tissue at any time point for any of the proteins assayed. In addition, no significant change in protein expression was noted for ANG-1, Tie2, phospho-Tie2, or VEGF proteins. Determination of the ratio of phospho-Tie2 to Tie2 protein for each sub-group also showed no change across the time course. Although not statistically significant, ANG-2 demonstrated a steady decrease in protein expression from 1d through 14d post-treatment. A second group of blots could not be analyzed statistically due to inadequate sample number but showed similar trends to the data presented. The lack of changes in
expression for any of the proteins studied was in contrast to the significant differences demonstrated by mRNA analysis when comparing between NSC and MC sub-groups and within the MC group.

*Immunostaining Method Development*

To assess possible changes in protein expression patterns that may not have been reflected in the quantitative data, immunostaining of tissue sections was attempted. A standard immunostaining protocol previously used in the Coffin laboratory was followed for initial testing of antibodies against ANG-1, ANG-2, Tie2, and phospho-Tie2 in tissue fixed in Tris-buffered zinc fixative (Tris/zinc). With the exception of staining of red blood cells (RBCs) in all tissue sections, no-primary controls for ANG-2 and phospho-Tie2 demonstrated a lack of non-specific staining; however, goat anti-human ANG-1 and mouse anti-human Tie2 antibodies showed significant levels of background. Tissue immunostained for either antibody had been blocked with normal horse serum (NHS); therefore, a new lot of NHS was diluted and tested. Non-specific staining was evidenced with serum from either batch suggesting that the blocking step was not the cause of the background staining.

The anti-Tie2 tested was a mouse monoclonal antibody, which suggested that binding of the secondary antibody to endogenous mouse immunoglobulin may have resulted in non-specific staining. Therefore, a commercially available mouse-on-mouse blocking kit was tested with the anti-Tie2 primary antibody. While treatment of the tissue to block mouse immunoglobulin did reduce background, non-specific staining was
Figure 7. Western blot analyses of ANG-1 and ANG-2 demonstrated no temporal change in protein expression. Densitometry values were normalized to NSC from the same blot prior to calculating mean and expressed as percentage of NSC +/- SEM (n = 4).

Statistically significant differences were not seen when comparing SC and MC groups at a given time-point or MC groups across the time course (p<0.05). Hyphenated (----) line indicates NSC (baseline) expression. MC = microcauterizer-treated, SC = surgical control, NSC = non-surgical control, d = days post-treatment
Figure 8. Western blot analyses of Tie2 and phospho-Tie2 showed no temporal change in protein expression. Densitometry values were normalized to NSC from the same blot prior to calculating mean and expressed as percentage of NSC +/- SEM (n = 4). Statistical differences were not seen when comparing SC and MC groups at a given time-point or MC groups across the time course (p<0.05). Hyphenated (---) line indicates NSC (baseline) expression. MC = microcauterizer-treated, SC = surgical control, NSC = non-surgical control, d = days post-treatment
FIGURE 9

[Diagram showing VEGF protein level (% of NSC) over timepoints (NSC, 1d, 4d, 7d, 10d, 14d) for SC and MC conditions.]
Figure 9. VEGF protein expression showed no change over time when assessed by ELISA. Values were expressed as percentage of NSC +/- SEM (n = 4). Statistical differences were not seen following multiple comparisons (p<0.05). Hyphenated (----) line indicates NSC (baseline) expression. MC = microcauterizer-treated, SC = surgical control, NSC = non-surgical control, d = days post-treatment
not eliminated nor was specific staining detected. A biotinylated anti-Tie2 was also
tested on the Tris/zinc fixed tissue and demonstrated no specific staining.

Antigen-retrieval techniques were not performed as part of the ANG-1 and Tie2
protocols; however, the relatively fragile nature of the Tris/zinc fixed tissue compared to
paraformaldehyde fixed tissue precluded the use of pepsin & trypsin treatments longer
than 3 min or citrate/heat treatment. In addition, previous immunostaining of
paraformaldehyde fixed tissue with the ANG-1 goat anti-human antibody showed no
staining with or without antigen retrieval. Testing of a second ANG-1 antibody on
Tris/zinc fixed tissue also demonstrated no specific staining.

Diaminobenzidine-only treatment of Tris/zinc fixed tissue sections showed
reactivity of RBCs suggesting that the standard peroxidase-blocking treatment was
inadequate. A battery of peroxidase-blocking techniques was tested and treatment of
tissue sections overnight in 0.3% H2O2/40% MeOH in PBS was found to successfully
block the endogenous peroxidase activity of the red blood cells; the addition of this
blocking step to the immunostaining protocol eliminated non-specific staining
(previously seen in RBCs) in no-primary controls for ANG-2 and phospho-Tie2.
Unsuccessful peroxidase-blocking techniques included 3% H2O2/ H2O for 10 min, 0.3%
H2O2/MeOH for 20 min, 100% EtOH followed by 0.075% HCl for 15 min, and 0.01%
periodic acid for 10 min followed by 0.1 mg/ml sodium borohydride for 2 min. ANG-2
and phospho-Tie2 were carried out following the standard protocol with the replacement
of the 10 min 3% H2O2/H2O treatment with incubation overnight in 0.3% H2O2/40%
MeOH in PBS.

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Angiopoietin-2 Immunohistochemistry

Angiopoietin (ANG)-2 immunohistochemistry on infarcted tissue (Fig. 10) showed no notable change in labeling within the wound site when comparing 4d, 7d, 10d, and 14d PT hearts. Positive cells were visible within the infarct at each time point; however, no change in the pattern of expression was clearly discernible. In addition, no vessels within the wound site demonstrated positive labeling around the entire circumference of the vessel at any time-point. Similar staining of cells and vessels was seen in non-infarcted tissue (at all time points) and NSC. ANG-2 immunostaining showed no appreciable differences in staining patterns within or between groups consistent with the results of Western blot analysis for the protein.

Phospho-Tie2 Immunostaining

Immunostaining for phosphorylated Tie2 (Fig. 11) revealed distinct patterns of expression in the infarcted region at each of the time points studied. At 4d PT (Fig. 11B) numerous cells, microvessels, and vascular chords exhibited positive staining for phospho-Tie2 in the periphery of the injured tissue; however only a few positive cells or microvessels were seen in the center of the infarct. Examination of 7d PT tissue (Fig. 11C) showed immunostaining resembling that of the 4d tissue, however positively stained vessels were seen throughout the wound site. In addition, anti-phospho-Tie2 positive vessels of increasing diameter were also noted. At 10d post-infarction (Fig. 11D), staining was seen throughout the infarcted area with vascular chords, microvessels, and vessels of small diameter demonstrating the most intense reactivity. Large-caliber vessels within the infarct showed weak or no phospho-Tie2 reactivity. Phospho-Tie2
labeling of 14d infarcted tissue (Fig. 11E) revealed minimal staining of the large vessels that dominated the site; positive staining did not cover the entire circumference of the vessels and was often weak. Phospho-Tie2 staining of cells and vessels of various diameters, especially microvessels, was also seen in non-infarcted tissue (Fig. 11A) at all time points. In contrast to the quantitative Western data, phospho-Tie2 immunostaining demonstrated changes in expression between the time-points suggesting that the differences in patterns of expression may have resulted in no net change in phospho-Tie2.
FIGURE 10

A
SC
B
4d
C
7d
D
16d
E
F
14d (inset)

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Figure 10. Alternate serial sections were immunolabeled for angiopoietin (ANG)-2 at 4, 7, 10, and 14d post-microcauterizer-treatment. Comparison of staining from each time-point (B-E) showed no difference in expression pattern. Similar ANG-2 staining of cells and occasional vessels was seen in surgical control (A) and non-surgical control (not shown) tissue. Arrows indicate ANG-2 positive cells; arrowheads designate vessels positive for ANG-2. Asterisk (*) indicates reference vessel for comparison to trichrome staining. Infarct area in (C) is region below line; non-infarcted myocardium is above the line. Representative images are shown. 400X magnification; inset = 1000X magnification
Figure 11. Immunolabeling of infarcted tissue for phospho-Tie2 demonstrated differences in expression pattern between the time-points. Numerous positively-labeled microvessels and chords are seen at 4 (B), 7 (C), and 10d (D) PT with progression of the vessels from the periphery at 4d post-infarction into the center of the infarct area by 7d PT. Large-caliber vessels at 10 and 14d (E) PT indicate weak phospho-Tie2 labeling. Phospho-Tie2 staining of microvessels, chords, cells, and partial staining of larger vessels was also seen in surgical control (A) and non-surgical control (not shown) tissue. Arrows indicate phospho-Tie2 positive microvessels, vascular chords, and cells. Arrowheads designate vessels demonstrating relatively weak phospho-Tie2 immunoreactivity. Asterisk (*) indicates reference vessel for comparison to trichrome staining. Infarct area in (C) is region below line; non-infarcted myocardium is above the line. Representative images are shown. 400X magnification; inset = 1000X
DISCUSSION

Cardiovascular disease is the cause of death of almost 1 million individuals in the United States each year. Reduction in blood flow due to coronary artery disease results in deprivation of oxygen and nutrients to the myocardium causing severe tissue damage. The development of novel and effective therapeutic angiogenesis strategies is contingent upon understanding the regulation of angiogenesis and vascular remodeling in the adult heart. Previous research has demonstrated that the mechanisms regulating these processes are complex and multi-factorial, though not clearly defined. Furthermore, models attempting to characterize the roles of angiogenic regulatory molecules often fail to place these factors within a morphologically defined model of vascular growth and remodeling. Placement of regulatory data within the framework of a well-characterized angiogenic model will significantly strengthen its contribution to the design of rational therapeutic strategies. The overall objectives of this study were to characterize the transition between angiogenesis and vascular remodeling during tissue remodeling in the ischemic murine heart and establish temporal expression patterns of the angiogenic regulatory molecules ANG-1, ANG-2, Tie2 receptor, VEGF, and the phosphorylated Tie2 receptor within the context of a model of vascular growth and remodeling.

Angiogenesis and Vascular Remodeling in the Infarcted Murine Heart

The ability to morphologically assess the complete angiogenic response, comprised of both growth and remodeling phases, is a key factor in determining the

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regulatory mechanisms of each aspect of the process. Tie2LacZ transgenic mice provide an attractive experimental system for histologically defining angiogenic growth and remodeling. Microcauterizer-treatment of the heart to generate a myocardial infarction serves as a means of stimulating a complete angiogenic response. Tissue remodeling associated with the microcauterizer-generated infarct is consistent with that evidenced in a murine model of myocardial infarction induced by permanent left anterior descending coronary artery occlusion [76]. Whole mount β-galactosidase staining of the tissue provides a global view of morphological changes in the vasculature. Unlike previous systems using fluorescein-dextran perfusion of the whole mount heart [75], the Tie2LacZ model allows detection of microvessels surrounding the infarct as early as 1d PT with growth most readily visible at 4d and 7d PT. In addition, early signs of vascular remodeling are evident at the 7d time-point in contrast to the fluorescein-perfused heart in which an apparently avascular area was noted at 7d PT. The Tie2LacZ model shows gross evidence of remodeling at 10d and 14d PT with the presence of a substantial number of larger vessels traversing the infarct area. In addition, the presence of larger remodeled vessels and a small number of fine microvessels at 10d PT, suggests a transitional phase between growth and remodeling.

The whole mount data is further substantiated by PECAM and smooth muscle α-actin (SMA; for smooth muscle cells and pericytes) labeling of infarcted tissue from each time point. Bluo-Gal staining was determined to be the preferred method for whole mount characterization of the tissue; however, based on preliminary studies and a published report which found PECAM immunohistochemistry to be superior to β-galactosidase staining of sectioned cardiac tissue [91], the former method was selected.
for the current study. The appearance of PECAM-labeled vascular chords in the periphery of the 4d PT infarct is consistent with the early Bluo-Gal stained capillary plexus surrounding the infarcted region. Furthermore, the broadening of the vascular zone around the damaged area and initial appearance of larger vessels at 7d PT is consistent with the expansion of PECAM-stained microvessels into the central area of the infarct. The large Bluo-Gal stained vessels crossing the infarcted area at 10d PT correspond with the relative increase in large caliber, PECAM-labeled vessels within the site, which in addition to SMA staining surrounding the vessels, suggests a transition to early maturation and stabilization of the neovasculature. Additionally, the decreased PECAM-staining of large vessels and irregular vascular borders seen at 10d and 14d PT raises the possibility that vascular regression may also be taking place as part of the remodeling process. Regression is believed to occur in part through endothelial cell apoptosis [7, 11], which would result in an apparent reduction in PECAM-staining. Remodeling of infarct-specific neovasculature is most predominant at 14d PT as evidenced by the large Bluo-Gal stained vessels traversing the site as well as the prevalence of double-labeled vessels when studied in cross-section.

The proposed time course for angiogenesis and vascular remodeling is consistent with a report by Sephel et al in a study of basement membrane components in wound angiogenesis [93]. In the space-filling sponge implant model, capillary growth was observed between days 3 and 5 with maturation by day 12, as indicated by lumen diameter. Furthermore, a maturation phase extending from 14 days post-occlusion was described in a rabbit hind-limb model of collateral growth [94]. While the model proposed by Scholz et al delineates the cellular and proteolytic activity taking place
during the maturation phase, it does not describe the remodeling process in terms of stabilization of vascular structure. A number of other models also describe the expression of a variety of molecular signals and the activity of specific cell types, including endothelial and smooth muscle cells, over a period of time [95-98]. However, while these models suggest a time course for the angiogenic process, little emphasis is placed on the importance of vascular remodeling in this process.

Remodeling results in the pruning, stabilization, and maturation of the vascular network. To this point, the focus of therapeutic angiogenesis strategies has remained primarily on the stimulation of new blood vessel growth. While an increase in the number of vessels may be a necessary element of collateral circulation formation, the remodeling process has been a frequently overlooked component that may in fact be essential to the development of effective angiogenic therapies in the heart. Gene therapy using angiogenic factors to stimulate coronary angiogenesis has become a highly desirable means of treating cardiovascular disease. However, in order to provide long-term benefits, therapeutic angiogenesis strategies must result in patent, functional vessels that increase the overall perfusion of the affected area. Using Tie2LacZ mice, we have described the morphological changes characteristic of angiogenesis and the transition to vascular remodeling. This model allows the ability to selectively study time-points demonstrating desired characteristics of growth or remodeling \textit{in vivo} enabling a more focused analysis of the regulatory mechanisms controlling a specific aspect of the overall process. For example, a focus on the 4d time-point would enable a better understanding of the specific cellular mechanisms involved in regulating the formation of new vessels on the periphery of a myocardial infarction prior to scar formation.
mRNA Expression of ANG-1, ANG-2, Tie2, and VEGF

Existing therapeutic angiogenesis strategies have focused primarily on the VEGF and fibroblast growth factor families, due largely to their ability to stimulate angiogenesis in animal models and in vitro. A number of gene and protein therapy studies utilizing these factors have demonstrated increases in perfusion and improvement in angina or objective measures of function. Nonetheless, side effects, such as edema, and yet to be answered questions about long-term stability of the neovasculature have led to the hypothesis that a single growth factor may not be adequate to stimulate the development of a stable, functional vasculature capable of effectively perfusing the target tissue. As a result, additional factors, including ANG-1 and HIF-1α, are under investigation. In addition to other favorable characteristics, in vivo models utilizing either factor have stimulated a vascular response without the leaky vessels attributed to VEGF-stimulation of vascular permeability [71, 72]. However, the selection of these factors for therapeutic angiogenesis has been based largely on knowledge gained from non-cardiac systems, such as embryonic development, the skin, and in vitro studies. Knowledge of mechanisms regulating angiogenesis and vascular remodeling in the mammalian heart will facilitate the refinement of therapeutic strategies for myocardial ischemia.

In order to better understand the molecular regulation of the morphological changes associated with vascular growth and remodeling, real-time RT-PCR was performed to assess ANG-1, ANG-2, Tie2, and VEGF mRNA levels. The real-time data suggest a possible role for the ANG/Tie2 system and VEGF in establishment and maturation of the neovasculature. Comparison of infarcted tissue to NSC showed significant increases in ANG-2, Tie2, and VEGF mRNA at 10d PT corresponding to the
transition to remodeling demonstrated morphologically. A rise in ANG-1, although not achieving statistical significance, was also evident at the 10d time-point. In addition, significant increases in ANG-2 and VEGF were also demonstrated at 4d PT suggesting a potential role for these factors in the growth of the neovasculature. These data suggest that the ANG/Tie2 and VEGF systems could be involved in coordinately regulating the growth and later stabilization of the newly formed vasculature.

A coordinate role for ANG-2 and VEGF in vascular growth and/or remodeling has been previously suggested based on a variety of evidence. Coexpression of ANG-2 and VEGF has been noted at sites of angiogenesis, including the corpus luteum and vascularized tumors [48, 65]. In addition, stimulation of ANG-2 expression by VEGF has been demonstrated in vitro [47]. In vivo administration of ANG-2 with VEGF results in a proangiogenic effect in corneal micropocket and pupillary membrane models [54, 66]. Furthermore, ANG-2 and VEGF protein levels peaked at 3d post-treatment in a canine model in which animals were subjected to repetitive episodes of myocardial ischemia by brief occlusion of the left anterior descending artery (LAD) [77]. The similar biphasic expression patterns of ANG-2 and VEGF mRNA noted in the current study combined with evidence from prior studies provides support for a role of these factors in the vascular response to cardiac ischemia.

A critical concern regarding the RNA expression data is the lack of significant differences between microcauterizer-treated and surgical control tissue at any time-point for any of the factors studied. The morphological changes described provide clear evidence that a difference does exist between the microcauterizer-treated and surgical control tissue. The essential question is whether changes in expression between NSC and

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MC groups or between time-points within the MC groups are relevant in light of the surgical control data.

Existing evidence suggests that hypoxia, which has been shown to stimulate VEGF, ANG-2, and Tie2 expression in vitro, may be induced by thoracotomy alone [99]. In addition, the thoracotomy itself is likely to trigger a wound-repair response, which includes angiogenesis, as a result of the incisions made to the skin and muscle. While growth factors are known to act on a local, rather than systemic, level, it is conceivable that activation of cytokines and other circulating factors by thoracotomy, or more specifically the wound healing response, may result in up- or down-regulation of local angiogenic factors elsewhere in the thorax.

Additional evidence suggests that an increase in growth factor expression alone is not sufficient to stimulate vascular growth. Intramuscular gene transfer of VEGF DNA into ischemic limb resulted in neovascularization only in the ischemic limb in spite of elevated serum VEGF levels and edema in the non-ischemic limb [69]. These data suggest that a local hypoxic stimulus, or other insult, must be coupled with modulation of growth factor expression to trigger an angiogenic response; this information is consistent with the lack of vascular response in the SC animals in spite of elevation of growth factor levels over NSC. Therefore, changes in mRNA expression, potentially resulting from thoracotomy, may integrate with biochemical changes triggered by microcauterizer-treatment of the heart to initiate the vascular response evidenced morphologically. The following experimental plan is designed to elucidate a potential mechanism for the up-or down-regulation of angiogenic factors in the heart following thoracotomy.
Experimental Plan for Analysis of the Affect of Thoracotomy Treatment

The quantitative mRNA and protein data presented suggest that the surgical control treatment stimulates changes in expression of angiogenic factors in the heart. Opening of the chest wall causes a wound healing response characterized by both inflammation and angiogenesis. This response is believed to stimulate circulating factors, such as cytokines, which may cause downstream effects in the cardiac tissue. Based on previous studies demonstrating stimulation of angiogenesis and/or regulation of angiogenic factors [79, 100-102], TNF-α, IL-6, and IL-8 are candidates for modulating the expression of angiogenic factors in response to thoracotomy. The proposed aims will test the hypothesis that thoracotomy initiates a change in angiogenic factor expression in the heart by stimulation of circulating cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-8.

**Specific Aim I:** Establish protein expression profiles of circulating TNF-α, IL-6, and IL-8 following thoracotomy. Tie2LacZ transgenic mice will undergo thoracotomy only, and serum will be collected prior to thoracotomy and at 1, 4, 7, and 10 days post-treatment prior to sacrifice at day 10; serum will also be collected from age-matched non-surgical control mice. ELISA will be used to measure serum protein levels of each cytokine. A significant increase or decrease in serum levels of any of the cytokines studied compared to non-surgical mice will suggest that thoracotomy treatment stimulates inflammatory response involving said factor; such factors will be investigated further in Aim II. No change in the expression profile of TNF-α, IL-6, or IL-8 suggests that these factors are not stimulated in the circulation following thoracotomy but does not preclude the involvement of other circulating cytokines.
**Specific Aim II:** *Determine the effect of cytokines identified in Aim I on VEGF, ANG-1, ANG-2, and Tie2 mRNA levels in vitro.* Real-time RT-PCR for VEGF, ANG-1, ANG-2, and Tie2 will be used to quantify changes in mRNA expression following treatment of a vascular smooth muscle cell/endothelial cell co-culture with recombinant cytokine (i.e. TNF-α); cytokine(s) demonstrating up- or down-regulation in the serum of animals from Aim I will be assessed in vitro. Subsequently, angiogenic factor expression will be assessed following administration of cytokine and anti-cytokine receptor antibody to block signaling of the protein. TNF-α, IL-6, or IL-8 is expected to stimulate an increase in mRNA expression of VEGF, ANG-1, ANG-2, and Tie2 in smooth muscle and endothelial cell co-culture. Addition of anti-cytokine receptor antibody is expected to block the increase demonstrating that the change in mRNA is cytokine-dependent. An increase in angiogenic factor expression in spite of the administration of blocking antibody will suggest that the change is cytokine-independent and may in fact be due to up-regulation of one of the other angiogenic factors.

**Specific Aim III:** *Assess the effect of disruption of cytokine signaling on ANG-1, ANG-2, VEGF, and Tie2 mRNA levels in vivo.* Investigation of cytokines in Aim II will be continued in Aim III. Prior to thoracotomy treatment, Tie2LacZ transgenic mice will be injected systemically with anti-cytokine receptor antibody; non-surgical controls will also be injected with the blocking antibody. In addition, a species-specific isotype control antibody will be injected prior to thoracotomy treatment to ensure the effects seen in the primary treatment group are not caused by immunoglobulin molecules. Serum will be collected from all groups prior to antibody injection and at 1, 4, 7, and 10 days post-injection to monitor circulating cytokine levels. ELISA will be used to assess serum...
protein levels of the cytokine. In the thoracotomy and control groups receiving the blocking antibody, a decrease in circulating cytokine is likely to result from autocrine feedback mechanisms. No change in cytokine expression should be seen in the isotype control group. At 10 days post-injection, heart tissue will be harvested from all groups, and ANG-1, ANG-2, VEGF, and Tie2 mRNA levels assessed by real-time RT-PCR. No change in ANG-1, ANG-2, VEGF, or Tie2 mRNA levels is expected in the hearts of animals receiving blocking antibody prior to thoracotomy treatment upon comparison to non-surgical controls. Messenger RNA levels of angiogenic factors in the heart should be stimulated in the isotype control as seen in prior experiments. A change in angiogenic factor expression in the heart following thoracotomy, in spite of disruption of signaling of the targeted cytokine, would suggest that one or more different circulating factors may act to initiate downstream changes in the heart or that the mechanism for modulation of angiogenic factors following thoracotomy is cytokine-independent.

ANG-1, ANG-2, Tie2, and Phosphorylated Tie2 Protein Analysis

In an attempt to characterize the regulatory mechanisms of angiogenesis and vascular remodeling at the protein level, ELISA for VEGF and Western blot analyses for ANG-1, ANG-2, Tie2, and the phosphorylated Tie2 receptor were carried out. However, the changes in expression evident by real-time RT-PCR were not reflected in the protein data. While the use of the NSC as a blot-to-blot normalization standard precluded statistical comparison of Western blot data from microcauterized samples and NSC, graphical interpretation of the data suggested no difference. Furthermore, comparisons within the MC group showed no significant differences suggesting the concept that
neither the VEGF nor the ANG system is involved in the modulation of vascular growth and remodeling associated with the microcauterizer-generated infarct.

Several possible explanations may account for the disparity between the RNA and protein data. First, the growth factors analyzed may not play the predominant role in this system. While a broad range of \textit{in vivo} and \textit{in vitro} studies provides support for the involvement of the VEGF and ANG/Tie2 systems in vascular growth and remodeling, previous cardiovascular studies have not provided consistent evidence for a clearly defined role of any of these factors or receptors in the formation and remodeling of vasculature in the adult heart. The likelihood that the role of these factors is system and context dependent is highlighted by the disparity in results when comparing similar cardiovascular models. The canine repetitive occlusion model demonstrated an increase in ANG-1 mRNA at 21d of repetitive occlusion with a decrease in ANG-2 at the same time-point [77]. In contrast, a rat model of permanent LAD occlusion showed no change in ANG-1 mRNA but an increase in ANG-2 at three weeks post-infarction [78].

In addition, an essential difference between the current study and the occlusion models described is the use of time-point surgical controls. Both Matsunaga et al and Shyu et al compared infarcted tissue to a single time-point surgical control rather than a control at each time-point [77, 78]. As seen in Fig. 5, 6, and 9, apparent differences in expression between time-points and between NSC and MC groups are raised into question when the SC for that time-point is considered. Therefore, the possibility exists that the interpretation of the data from Matsunaga et al and Shyu et al may have been altered if time-point surgical controls had been included in the studies.
An alternative explanation for the lack of detectable changes in protein expression over time is the limitations of the methods used to quantify such differences. While the VEGF ELISA may detect as little as 5 pg VEGF per 1 ml total protein, the technique specifically measures only the VEGF-164 isoform. A previous study using real-time RT-PCR to detect total VEGF mRNA and the VEGF-164 ELISA showed an increase in total mRNA expression compared to non-transgenic littermates, but no corresponding increase in VEGF protein expression [71] consistent with the current study. Additional studies have demonstrated differential regulation of the VEGF isoforms [103-105]. Together, these findings suggest that assessment of only VEGF-164 protein may not reflect changes in total VEGF protein expression. In addition, the sensitivity of Western blotting varies from protein to protein and is highly dependent on a number of factors, including transfer of proteins from gel to membrane and antibody specificity and sensitivity. For the ANG/Tie system, Western blot analysis may have limited utility as a quantitative method to detect small changes in protein expression.

Post-transcriptional regulation of the factors and receptor in question is a third possible explanation for the lack of change in protein levels. Little is known about the post-transcriptional regulation of any of the molecules investigated in this study. Hypoxia has been shown to mediate VEGF expression by increasing stability of the RNA [29, 30]. While previous evidence has demonstrated stimulation of ANG-2 and Tie2 by hypoxia, post-transcriptional regulation of the angiopoietins and their receptor is currently unknown.

Finally, the Western blot and ELISA analyses showed no quantitative differences in ANG-1, ANG-2, Tie2, phospho-Tie2, or VEGF protein. However, this does not
exclude the possibility that the proteins of interest may be expressed by only a small subset of cells, which may be diluted by excess tissue from within or outside of the infarcted region. This hypothesis was tested by immunohistochemical analysis of sectioned tissue containing the infarcted area.

**Immunohistochemical Analysis of ANG-2 and phospho-Tie2**

Equally important as the quantity of a given protein is the spatial distribution of that protein. In order to ascertain whether changes in protein expression across the time course were non-existent or not measurable by Western blot or ELISA, immunostaining was attempted for ANG-1, ANG-2, Tie2, and phospho-Tie2. Labeling for ANG-1 and Tie2 were unsuccessful with the antibodies tested therefore the tissue distribution of these proteins remains undetermined in this system.

While ANG-2 immunolabeling was successful, comparison of infarcted tissue within the MC group or against non-infarcted tissue showed no appreciable differences in staining patterns. These findings confirm the lack of measurable differences between SC and MC tissues by Western blot. The immunostaining data suggest that significant differences at the mRNA level seen when comparing within the MC group may not have been translated into protein changes due to post-transcriptional regulation. In addition, consistent with the observations of Ray et al [73], ANG-2 expression appears to be localized to the smooth muscle layer of vessels rather than the vascular endothelium. This finding suggests that information acquired from *in vivo* and *in vitro* studies demonstrating ANG-2 expression by endothelial cells may be of limited use in understanding the role of ANG-2 in the cardiac vasculature.
Consistent with prior data [43], vessels exhibiting positive immunolabeling for phosphorylated Tie2 were seen throughout the heart. However, the expression, most evident at 4d and 7d, of phospho-Tie2 by vascular chords and microvessels generated in response to microcauterizer-treatment suggests a further role for the Tie2 system in the vascular response to infarction. Previous in vivo data have suggested a role for ANG-1/Tie2 in maturation and stabilization of vessels by recruitment of perivascular support cells [53, 54]. However, the progression from angiogenesis to vascular remodeling evidenced morphologically suggests that microvessels seen at the 4d and 7d time-points are unlikely to be supported by pericytes or smooth muscle cells. While the ANG-1/Tie2 system may play a role in temporary stabilization of newly formed vessels, an alternative function may be the promotion of endothelial cell migration and tube formation. In vitro evidence has shown that treatment of ECs with ANG-1 results in both a chemotactic response [49, 50] and tubule formation [51]. Additionally, an anti-apoptotic effect has been demonstrated for ANG-1 acting via Tie2 [40, 49-52] suggesting that activation of the Tie2 receptor may protect newly formed vessels from undergoing premature regression prior to completion of remodeling.

Furthermore, the phospho-Tie2 immunostaining provides evidence that the localization of the protein and/or organization of cells expressing the activated receptor may prove of greater importance than the measurable quantity of the protein. As with ANG-2, the expression of phospho-Tie2 throughout the heart tissue may explain the absence of a quantitative difference in expression when microcauterized tissue was compared to surgical controls. However, there is a clear difference in the pattern of expression when examining the infarct of 4, 7, 10, and 14d tissue suggesting that
rearrangement of cells and/or vessels positive for activated Tie2 may result in no net change in phospho-Tie2. In addition, while ANG-1 has been shown to induce migration and EC survival via PI3-kinase pathways, the signaling pathways leading to putative perivascular cell recruitment and tube formation are currently unknown [106, 107]. This suggests the possibility that unknown permissive stimuli may result in a shift in the downstream signaling of the cell avoiding the need for an increase in the overall amount of ligand or receptor.

Revised Model for Regulation of Angiogenesis and Vascular Remodeling

The proposed model, shown in Fig. 12, has been refined to reflect the data obtained through these experiments. While Western blot and ELISA analyses showed no

![Diagram of Vascular Growth and Remodeling](image)

Figure 12. Revised regulatory model for vascular growth and remodeling. (artwork adapted from Yancopoulos et al [18])
changes in ANG-1, VEGF, and total Tie2 expression, the model indicates putative roles for ANG-1 and VEGF, which will be discussed in detail below. In addition, the role of Tie2 is based on the results of the phospho-Tie2 immunostaining, as the effects of the receptor are believed to require prior autophosphorylation.

While a small number of *in vitro* studies have demonstrated that ANG-2 may stimulate phosphorylation of Tie2 under unique conditions [48, 62, 63], immunostaining for ANG-2 suggests that is unlikely in this model given the distinct pattern of expression compared to phospho-Tie2. In addition, the ANG-2 Western blot and immunohistochemistry indicate that ANG-2 is not involved in destabilization of the existing vasculature as proposed, or any other aspect of the angiogenic response to microcauterizer-generated infarction at the time-points studied.

While VEGF ELISA analysis did not demonstrate differences in expression of the VEGF-164 isoform at any of the time-points studied, the possibility exists that the VEGF-120 or -188 isoforms may be elevated as a component of the vascular response. Prior evidence for differential expression of VEGF isoforms following myocardial infarction was provided by semi-quantitative RT-PCR analysis of rat cardiac tissue. Relative levels of the VEGF-120, -164, and -188 isoforms varied at the time-points studied, with VEGF-120 and -188 demonstrating greater change over time than VEGF-164 [105]. Furthermore, significant evidence suggests a role for VEGF in vascular formation.

The ability of VEGF to stimulate endothelial cell proliferation and migration has been extensively documented *in vitro* [19, 22]. In addition, numerous studies have shown changes in VEGF expression associated with myocardial ischemia/infarction [77, 78, 80].
Protein and gene therapy studies in myocardial and critical limb ischemia have routinely demonstrated increases in capillary density and improvement in hemodynamic measures in response to VEGF administration providing evidence that the growth factor can indeed stimulate neovascularization [69, 72, 81-85]. The studies described combined with the limitations of the methods used to detect VEGF protein expression in this study suggest that VEGF may still play a role in angiogenic growth.

While a change in ANG-1 protein expression was not quantifiable by Western blot analysis, this does not preclude a role for the growth factor in the vascular growth and remodeling evidenced morphologically. As previously discussed, ANG-1 is the only known ligand for Tie2 capable of consistently stimulating autophosphorylation of the receptor. This strongly suggests that ANG-1 immunostaining would mimic the patterns of phospho-Tie2 expression seen in the microcauterized and control tissue. As with phospho-Tie2, the localization of the protein and/or pattern of expression may be of greater significance than the quantity of the protein.

Previous studies have suggested a direct role for ANG-1/Tie2 in recruiting smooth muscle cells and/or pericytes to the vascular endothelium to facilitate maturation of the vessel. However, the model depicted in Fig. 12 proposes that activation of Tie2 by ANG-1 may instead stabilize the vascular endothelium, inhibiting permeability and promoting endothelial cell survival, providing a permissive signal for recruitment of perivascular support cells by other factors, such as platelet derived growth factor (PDGF). This hypothesis is supported by data from a postnatal murine retinal model. Systemic administration of an anti-PDGF receptor-β antibody blocked mural cell recruitment to the developing retinal vasculature [89]. Injection of exogenous ANG-1 into the vitreous
cavity restored the hierarchical organization of the vasculature and rescued vascular edema but was not capable of stimulating perivascular cell recruitment in the absence of PDGF signaling. Furthermore, this data supports a role for ANG-1/Tie2 in tube formation and differentiation of the vasculature into a network of vessels of varying diameter; the involvement of ANG-1 in formation a hierarchical network was first suggested by the phenotype of the ANG-1 null mouse [53]. Finally, previous models have suggested that binding of ANG-1 to Tie2 may stimulate the release of factors responsible for the recruitment of perivascular cells to the immature vasculature [44, 53]. However, to this point, no such factors have been identified providing further evidence that the ANG-1/Tie2 system may be involved in stabilizing the vascular endothelium rather than in the direct recruitment of mural cells.

_Potential Improvements in the Experimental Design_

While the Tie2LacZ microcauterizer model has allowed morphological determination of a transition from angiogenesis to vascular remodeling, several potential improvements in the model should be considered. Utilization of a LAD occlusion model would eliminate possible questions regarding the impact of generating an infarct with a heat probe. As previously mentioned, the tissue remodeling resulting from microcauterizer-generation of the infarct is equivalent with that following LAD occlusion. However, destruction of tissue and cauterization of vessels in this manner may initiate distinct wound responses, which may inhibit or promote additional factors involved in the process of neovascularization. In the same regard, it is impossible to
distinguish between tissue response(s) that may be resulting from the infarct and those from the burn trauma.

In addition, the LAD model, while not capable of eliminating biological variability inherent in an in vivo system, would enhance the consistency of the infarct in terms of both placement and severity. While every effort was made to achieve consistency in microcauterizer-generation of the infarct, a number of factors affected the depth and diameter of the infarct and where the infarct was generated on the left ventricle of the heart. Ideally, the infarct was generated on the apical left ventricle; however, biological variables, including situation of the heart in the chest cavity and rate of heartbeat, typically caused variation in the exact placement of the wound. In addition, the battery-operated microcauterizer was manually controlled therefore the heat of the cauterity tip and steadiness of the operator's hand, as well as the biological variables already mentioned, may have led to variation in the length of time the tip was in contact with the tissue, which may in turn have affected the depth and diameter of the infarct. Even slight differences may conceivably have resulted in greater deviation in the results of the biochemical analysis of the tissue. While the precise anatomy of each mouse is unique, placement of the LAD ligature around the same portion of the vessel would likely result in greater consistency than the current method [108].

A further consideration is the method of harvesting the tissue for RNA and protein biochemistry. The current method of extracting a 3 mm punch of tissue was designed to reduce dilution of RNA or protein changes within the infarct by non-infarcted tissue. However, several factors may lead to increased deviation in the results. First, the location of the infarct and plane of bisection generates variability in the quantity of tissue
harvested. Because the placement of the infarct is unique from heart to heart, bisection of the heart followed by tissue punch may result in unequal amounts of non-infarcted tissue captured within the punch.

Two possible alternatives have been demonstrated in rat myocardial infarction models. The first is dissection of the left ventricle to the interventricular septum [78]. While the size of the dissected left ventricle will vary from animal to animal, the technique should result in increased consistency of the amount of damaged relative to undamaged tissue. The second option is dissection of the infarcted area away from the uninfarcted tissue. Li et al demonstrated the ability to separate the tissue based on visual appearance of the heart muscle [80]. For either alternative, the possibility exists that the size of the murine heart relative to the rat heart may preclude use of the method.

A third consideration is quantitative assessment of ANG-1, ANG-2, Tie2, phospho-Tie2, and VEGF protein levels. While ELISAs are known to be a more specific and sensitive method of assessing protein expression, their development, with the exception of VEGF, is currently hindered by availability of murine-specific antibodies. As antibodies become available, ELISAs can be developed following the protocol of Chong et al [109]. In addition, antibodies against alternative epitopes, such as those containing tyrosine 992 or a second antibody containing tyrosine 1108 may confirm phospho-Tie2 expression. Finally, use of a VEGF ELISA capable of detecting all isoforms of the protein will enable quantification of changes in total VEGF protein expression.
In summary, the regulation of angiogenesis and vascular remodeling is a complex, multi-factorial process, involving a delicate balance between a variety of inducers and inhibitors of the process(es) [110]. Understanding the interplay between these factors in a specific in vivo system is necessary to effectively exploit the roles of these molecules in a therapeutic setting. Furthermore, determination of temporal expression patterns of angiogenic regulatory factors will facilitate combinatorial or sequential administration in the treatment of cardiac hypoxia. A well-characterized angiogenic model also provides opportunities for the identification of yet unknown factors, such as those suggested in Fig. 12, which may be critical to the regulatory cascade. The results presented demonstrate that the angiogenic response following microcauterizer generation of an infarct in the Tie2LacZ mouse provides a histologically and temporally defined model for the study of vascular growth and remodeling processes in the heart in addition to the characterization of the regulatory pathways mediating these phases. The results of the angiopoietin/Tie2 and VEGF expression studies reiterate the potential complexity of determining the specific roles of these factors in the adult cardiac vasculature. Furthermore, the data show the need for additional research into the growth/remodeling transition with greater emphasis on the role of remodeling in coronary angiogenesis.
REFERENCES


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