INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

THERMAL INJURY INCREASES TMR INDUCED ANGIOGENESIS IN THE ISCHEMIC MYOCARDIUM

Department of Experimental Surgery
McGill University
Nicolas Bousette
9949056
Supervisor: Dr Hani Shennib

Submitted to the Faculty of Graduate studies as partial fulfillment of requirements for degree of Master of Science.

ABSTRACT

Background: A growing number of patients suffering from ischemic cardiomyopathy are not eligible for conventional revascularization. This has prompted new research in the field of angiogenesis. This study hypothesized that since inflammation is probably the mechanism behind TMR induced angiogenesis; a larger inflammatory response induced by thermal injury may lead to increased angiogenesis.

Methods: The model used for this study was coronary artery ligation in the Rat. Four groups of animals were used to compare the novel experimental approach with conventional TMR and with ischemia alone. Neovascularization was determined by immunohistochemical techniques using anti-Factor VIII antibody. Evaluation ot VEGF, Ang-1 and Ang-2 expression was also carried out using immunohistochemistry.

Results: The experimental "HOT" TMR technique resulted in significantly increased angiogenesis presumably due to the thermal injury induced by the novel technique. Also a significant increase in VEGF expression was observed in all ischemic groups. Ang-1 expression was decreased in the experimental group while it was similar in the other groups. Finally Ang-2 was induced by ischemia as evidenced by increased expression among all ischemic groups. However Ang-2 expression did not significantly vary among ischemic groups.

Conclusions: The addition of thermal injury by heating of the needle led to an increased angiogenic response compared to ischemia alone and compared to conventional TMR. This increased angiogenesis was associated with increased VEGF expression at one week, however there was a significant inverse correlation between VEGF expression and angiogenesis among the ischemic groups. Also angiopoietin expression was in agreement with expression characteristics described in the literature.

ABSTRAIT

Introduction :Un nombre de plus en plus important de patients souffrant de cardiomyopathie ischemique ne sont pas eligibles pour une revascularisation conventionnelle. Ceci a commande une nouvelle recherche dans le domaine de l'angiogenesis. Cette etude suggere que puisque l'inflammation est probablement le mechanisme derriere l'angioigenesis genere par TMR, et de ce fait, une reponse inflammatoire plus large devrait conduire a une effet angiogenique plus important.

Methode : Le modèle utilisé pour cette etude etait une ligation de l'artere coronaire chez le rat. Quatre groupes d'animaux furent utilisé pour comparer la nouvelle approche experimentale avec la TMR conventionnelle et avec ischemia seule. Neovascularsation etait determine par des techniques d'immunohistochimique utilisant le antibody anti-Factor VIII. L'Evaluation de VEGF, Ang-1, et Ang-2 expression furent aussi conduite par utilisation de l'immunohistochimie.

Resultats : La technique TMR chaude experimentale a resulté en une accroissement signifiant de angiogenesis probablement dû à la blessure thermale produite par la nouvelle technique. Egalement, le developpment de neovascularisation etait associe de facon signifiante avec une augmentation de l'expression de VEGF apres une semaine. L'expression de Ang-1 a diminué dans le groupe experimentale tandis qu'il etait similaire dans les autres groupes. Finalement, Ang-2 fut introduit par ischemia comme evidence en augmentant l'expression dans tous les groupes ischemique. Cependant ils faut noter que l'expression Ang-2 n'a pas varier de facon signifiante parmi les groupes ischemiques.

Conclusions :L'addition de blessures thermales par l'echauffement de l'aiguille a conduit a une reponse angiogenique accrue comparé a l'ischemie seul et comparé a la TMR conventionelle. Cette angiogenesis accrue etait associé avec une expression VEGF augmenté, mais avait une correlation inverse de l'expression de VEGF entre les groupes ischemiques apres une semaine..

Egalement l'expression Angiopoietin etait en accord avec les caracteristes d'expressions decrites dans la litterature.

PREFACE

This study was performed as a partial requirement for the degree of Master of Science at McGill University. The surgical experiments for this study were conducted in the McGill University surgical clinic within the Montreal General Hospital. This study was funded by the Medical Research Council of Canada, and the Quebec Heart and Stroke Foundation.

ACKNOWLEDGEMENTS:

I would like to begin by thanking Dr. Hani Shennib. I have never before encountered such a hard working and dedicated individual. I would like to thank him not only for all the time he spent helping me and guiding me through my Master's degree but also for being my mentor. He taught much more than just surgical techniques, he taught me the value of truly hard work. I can only hope that one day I will be able to be that kind of role model for someone else.

My gratitude is also extended to Dr. Adel Giaid, for he too acted as a mentor for me. His positive attitude always motivated me to work harder. Although he is a renowned researcher he always showed the utmost respect and treated me as a colleague, instead of just as his student. He was always available for any questions I might have had and I must thank him for his invaluable guidance, which saved me a vast amount of time.

I would also like to thank everyone in the McGill University surgical clinic beginning with Allana Melanson who helped me on numerous occasions with a variety of problems. Also I would like to thank Diane Murray and Marlene Brydon, who not only helped me with the surgeries but also taught me how to work with the animals in a more humane manner, an important aspect in surgical research. Irene Sidorenko has my deepest gratitude for helping through the administrative aspects of my Master's degree.

Dr Takayuki Saito has my utmost gratitude and respect for teaching me the surgical techniques I needed to carry out my research. I must also thank Dr. Edward Chedrawy whose technical expertise was of great help. I cannot forget how Lara Tayara also helped me with the laboratory aspects of my research and for this I thank her. Finally I would like to thank Vivianne Korah who was extremely nice and helpful to me throughout my Master's studies.

List of Abbreviations:

- **ACE.....Angiotensin converting enzyme**
- **Ang.....Angiopoietin**
- **bFGF.....basic Fibroblast Growth Factor**
- **CABG.....Coronary Artery Bypass Graft**
- **CO2.....Carbon Dioxide**
- **CuSO4.....Copper Sulfate**
- **DNA.....Deoxyribonucleic Acid**
- **EC.....Endothelial Cell**
- **ECM.....Extracellular Matrix**
- **EGF.....Epidermal Growth Factor**
- **HCPTP.....Human Cellular Protein Tyrosine Phosphatase**
- **HIF-1.....Hypoxia Inducible Factor**
- **IL.....Interleukin**
- **kDa.....KiloDalton**
- **MCP-1.....Monocyte Chemo-attractant Protein**
- **mRNA.....Messenger Ribonucleic Acid**
- **NMR.....Nuclear Magnetic Resonance**
- **NO.....Nitric Oxide**
- **PDGF.....Platelet Derived Growth Factor**
- **PI-3.....Phosphatidyl Inositol**
- **RNA..... Ribonucleic Acid**
- **SMC.....Smooth Muscle Cell**
- **SPECT.....Single Photon Emmision Computer Tomography**
- **TGF.....Transforming Growth Factor**
- **TIE-2.....Tyrosine Kinase with Immunoglobulin and Epidermal Growth Factor Domain**
- **TMR.....Transmyocardial Revascularization**
- **TNF.....Tumor Necrosis Factor**
- **VEGF.....Vascular Endotheial Growth Factor**
- **VEGFR-2..... Vascular Endotheial Growth Factor Receptor**
- **VEPTP.....Vascualr Endothelial Protein Tyrosine Phosphatase**
- **VRAP.....Vascular Endotheial Growth Factor Receptor Associated Protein**

TABLE OF CONTENTS: **Pages**

1.0 INTRODUCTION

Heart disease is the leading killer in North America today, although it has many varying etiologies, myocardial ischemia is the most common cause. Ischemic heart disease is characterized by a reduction in blood flow through the coronary vasculature of the myocardium and is caused by coronary atherosclerosis, which is a narrowing or stenosis of the arteries. This decrease in blood flow leads the myocardial tissue to become hypoxic. The myocardium is thus "starved " for oxygen and cannot sustain optimal contraction, becoming dyskinetic and resulting in reduced cardiac output. If this ischemia persists it will lead to both apoptotic and necrotic cell death, thus resulting in ischemic heart disease.

Efforts to improve blood flow to the myocardium began as early as 1935 when Beck attempted to induce angiogenesis using omentum [1]. Also Vineberg attempted to increase perfusion by grafting the internal mammary artery to the epicardium [2]. Sen et al. were among the first to attempt blood delivery to the myocardium directly from the ventricular lumen in a technique termed transmyocardial acupuncture [3]. However, these techniques were later supplanted by newer medical and surgical treatments.

Today, medical therapies are aimed at reducing the workload of the heart. This is done using drugs such as diuretics and vaso-dilators, which decrease systolic resistance. On the other hand, surgical therapy consists of directly revascularizing the heart using autologous vessels, and is termed coronary artery bypass grafting (CABG). Coronary bypass grafting is the gold standard in surgical therapy, although it is still associated with a significant degree of morbidity including neurological and renal deficits post-operatively.

Also, many catheter-based techniques such as angioplasty and stenting are being performed today. Angioplasty has great therapeutic potential as it leads to immediate reperfusion by a minimally invasive procedure, however it is hindered by a relatively high rate of restenosis.

Although these treatments are relatively effective there is a growing population of patients for which both medical and surgical therapies are contraindicated and/or ineffective. Such non-bypassable or non-stentable patients have prompted research in alternative revascularization methods. There are two main research avenues presently being pursued, which include therapeutic angiogenesis (gene therapy) and TMR (transmyocardial revascularization).

1.1 Therapeutic Angiogenesis

Therapeutic angiogenesis via gene therapy is presently a popular research field owing to its exciting potential. The gene therapy can be delivered by a number of routes including intravenous, intra-coronary, intra-myocardial, trans-endocardial and intra-pericardial. In addition to the various routes of administration, there are also several different vectors that can be used including viral vectors (usually adenovirus), plasmid vectors, or simply direct protein transfer. Sustained release polymers are also potentially useful vectors as they can be surgically implanted into the epicardial fat pads and allow for chronic release of protein. Although adenoviral vectors have the benefit of prolonged protein production, they may provoke an immunological response [4].

In a study evaluating myocardial uptake of radio-labeled basic Fibroblast Growth Factor (bFGF) it was found that pericardial administration resulted in high uptake (19%), compared with an intra-coronary (3-5%), left atrial (1.3%), or swan-ganz (0.5%)

approach [5]. One reason why intravenous administration led to such low myocardial uptake could be due to bFGF binding to the heparin sulfate receptors in the lung.

A study performed by Lee et al. showed that intra-myocardial administration of an adenovirus vector that coded for Vascular Endothelial Growth Factor (VEGF) was taken up more efficiently than intra-coronary administration [6]. It was also demonstrated that the location of VEGF remained specific to the injection site, whereas with the intra-coronary administration it equalized throughout the myocardium. Since both bFGF and VEGF are vasodilators, the maximum doses are limited by their hypotensive side effects [5].

1.1.1 Basic Research

One study involving a rabbit ischemic hind-limb model showed increased capillary density and increased perfusion to the ischemic limb by intra-muscular injection of Angiopoietin-1 (Ang-1) encoded plasmid DNA [7]. The capillary density and perfusion scores in the Ang-1 plasmid treated animals were significantly increased over naked plasmid and saline controls. Interestingly, in the same study, they determined that Angiopoietin-2 (Ang-2) when injected alone did not have an angiogenic effect. Surprisingly, another study showed that Ang-1 and Ang-2 led to an increase in vascularity only if co-administered with VEGF, and that this effect was additive when compared to the administration of VEGF alone [8]. This study used the introduction of pellets containing VEGF, Ang-1, or Ang-2 (or a combination of these) in a mouse corneal pocket assay. The angiogenic effect induced by VEGF/Ang-1 differed from VEGF/Ang-2 by the fact that the former resulted in increased vascular density with patent vessels and

surrounding peri-endothelial cells, whereas the latter caused more of a simple proliferation of endothelial cells leading to increase vessel length rather than size [9].

A study using an acute myocardial infarction model in the canine was used to evaluate the angiogenic potential of intra-myocardial administration of bFGF protein [10]. Using colored microspheres it was found that epicardial infarct and peri-infarct endocardial zones had increased blood flow at seven days. Also, ejection fraction was increased and ventricular thinning was decreased in bFGF group when compared to control group after seven days. In a similar study conducted by Laham et al. in which ameroid constrictors, (which decrease vessel diameter over a period of weeks) were used to induce chronic myocardial ischemia in the pig, it was found that intra-pericardial administration of bFGF led to increased myocardial blood flow, and that this effect was dose dependent. However, a certain degree of fibrosis was observed in all bFGF groups [11].

Although tissue hypoxia is an important stimulator for angiogenesis, it is not absolutely essential, as it was found that normoperfused skeletal muscle could undergo angiogenesis via the up-regulation of VEGF. Gowdak et al., demonstrated using angiography and radioactive microspheres, that intra-muscular injection with VEGF encoded adenovirus increased vasculature in a normal limb. These newly formed vessels allowed for improved perfusion after induction of ischemia as determined by tissue metabolite levels, which were evaluated using P31-NMR spectroscopy [12].

1.1.2 Clinical Research

A phase I clinical trial using recombinant human VEGF via intra-coronary administration showed tolerance for doses up to 0.167μg/kg [13]. Also, significantly

improved resting myocardial perfusion was observed with the higher doses as determined by single photon emission computer tomography (SPECT). Another phase I trial tested the safety of placing bFGF containing sustained release heparin-alginate microcapsules in non-graftable regions of patients undergoing conventional revascularization [14]. Patients in the high dose group (100μg) experienced a significant reduction in the defect size as determined by stress nuclear perfusion imaging. Also, all high dose group patients were free of angina post-operatively, whereas 3/8 control and 1/8 low dose patients experienced angina post-operatively.

A study done by Symes et al. looked at the safety of intra-myocardial injection of plasmid encoding VEGF gene [4]. The plasmid was well tolerated and significant symptomatic improvements were observed including a decrease in weekly nitroglycerin use. Also 70% (7/10) of patients were free of angina at six months. In addition 13/17 patients showed significantly reduced ischemic defects using SPECT scans. Although this was even more remarkable in the high dose group where 7/8 patients showed reduced ischemic defects.

Although gene therapy has fantastic potential in improving myocardial perfusion and thus reducing ischemic heart disease, it may also have the additional negative effect of aggravating atherosclerosis. This speculation is based on the observation that advanced atherosclerotic lesions express VEGF at high levels [15]. Intra-plaque neovascularization may further increase neointimal hyperplasia hence increasing occlusive lesions. In fact, in a canine femoral balloon injury model it was shown that systemic administration of VEGF exaggerated intimal thickening [5]. Plaque

neovascularization may also lead to plaque instability thus leading to an increased potential for thrombo-embolic disease [5].

In addition to the possible aggravation of atherosclerosis there is also potential to induce or worsen other neovascular diseases such as metastatic cancers, diabetic retinopathies, and rheumatoid arthritis [5]. Thus, patients to be accepted into clinical trials for these new treatment modalities must be free of malignancies, retinopathies, renal insufficiency, in addition they must not be eligible for conventional "gold standard" revascularization [16].

1.2 Transmyocardial Revascularization

Transmyocardial revascularization (TMR) is the process of making transmyocardial channels in the ventricles of ischemic hearts. It was initially done in hopes of replicating reptilian myocardial perfusion in which the myocardium is perfused with blood coming directly from the ventricular lumen. However several reports indicated that these channels do not remain patent [17]. Nevertheless, a definite clinical effect has been observed as patients experience reduced angina after undergoing TMR. Thus the alternative hypothesis of angiogenesis arose as the mechanism of anginal relief upon the observation of neovascularization surrounding the TMR induced scar tissue. Numerous studies have shown the effectiveness of TMR techniques at inducing angiogenesis [18,19,20,21,22,23]. Therefore, efforts have been aimed at proving that the beneficial effects of TMR are the result of increased perfusion due to neovascularization. Alternatively, it has been hypothesized that the reduction in anginal pain observed

clinically was the result of denervation of the myocardium. Evidence contradicting this theory has been put forth [24], but more studies are needed to completely rule it out.

TMR can be carried out with either a sophisticated laser system or a simple hypodermic needle. Though Chu et al. demonstrated that there was no difference in angiogenesis between laser TMR and needle (mechanical) TMR [20]. There are three main laser types including the carbon dioxide, the Excimer, and the YAG: holium lasers, all of which have shown some success at inducing angiogenesis. However Lansing [25] described the CO2 laser as better for both anginal relief and increased perfusion.

It is known that hypoxia induced inflammation inherently results in some degree of compensatory angiogenesis. Interestingly, there is a growing consensus that inflammation is the mechanism behind TMR mediated angiogenesis. Although there have been no studies directly demonstrating this, several articles have suggested that inflammation is the causative factor [20,26,27,18,19]. In one study, Bortone et al. showed a significant increase in neutrophil migration specifically to the lased areas of the myocardium after percutaneous transmyocardial revascularization [27]. This neutrophil infiltration coincided with a systemic increase in procalcitonin, a marker of acute inflammation. Based on these results, they speculated that inflammation was the link for TMR mediated angiogenesis. Also, in a post mortem study on TMR patients, capillary networks and a monocyte/macrophage infiltration were observed within granular tissue, a hallmark of inflammation.

1.21 Basic research

One study using the coronary ligation model in the rat showed that vascular density increased significantly as a result of TMR. In addition, TGF-β, and bFGF were

expressed significantly higher in the TMR group as compared to the negative control in which there was only coronary ligation [18]. Interestingly though, there were no differences in VEGF expression between the TMR group and the negative control in that study. All growth factors peaked at one week and declined thereafter along with vascular density [18]. The same group conducted another study using needle TMR on the chronically ischemic porcine model [20]. In this study, significantly increased neovascularization was also observed in the TMR group. In addition, VEGF, bFGF, and TGF-β were all significantly increased 1-week post-TMR. Again, angiogenic factor expression decreased at 4 weeks but was still significantly higher for VEGF, and TGF-B, but not bFGF. The differences in VEGF expression between the two studies may be due to interspecies differences. Horvath et al. performed a very similar study in which TMR was performed again on a porcine chronic ischemia model, however they used a CO2 laser system as opposed to the needle technique [21]. It was found that this also led to increased VEGF levels and that this in turn was the cause of the increased angiogenesis observed.

Another study showed that TMR induced by a laser resulted in significantly higher number of neovessels in the ischemic myocardium in a porcine model [19]. In this study, it was found that the laser-induced channels were replaced with connective tissue, but there was apparent neovascularization in the periphery of the "old" channels.

1.2.2 Clinical research

Clinically, Bortone et al. showed improvements at a one-year follow-up using exercise SPECT-perfusion scans, for patients who had undergone percutaneous TMR

[28]. Recently, two other major multi-center randomized clinical trials were completed both showing significant improvement in Canadian Cardiovascular class status, but only one managed to demonstrate increased myocardial perfusion [29,30]. Another study showed significant improvement in long-term clinical status and exercise tolerance, but they failed to provide evidence for increased perfusion [31]. Lee et al. also reported a reduction in symptomalogy in a one-year follow-up study [32]. In contrast, De Carlo et al questioned long-term symptomatic relief after a follow-up study was conducted on TMR patients due to the high recurrence of either angina or other cardiac events [33]. Several other studies conclude definite symptomatic improvements in patients who have undergone TMR [25,32,34], however not all studies report increased perfusion. Thus further studies are being conducted to completely elucidate the mechanism of symptomatic improvement.

2.0 LITERATURE REVIEW

2.1 Myocardial Inflammation and Angiogenesis

Upon occlusion of a major coronary artery, a substantial amount of myocardial tissue will become hypoxic. Such hypoxia will lead to a characteristic inflammatory response, including inflammatory cell infiltration and up-regulation of growth factors and cytokines. This was demonstrated in a porcine embolization model, in which characteristic infiltrates consisting of macrophages/monocytes, fibroblasts, and neutrophils were observed [35]. Another study, using a porcine micro-embolization model demonstrated that monocyte secretion of Insulin-like growth Factor-1 was an important link to inflammation-mediated angiogenesis [36]. In fact, they also observed that endothelial cell mitosis occurred only when in close proximity to macrophages within the ischemic myocardium [36]. It is well known that a large part of hypoxia-induced angiogenesis is a consequence of up-regulation of Hypoxia inducible factor-1 (HIF-1) [22]. This transcription factor specifically up-regulates VEGF [37]. Recently, it was demonstrated that a macrophage derived peptide termed PR39 led to increased vascularity in a mouse ischemic myocardium [38]. The mechanism behind this angiogenic activity was found to be inhibition of ubiquitin-mediated proteolysis of HIF-1.

Angiogenesis is a term used broadly for the formation of new blood vessels. However, it specifically refers to the formation of new blood vessels from a pre-existing vasculature. In order for angiogenesis to occur, a stimulus is required, such as hypoxia-induced inflammation. This stimulus will induce the release of several cytokines and growth factors by nearby endothelial cells, which will in turn induce the migration of

inflammatory cells such as neutrophils. Interestingly, an inherent effect of hypoxia is to induce neutrophil adherence to nearby endothelial cells [39]. Neutrophils have recently been implicated as facilitators of angiogenesis by inducing the release of VEGF, and by the formation of reactive oxygen species [41,42]. The adherence process involves several adhesion molecules including Selectins and Integrins. The adhered neutrophil will then pass through the vascular wall through fenestrations, towards the hypoxic stimulus in a process termed diapedesis. Vascular fenestrations are formed as a result of VEGF activity [40].

Over a period of days, the inflammatory infiltrate will change from consisting of primarily neutrophils to mostly macrophages. Not surprisingly, macrophages have also been implicated as integral mediators in the angiogenic process. In fact, they were found to have an impact on every stage of angiogenesis based on the huge repertoire of secretory products including proteases, growth factors and cytokines [43]. In agreement with this, a study conducted by Moldovan et al. suggests that monocytes and/or macrophages may play a significant role in "compensatory Neovascularization" [18]. In that study, they over-expressed MCP-1 (monocyte chemo-attractant protein-1) in the myocardium. This consequently led to a monocyte/macrophage infiltration, which resulted in the formation of many non-endothelialized channels. They speculated that this may have directed formation of neo-vessels in ischemic tissue, and as such the macrophages acted by "drilling" channels through the myocardium. In another study evaluating the effects of macrophages on tumor angiogenesis, it was found that there was a direct relationship between macrophage infiltration and degree of angiogenesis. This relationship was mediated by macrophage release of TNF-α and IL-1α [44]. Thus, the

inflammatory infiltrate most likely leads to a massive up-regulation of growth factors and cytokines, which ultimately may lead to a substantial angiogenic stimulus. Fibroblasts are also integral players in the infiltration process as they are involved in fibrin deposition necessary for endothelial cell migration [45].

Once the inflammatory stimulus has been established, whether it is hypoxic or injury induced, there is a requirement of vascular cell migration towards the stimulus in order for angiogenesis to ensue. Firstly, endothelial cells must form the primary capillary plexus and thus are likely the first cells to migrate, however there is evidence pointing to pericytes (which are discussed below) as the first to differentiate and actually direct endothelial cell migration [46]. This was demonstrated in a study which showed pericytes at the leading tip of vessel sprouts, in addition they acted to bridge the gap between two oncoming sprouts [47]. Migrating endothelial cells, regardless of whether they are directed by pericytes or whether they simply migrate based on a VEGF gradient, must arise from either pre-existing vasculature or from circulating angioblasts. Endothelial cells originating from other vessels must have had a stimulus for their release from cellular and extracellular-matrix (ECM) contacts. This loosening of cellular contacts is thought to be mediated by Angiopoetin-2 (Ang-2), a factor known to competitively inhibit Angiopoietin-1, which is a vessel stabilizer [37]. Thus, Ang-2 leads to a destabilization of endothelial interactions with peri-endothelial cells. This allows for VEGF mediated proliferation and migration of endothelial cells. Alternatively the endothelial cells originate from angioblastic precursors via the action of VEGF, which in turn originate from mesenchymal stem cells via the action bFGF [48].

Once the primary capillary plexus is formed, peri-endothelial cells are induced to migrate, including pericytes and smooth muscle cells. It is believed that N-Cadherin is the major adhesion molecule involved in endothelial cell/ smooth muscle cell interaction [56]. Peri-endothelial cells function not only as support cells, but also by fibrinolysis and proteolytic degradation of the ECM during migration [50]. Interestingly, different studies by Nicosia et al. and Doherty et al. have provided evidence of smooth muscle cell differentiation into pericytes and pericyte differentiation into smooth muscle cells, respectively [51,52]. However, there is also evidence that suggests that endothelial cells recruit mesenchymal cells and induce them to differentiate into pericytes [53]. It is believed that this occurs via the action of Platelet-Derived Growth Factor (PDGF) [49,46]. One study conducted by Grosskreutz et al. actually suggested that peri-endothelial cell migration occurs via VEGF [54]. In that study they showed that smooth muscle cells actually expressed VEGF receptors, which is contradiction to the longstanding dogma in which VEGF acts only on endothelial cells. These VEGFR-2 expressing smooth muscle cells were shown to have a dose dependent migratory response to VEGF.

Located abluminally on capillaries and venules, pericytes are integral parts of the microvasculature and are likely to have a prominent role in vascular maintenance. Pericytes have been shown to inhibit endothelial proliferation via a heterogenic contact inhibition mechanism involving TGF-β and as such are in vivo regulators of endothelial cells [49,46]. In addition to SMCs, pericytes have also been implicated to have some contractile function [53]. It was shown by Verbeek et al. that TGF-β1 actually induced the expression of smooth muscle cell-actin in pericytes [55]. The muscularized coat of

the neovessels therefore acts to inhibit further endothelial proliferation in addition to induce extracellular matrix deposition. The latter is believed to occur by the activity of TGF-β [48].

In contrast to either vasculogenesis or angiogenesis in which new vascular sprouts are produced, arteriogenesis results in the enlargement of pre-existing arteries. The stimulus for this process is usually an increase in shear stress in a co-lateral artery due to occlusion of a major supply artery. The process involves an increase in monocyte adhesion followed by destruction of the medial layer by proteinases and pro-apoptotic factors, such as TNF-α [56]. Following this an endothelial proliferation must take place leading to an increased lumen size. Ultimately the increased expression of growth factors such as bFGF leads to the re-muscularization of the artery with smooth muscle cells. However the entire process is dynamic and each step most likely overlaps with others.

2.2 Angiogenic Growth factors

2.2.1 VEGF

VEGF is one of the most prominent angiogenic factors, not only because it is absolutely essential, but also because it stimulates the expression of other angiogenic factors. VEGF is a 34-46 kDa basic heparin binding protein [6,57]. The VEGF gene is located on the chromosome 6p21.3 [57]. The are five different iso-forms of VEGF including VEGF-A, -B, -C, -D, PlGF which are formed by alternate splicing of the same gene [58]. These five different VEGF iso-forms interact with three different subtypes of VEGF receptors VEGF-R1, -R2, -R3 (also called Flt-1, KDR/Flk-1, and Flt-4

respectively) [59]. VEGF receptors are tyrosine kinase receptors with seven extracellular immunoglobulin domains [58]. VEGF-R1 and R2 are expressed primarily on vascular endothelial cells; VEGF-R3 is found more on the lymphatic endothelium [59]. VEGFR-2 null mutation leads to a complete absence of endothelial cells and is thus a lethal mutation [58]. The VEGF-R1 may be responsible for the down-regulation of VEGF activity with respect to endothelial cell growth. This is evidenced by the fact that the VEGF-R1 null mutant embryo showed abnormally high levels of endothelial cells and poor vessel assembly [59,58]. In contrast, VEGFR-1 mutants, which only had a functional binding domain, showed a normal phenotype thus suggesting a role of sequestering VEGF [59].

VEGF is known to be up-regulated by both hypoxia and hypoglycemia [37,60]. The mechanism involves the binding of Hypoxia Inducible Factor (HIF) to a 5' flanking sequence in the operator region of the VEGF gene [58]. In fact, it has been demonstrated both clinically and in animal models that circulating VEGF levels are significantly increased after an acute myocardial infarction [61,62]. In accordance with this, it was found that it is highly expressed in the ischemic myocardium [63]. A study was also conducted on children who have cyanotic congenital heart disease, and it showed that they too had elevated circulating levels of VEGF in comparison with children who had acyanotic congenital heart disease [64].

Hypoxic tumor cells have also been shown to up-regulate VEGF expression, while endothelial cells within these tumors up-regulate VEGF receptors [49]. In addition to hypoxia, several cytokines induce the expression of VEGF including EGF, TGF-α, TGF-β, Il-1, and Il-6 [58]. Interestingly, it is known that VEGF mRNA is more stable in

hypoxic as opposed to normoxic conditions [62]. Cells expressing VEGF include cardiomyocytes, vascular smooth muscle cells (SMC), lymphocytes, macrophages, and neutrophils [61].

VEGF is a potent stimulator of endothelial cell growth [37] whose dose dependency has been demonstrated by the fact that a single null VEGF-A allele is lethal [59]. VEGF has been reported to have a substantial proliferative effect on endothelial cells [37]. This is in addition to its activity in endothelial chemotaxis and extracellular matrix (ECM) breakdown [65]. VEGF stimulates matrix degradation indirectly by stimulating plasminogen activators as well as collagenases [57]. VEGF also acts to increase vascular permeability by increasing vascular fenestrations and as such VEGF is also termed vascular permeability factor [57,58]. The latter VEGF activity was demonstrated in a mouse model in which over expression of VEGF led to tissue edema and hemorrhage [66]. Finally, VEGF is also a vasodilator, probably via the formation of nitric oxide [57]. Interestingly, it was found in one study that the angiogenic effect of VEGF is likely mediated by nitric oxide [60]. This was determined by the fact that mice bearing VEGF containing corneal implants lacked an angiogenic response if L-NAME, a nitric oxide synthase inhibitor was administered systemically. Also LY 83583 an inhibitor of Guanylate cyclase, a downstream enzyme in the nitric oxide pathway also inhibited VEGF mediated migration and DNA synthesis of endothelial cells [60]. In contrast the L-NAME did not prevent bFGF containing corneal implant mediated angiogenesis. On the other hand, SQ 22536, an adenylate cyclase inhibitor, actually enhanced the VEGF mitogenic response. In addition to its own capacity to promote angiogenesis, VEGF also induces the expression of angiopoietin 2 (Ang-2).

It has been found that phosphatidylinositol 3-kinase was necessary for VEGF mediated proliferation of human umbilical vein endothelial cells (HUVECS) [67]. Interestingly, P-85, the regulatory subunit of PI-3 kinase was found to be constitutively bound to VEGFR-2 (otherwise known as Flk-1/KDR) [67]. There is evidence that supports MAP-K as another downstream signaling enzyme involved in the proliferation of endothelial cells [68]. Yet another protein found to bind to VEGFR-2 is VRAP (VEGF receptor associated protein) [69]. It has been shown that the activation of VEGFR-2 via VEGF binding induced the intracellular binding of VRAP to VEGFR-2 [69]. Effector enzymes of the VEGF pathway such as Phospholipase C gamma and PI 3-kinase were found to be constitutively bound to VRAP, hence suggesting the role of adapter protein for VRAP.

It has been determined that VEGF is regulated at least in part by the action of TNF. A study showed that TNF inhibited the phosphorylation and thus activation of KDR, otherwise known as VEGFR-2 [68]. This inhibitory action is believed to be mediated through the action of a protein tyrosine phosphatase. This was deduced by the fact that the addition of Sodium Orthovanadate, a general phosphatase inhibitor, abrogated the effect of TNF. It is thought that Shp-2 may be the major phosphatase in this mechanism since there is an increase in Shp-2 association with VEGFR-2 upon exposure of TNF [68]. It has been demonstrated that chronic exposure of TNF causes a down-regulation of VEGF receptors [68], and induces endothelial cell apoptosis [70]. Another protein tyrosine phosphatase termed HCPTP (human cellular-PTP) is believed to interact with the VEGFR-2 receptor in vivo [71]. This is evidenced by the fact that

HCPTP over-expression abrogated the angiogenic effect of VEGF. This phosphatase is also said to bind Tie-2 and PDGF receptors [71].

The importance of VEGF regulation was underscored in a study in which continuous up-regulation of VEGF led to serious deleterious effects [72]. The unregulated expression driven by implanted VEGF expressing myoblasts resulted in failure to thrive (in 6/11 mice) or death (in 5/11 mice). Hemangioma resembling vascular tumors was observed in all 6 surviving mice. On the other hand the control mice, which received myoblasts expressing only the B-gal. reporter gene were all normal.

2.2.2 ANGIOPOIETINS

Angiopoietins are necessary factors in normal vascular development as demonstrated by the fact that Ang-1 null mutants die at embryonic stage E10.5 [59]. Ang-1 is a glycosylated 70-kDa constitutive protein secreted from peri-endothelial cells [7,73]. All Angiopoietins share similar structures with three main domains including an N-terminal domain lacking homology with any known structure, an alpha-helical coiled-coil domain important in multimerization (required for activity), and a fibrinogen-like domain which is the most conserved portion of the Angiopoietins, necessary for receptor binding [74]. Interestingly, Ang-1 is believed to form hexamers resembling Tenascin-C, a chemo-attractant for SMCs that has sequence homology with Ang-1 [46]. The fibrinogen-like domain of the Angiopoietins dictates whether it is an agonist or antagonist [74].

It is believed that Ang-1 acts as an agent responsible for maintaining vessel stability. This is evidenced by the fact that Ang-1 mutants show immature disorganized

vessels with aberrantly round cells that do not associate with either the surrounding matrix or with peri-endothelial cells such as peri-cytes and smooth muscle cells [75]. This effect was also observed upon mutation of Ang-1's cognate receptor Tie-2 [9].

Unlike VEGF, Ang-1 did not show mitogenic activity with respect to endothelial cells [59]. However Ang-1 has shown anti-apoptotic effects towards endothelial cells in culture [76]. One study proposed that the anti-apoptotic mechanism involves the activation of the AKT protein (or protein kinase B) [77]. This study showed a significant increase in AKT phosphorylation by Ang-1. It was found that this AKT phosphorylation was mediated by PI 3-kinase since Wortmannin, a PI 3-kinase inhibitor prevented the AKT activation. This increased AKT phosphorylation was associated with an increase in the survival protein, Survivin, in endothelial cells. This survival effect of Ang-1 is probably not mediated by Bcl-2, another survival protein, since there was no observed changes in its expression upon Ang-1 stimulation [77]. Interestingly VEGF may also stimulate Survivin expression [77]. Also Ang-1 has chemo-attractant properties with respect to endothelial cells [7,75], as well as perivascular cells [78]. As such the role of Ang-1 is not limited to vessel stabilization. In fact, Ang-1 induces vessel sprouting in vitro, but does not stimulate endothelial cell proliferation on its own [9]. In accordance with this Ang-1 overexpressing mutants lead to a hypervascularization state [59,46,79,80]. This hypervascularization is characterized as an increase in both vessel size and branching pattern [79,66]. Mice who were overexpressing Ang-1 showed leakage-resistant vessels in which inflammatory agents such as mustard oil, which normally induce tissue edema, had little effect [66]. Also unlike VEGF, Ang-1 is not up-regulated, but may be down-regulated by hypoxic conditions [7]. This was exemplified

by the lack of Ang-1 expression in the hypoxic portion of a tumor, which showed substantial VEGF expression [76].

Ang-1 Null mutants (as well as Tie-2 null mutants) show irregularities at a later stage in comparison with VEGF null mutants indicating a temporal difference in expression of these factors during embryonic angiogenesis [7,59]. Interestingly the adverse effects of Ang-1 null mutations not only affected the endothelium of the vasculature but also of the endocardium. It was found that Ang-1 null mutant hearts had a very loosely associated endothelial layer. in addition to lacking the normal trabeculations usually found within the ventricles [59].

Using in situ hybridization Ang-1 mRNA was detected in mesenchymal cells of the human fetal heart and blood vessels [46] as well as in a mouse embryo [81]. Ang-1 was also expressed in all glioblastoma cells and the degree of staining was found to be proportional to the grade of the tumor [49].

Angiopoietin-2. also known to bind the Tie-2 receptor, has a 60% homology with Ang-1 [75]. However, as opposed to the constitutively expressed Ang-1, Ang-2 is only expressed at sites of either vascular degeneration or sprouting [59]. Ang-2 works in an autocrine fashion as it is both secreted by, and acts on endothelial cells [80]. Interestingly the normal brain does not express Ang-2 but it was observed in glioblastoma tumor vessels [76]. In another study of cell-type specific expression of glioblastoma cells it was found that Ang-2 was expressed in smaller vessels but absent in larger vessels of the tumor, suggesting its role to be limited to smaller type vessels [49].

Angiopoietin-2 (Ang-2) is believed to counteract or antagonize the role of Ang-1 [75]. The mechanism behind this antagonism is believed to be simple competitive

inhibition of the respective receptor. For instance it is known that both Ang-i and Ang-2 bind the same Tie-2 receptor (discussed later). However the difference in activities of Ang-1 and Ang-2 reside in the fact that Ang-1 causes receptor dimerization and subsequent phosphorylation whereas the binding of Ang-2 causes no such activity and thus probably exerts its effect by preventing the binding of Ang-1 (i.e. competitive inhibition). Interestingly, Ang-2 overexpression led to vessel disruption and death in mouse embryo [75,73]. VEGF was demonstrated to stimulate Ang-2 expression, but not Ang-1, nor Tie-2 [37]. Hypoxia also had a stimulatory effect on Ang-2 expression that was independent of VEGF [37].

Therefore with respect to angiogenesis it is believed that the down-regulation of Ang-1 and the concomitant up-regulation of Ang-2 stimulates vessel instability [65]. This is supported by the fact that Ang-2 is only expressed in sites of new vessel formation whereas Ang-1 is constitutively expressed in quiescent adult vessels [65]. Therefore the presence of VEGF induces the up-regulation of Ang-2 which leads to vessel instability and then allows for VEGF stimulated endothelial cell proliferation and migration to take place.

In addition to Ang-i & 2, Angiopoietins 3 & 4 have been discovered based on their homology with the first two [74]. Sequence homology suggests that Ang-3 and Ang-4 are mouse and human counterparts, respectively. However receptor binding assays indicate that they have diverged since Ang-4 induces Tie-2 phosphorylation whereas Ang-3 does not. Ang-3 is widely expressed in mice whereas high levels of Ang-4 have been detected in the human lung [74]. All four Angiopoietins bind Tie-2, and they all have a pattern of three closely linked cysteines in their fibrinogen-like domain. Yet

another two Angiopoietins have been discovered based on their sequence homology and they are termed Angiopoietin-related protein 1 & 2 [81]. They have the coiled-coil and fibrinogen-like domains in common with the other Angiopoietins however they lack the Tie-2 binding capacity [81].

2.2.3 TIE-2

The Tie-2 receptor is named as such because it is a Tyrosine kinase receptor with Immunoglobulin and Epidermal growth factor domains [58]. The immunoglobulin domain of Tie-2 is common among tyrosine kinase receptors that dimerize [46,58]. Tie-2 is expressed not only on endothelial cells but also on hematopoietic stem cells and megakaryoblasts [46,82].

The ultimate purpose of most tyrosine kinase receptors is to transmit signals across membranes. As Ang-1 binds Tie-2 it causes dimerization and autophosphorylation of the receptor, which is a requirement for activity [83]. Interestingly overexpression of Tie-2 led to ligand independent auto-phosphorylation [83]. Tie-2 phosphorylation results in the formation of "docking sites" for many intracellular ligands, including Shp-2, a tyrosine phosphatase known to interact with a VEGF receptor (VEGFR-2), and p85, a subunit of phosphatidylinositol 3-kinase (PI 3-kinase) a common signaling pathway enzyme [83]. This is in addition to several adapter proteins such as Grb-2, Grb-7, Grb-14 [83]. In fact both migratory and anti-apoptotic signaling pathways are known to involve PI 3-kinase activity [83]. In accordance with this, Wortmannin, a PI 3-kinase inhibitor reduced Ang-1/Tie-2 mediated migration and survival (anti-apoptosis activity) [83].

It has been determined that Tie-2 is also induced by hypoxia [84]. As such, Tie-2 up-regulation has been observed in the infarcted myocardium [84]. This effect may be

species and vessel specific as human coronary microvascular endothelial cells were stimulated to express Tie-2 under hypoxic conditions, however human umbilical vein or bovine aortic endothelial cells had no such response [84]. Although, unlike VEGF, Tie-2 was not up-regulated by substrate deprivation [84]. Both TNF-α and IL-1β also induced Tie-2 expression in endothelial cells [84].

Tie-2 is regulated by action of a specific tyrosine phosphatase termed VE-PTP (vascular endothelial protein tyrosine phosphatase) in mice and HPTP-B (human tyrosine phosphatase protein-B) in humans [85]. HPTP-B is up-regulated in cultured endothelial cells and is substantially expressed in mature vessels [85] as such it is implicated as a factor involved in contact inhibition.

The formation of the primary capillary plexus is independent of the presence of Tie-2 [58]. Tie-2 null mouse embryos had simple capillary branching but failed to invade the neuroectoderm [46]. Tie-2 null mutants not only lack branching of vessels but they also did not differentiate into small and large type vessels [8]. In addition to this there is also a decrease in SMC recruitment in Tie-2 mutants [8].

A closely related receptor called Tie-1 also exists, although it is an orphan receptor as it has no known ligands, it is however, expressed in the human fetal myocardium [86] and it is up-regulated in the vascular endothelium of tumors [49]. Normally, though it is believed to be down-regulated after birth except in sites of neovascularization [46]. Tie-1 null mice suffer from severe edema and hemorrhage [46,58]; as such it is believed that it is involved in the control of transcapillary fluid exchange [8]

2.2.4 PDGF

Platelet-Derived Growth Factor (PDGF) is another growth factor believed to be integral in the angiogenic process, in fact it is the chemotactic properties of PDGF which are believed to be important in the recruitment of peri-endothelial cells after the primary capillary plexus is formed [49,46]. PDGF consists of two different subunits from which it can make three different dimers. The subunit PDGF-A and the subunit PDGF-B can combine to form the three isoforms of PDGF, including PDGF-AA, BB, and AB [87]. Migration and proliferation of multi-potent mesenchymal cells is believed to occur, in large part, by the endothelial cell secretion of PDGF [87,49]. A study was done that found that the BB form of PDGF was the only subtype that significantly performed this function [87]. This chemo-attractant molecule thus acts as a recruitment factor of mesenchymal cells in angiogenesis. In agreement with this it is found that endothelial cells involved in new vessel formation express and secrete the PDGF-BB form and the mesenchymal cells express the PDGF-B receptor subtype [87]. These mesenchymal cells eventually differentiate into pericytes and/or smooth muscle cells [87]. In support of this it was found that mice with a PDGF null mutation lack pericytes in and around capillary beds [87].

2.2.5 TGF-β

Transforming Growth Factor-β is believed to become involved at later stages of the angiogenic process as the primary capillary plexus begins the process of maturation and the surrounding mesenchymal cells are commencing to differentiate into their pericyte and/or smooth muscle cell fate. There is evidence supporting the fact that TGF-

β is involved in the differentiation and maturation of these new vessels [87]. In fact it has been demonstrated that TGF-β caused a decrease in Ang-2 expression thereby promoting vessel stability and hence vessel maturation [65,46]. The initial step in maturation must be to halt proliferation; supportive of this is the fact that TGF-β has been shown to inhibit endothelial cell growth [87]. It is believed that TGF-β activity occurs only after EC-SMC interaction [48].

In contrast to this a rat myocardial infarct model demonstrated an increase in vasculature correlated well with the presence of TGF-β [18]. However, it was suspected that TGF-β functioned in an indirect manner by stimulating the migration of growth factor secreting cells such as monocytes and fibroblasts [18]. Not surprisingly TGF-β also induces matrix deposition [48].

3.2.6 Other Factors

There is now emerging evidence of a wide variety factors with angiogenic properties from proteins as distinct as leptin, a fat regulatory protein, to copper, a simple metal often used as a cofactor [89,90]. Leptin's role in angiogenesis is still unclear, although new evidence is emerging that it is involved in some manner. Chick embryos treated with Leptin showed marked angiogenesis in comparison with controls. It is known that Leptin is a protein which binds it's cognate receptor on endothelial cells and causes an increase in reactive oxygen species production, specifically an increase in hydrogen peroxide [91]. The resultant increase in reactive oxygen species may lead to increased VEGF expression, which may be the driving force behind Leptin induced angiogenesis.

Copper has shown angiogenic potential, as it is both necessary and sufficient for endothelial cell proliferation [89]. This copper induced endothelial cell proliferation occurred in serum free media and was dose dependent leveling off at 200mM CuSO4. The proliferative effect of copper was comparable to that induced by bFGF [89]. This effect was specific to endothelial cells as there was no effect on either SMCs or fibroblasts [89].

In fact many cancer treatments are now aimed at reducing angiogenesis in an attempt to starve the tumors. In light of these findings a phase I clinical trial was carried out evaluating the effects of anti-copper therapy for treatment of metastatic cancer using the copper binding agent, Tetrahydromolybdate [90]. This trial showed minimal side effects and significant reduction in tumor size if therapy was maintained for at least 90 days.

Certain medical therapies, including ACE inhibitors and calcium channel blockers are emerging as having angiogenic "side effects" in addition to their primary purpose. For example, Quiniliprat an ACE inhibitor has been shown to induce angiogenesis to a level comparable of VEGF [92]. The mechanism is believed to involve the formation of nitric oxide, which acts on B1 receptors in post-capillary venules to induce endothelial cell growth [92]. Also, MCI-154, a calcium channel sensitizer, increases cardiac contractile efficiency by making the sarcomeres more sensitive to calcium [63]. However an added benefit of increased microvascular density was found during a study on dilated cardiomyopathy in the mouse model. Specifically, Hamster hearts treated with MCI-154 showed an increase in total capillary density, in addition to an increase in VEGF mRNA expression as determined by RNA northern blot analysis [63].

3.0 HYPOTHESIS

It is known that TMR induces angiogenesis and there is evidence to suggest that this angiogenesis is mediated via an inflammatory response. This inflammation leads to an infiltration of neutrophils and macrophages, in addition to the up-regulation of many growth factors including VEGF, bFGF, as well as the Angiopoietins. Based on this, it is hypothesized that increasing the degree of inflammation will lead to increased angiogenesis as well as an increase in growth factor expression. Therefore in an attempt to increase the degree of inflammation associated with TMR, the hypodermic needle was heated in situ after puncturing the myocardium. It is believed that the induction of a thermal injury in addition to a mechanical injury would lead to a greater inflammatory response and therefore increased angiogenesis in a controlled animal study.

The objective was thus to show increased angiogenesis in animals undergoing "HOT" TMR in comparison with either regular TMR or hypoxic injury alone. We also aimed at determining the differential expression of VEGF and the Angiopoietins in the various study groups. The animal model chosen was the rat, both for its precedence in the literature for this type of study and for its cost efficiency.

4.0 MATERIALS & METHODS

4.1 Surgical Procedure

Four groups (group 1, n=4; grp.2; n=14, grp.3; n=11; grp.4, n=18) of male Lewis Rats weighing 250-300g were used as the model in this study. Number of animals used per group varied based on target number required (Group1, n=4; Groups 2-4, n=10) and mortality associated with that group.

Rats were initially anesthetized by placement in to a chamber containing 5% isoflurane (by volume) for three minutes (or to the surgical plane of depth of anesthesia). This allowed for the intubation procedure using 16 gauge catheters. Anesthesia was maintained by 2.5% isoflurane administered intra-tracheally. The left chest was then shaved and a 3cm incision using scissors was performed to enter the third intercostal space. The sub-cutaneous and muscular layers were then incised and the ribs were retracted finally exposing the heart. The left anterior descending artery (LAD) was then identified at which point a suture was used to encircle the artery and ligate it (i.e. occlude it). This consistently caused a myocardial infarction in the region of the LAD in the left ventricle. After ligating the LAD, the muscular and subcutaneous layers of the chest wall were closed and the skin layer was closed using absorbable 3-0 vicryl sutures. The rats were then allowed to recover for two weeks. During the critical period (i.e. 48 hours post-op.) the animals were monitored 2-3 times daily. After which time the rats were monitored once daily. During monitoring rats were observed for anorexia and/or weight loss (>15%), lethargy, cachexia, dehydration, dyspnea or wound infection. If any such findings were present the rat was immediately sacrificed.

Animals were divided into four groups. A Sham group (Group I) underwent a thoracotomy but neither LAD ligation nor treatment. Negative control group (Group II) had its LAD ligated but received no treatment. Group III received TMR as treatment two weeks after coronary ligation. And finally group IV received "HOT TMR" two weeks after coronary ligation.

Treatment groups were again anesthetized as described above. Re-thoracotomy was done as described before, however through the fourth intercostal space in order to avoid adhesions that developed due to healing of the first incision. Chest closure was performed as described above. The rats were then monitored during the recovery period as described above.

One week following treatment animals were euthanized by exsanguination after administering anesthesia and the heart was resected for histological examination. After every surgical intervention the animals were given analgesia (buprenorphine; 0.01-0.05 mg/kg BW) every 8-12 hours for 36-48h.

Sections were cut and fixed in 10% formalin for ~18 hrs. After which they were embedded in paraffin wax from which 5-micrometer sections were cut.

4.1.1 TMR Technique

Once the infarcted region was exposed a 28.5 Gauge needle was used to create 12 channels through the myocardium. This was followed by chest wall closure and the animal was allowed to recover.

4.1.2 "HOT" TMR Technique

Once the infarcted region was exposed a 28.5 gauge needle was used to puncture the myocardium at which point a disposable cauterizer was placed in contact with the

exposed portion of the needle. The contact was maintained for ~10 sec. which caused a substantial heating of the needle. After removal of the needle a 4x4 Gauze was placed for 2-3 minutes on the punctured myocardium to reduce blood loss. This was repeated 8-12 times depending on blood loss, followed by chest closure and animal recovery.

4.2 Immunohistochemistry

Sections were placed in toluene for 20 min. for dewaxing after which they were placed in 100%, 90%, 70%, and 50% ethanol for 2 min each. This was followed by a 5 min rinse in phosphate buffered saline (PBS). Sections were then permeabilized using 0.3% triton-X100 for 30 min. Next the samples were rinsed with PBS for five minutes thrice. Placing the samples in 5% hydrogen peroxide for 30 min. saturated endogenous peroxidase activity. Samples were then rinsed again with PBS. Normal goat serum (added as a blocking agent at 10% for 30 min) followed this. After which the primary antibody was added and left for incubation at 4° C for 18 hours.

Post incubation samples were rinsed with PBS. The secondary antibody (biotinylated IgG) was then added to samples for 45 min. This was followed by a PBS rinse. Staining reagents were then added for 45 min. followed by a PBS rinse. The samples were then exposed to DAB solution (3'3 diaminobenzidene 0.025 g/100 ml) for 2-3 minutes. Cover slips were then added and sections were ready for analysis.

4.3 Quantification

Analysis of staining was done using an *Olympus BX760* microscope connected to a video camera and a computer using the *Image Pro Plus* Program. Specifically, using a camera affixed to a microscope a picture of the sample was taken. The picture was then imported into the *Image-pro plus* program where it was digitized. Pixels were then counted as

positive, if dark brown in color. The positive counts were then imported into *Microsoft Excel* for summation. As such, relative area could be determined by counting number of positive pixels per image (P.P.I.). This was repeated five times per section. One representative section was chosen from each animal in each group.

4.4 Reagents

Antibodies for VEGF, Ang-1 and Ang-2 were obtained from *Santa Cruz Biotechnology* (Santa Cruz, CA.). Anti-Factor VIII antibody was obtained from *Dako* ltd. Secondary biotinylated antibodies were obtained from *Vector Laboratories* (Burlingame, CA.) as was the staining kit for immunohistochemistry and the normal goat serum.

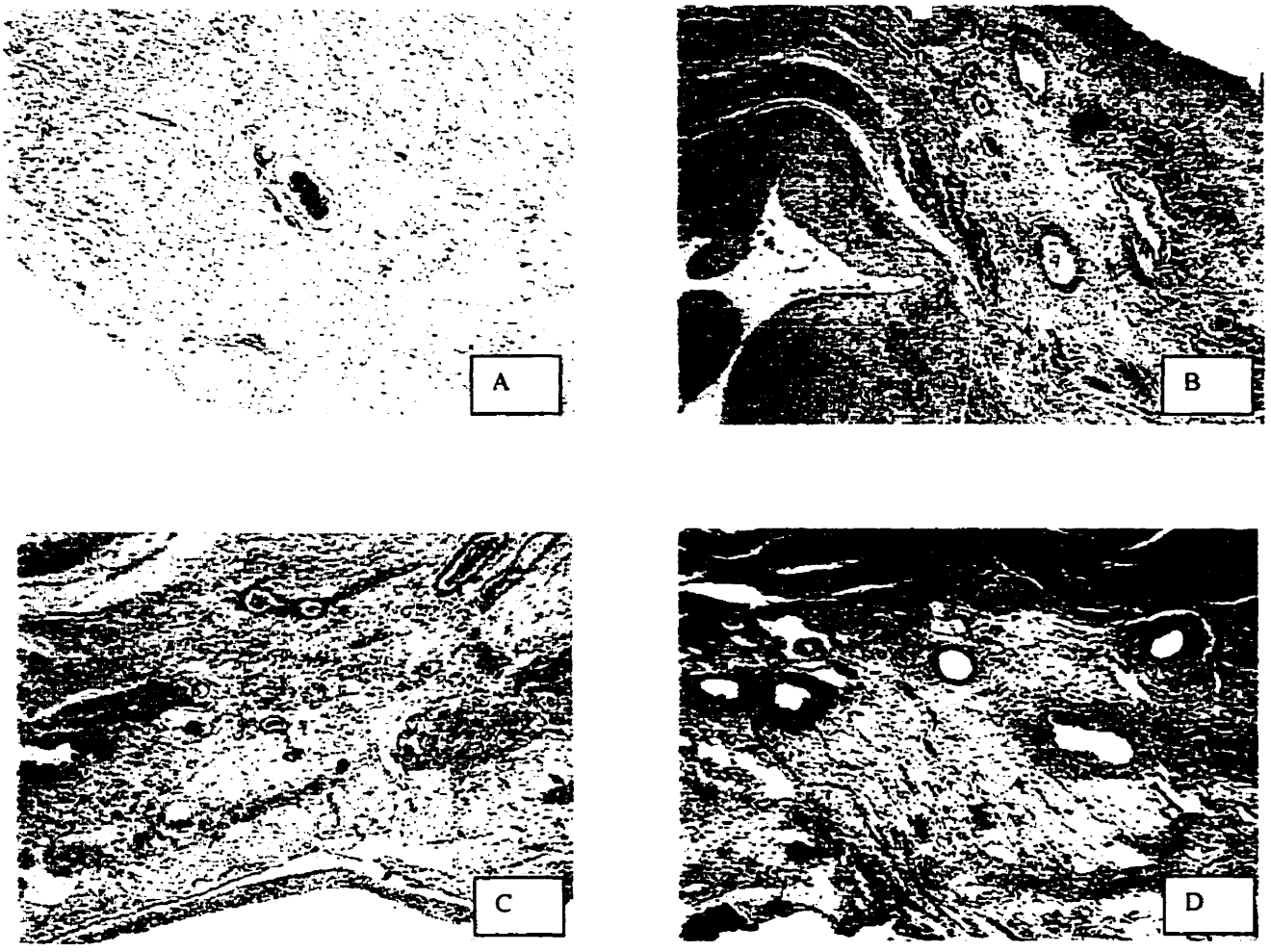
4.5 Statistics

Statistics were carried out using the *SYSTAT 8.0* statistical analysis program. Groups were compared using the Student's T-test for two groups. Meanwhile, correlation tests were used for to test for significant associations.

5.0 RESULTS

To assess the level of angiogenesis induced by this novel technique animals were divided into four groups. Group I (n=4) was a sham group, which was subjected only to a thoracotomy, not surprisingly there were no infarctions and no procedural deaths. Group 2 was subjected to thoracotomy followed by LAD ligation in which mortality was 30% (4/14). All surviving animals showed substantial infarcts. Group 3 was also subjected to LAD ligation however it received TMR as a treatment and it resulted in only 8% (1/11) mortality. 9 of the ten surviving animals (of group 3) experienced infarctions however 3 of them were not trans-mural infarcts. Group 4 was again subjected to LAD ligation but received "HOT" TMR as a treatment. This however led to a 44% (10/18) mortality rate. Again 9 of the 10 surviving animals (of group 4) experienced infarcts however two were not trans-mural infarcts. All animals that died prior to the sacrifice date were not included in the data analysis. Tissue samples with representative infarctions are shown in Figure 5.1

Figure 5.1



Representative myocardial sections from the four different groups stained with hematoxylin and eosin: (A) Group I. Negative control, the sample shows no sign of inflammation. (B) Group II. Coronary ligation only, this sample shows necrotic tissue surrounded by inflammatory infiltrates as well as increased blood vessels indicating signs of angiogenesis. (C) Group III. Coronary ligation + TMR, much like group II there are signs of inflammation and necrosis however the angiogenesis seems to be more prominent. (D) Group IV Coronary ligation +"HOT"TMR, again similar findings as in groups II & III however perhaps even more angiogenesis. (100 X magnification)

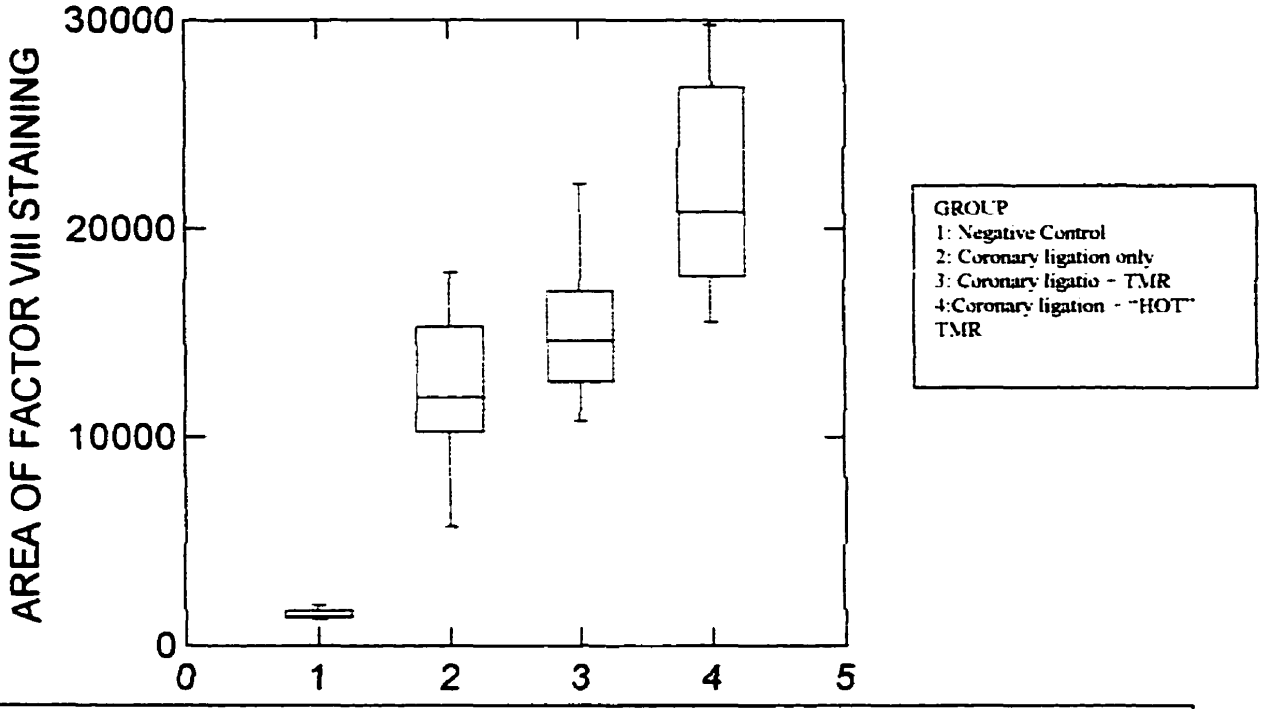
5.1 Neovascularization

The degree of neovascularization was determined indirectly by quantification of Factor VIII expression within the myocardial tissue sections. Factor VIII is a well-known endothelial cell marker. Factor VIII expression was quantified as relative area of staining per section but was not quantified with respect to varying staining intensities. The image of the tissue section was converted to a digital image and pixels within that image were counted as either positive or negative for Factor VIII staining. This prevented confusing varying endothelial cell numbers with differential expression of the protein.

It was found that animals from groups 2, 3, and 4 had significantly elevated levels of Factor VIII expression in comparison with group 1 the negative control ($p<0.01$) (see Figure 5.2). Specifically the degree of staining measured, as relative area per five high power fields was 12,129 ± 3,516 positive pixels per image (P.P.I.) for Group 2, which only received the coronary ligation. Group 3 on the other hand, which had TMR treatment in addition to coronary ligation showed 15,172 ± 3,594 P.P.I. Finally, Group 4 the experimental group which received coronary ligation followed by the "HOT" TMR treatment resulted in 21,983 ± 5,350 P.P.I. Whereas the negative control (Group 1) showed barely detectable levels (1,513 ± 297 P.P.I.). Representative pictures are shown in figure 5.3.

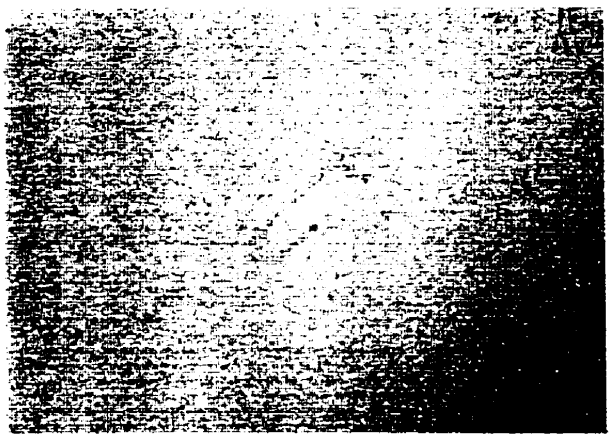
Figure 5.2

FACTOR VIII IMMUNOSTAINING



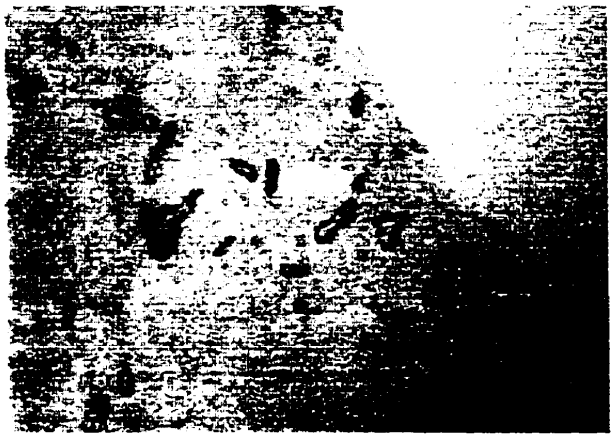
This graph demonstrates the degree of Factor VIII staining among the different groups using a box plot. Groups 2, 3, and 4 are all significantly ($p<0.01$) increased over the negative control group (Group 1). Group 4 is also significantly increased over Group 2 ($p=0.003$) and Group 3($p=0.004$). However Group 3 only showed a non-significant trend towards increased Factor VIII staining over Group 2 ($p=0.136$). The boxes represent the 2nd and 3rd quartiles of data separated by the median. While the lines extending from the boxes represent the 1st and 4th quartiles of data.

Figure 5.3

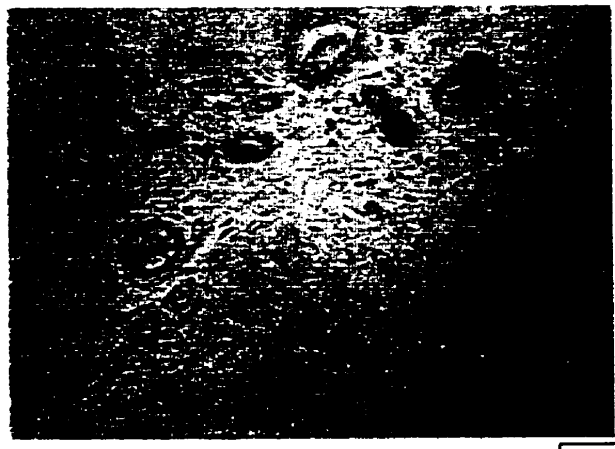


A

B



C



D

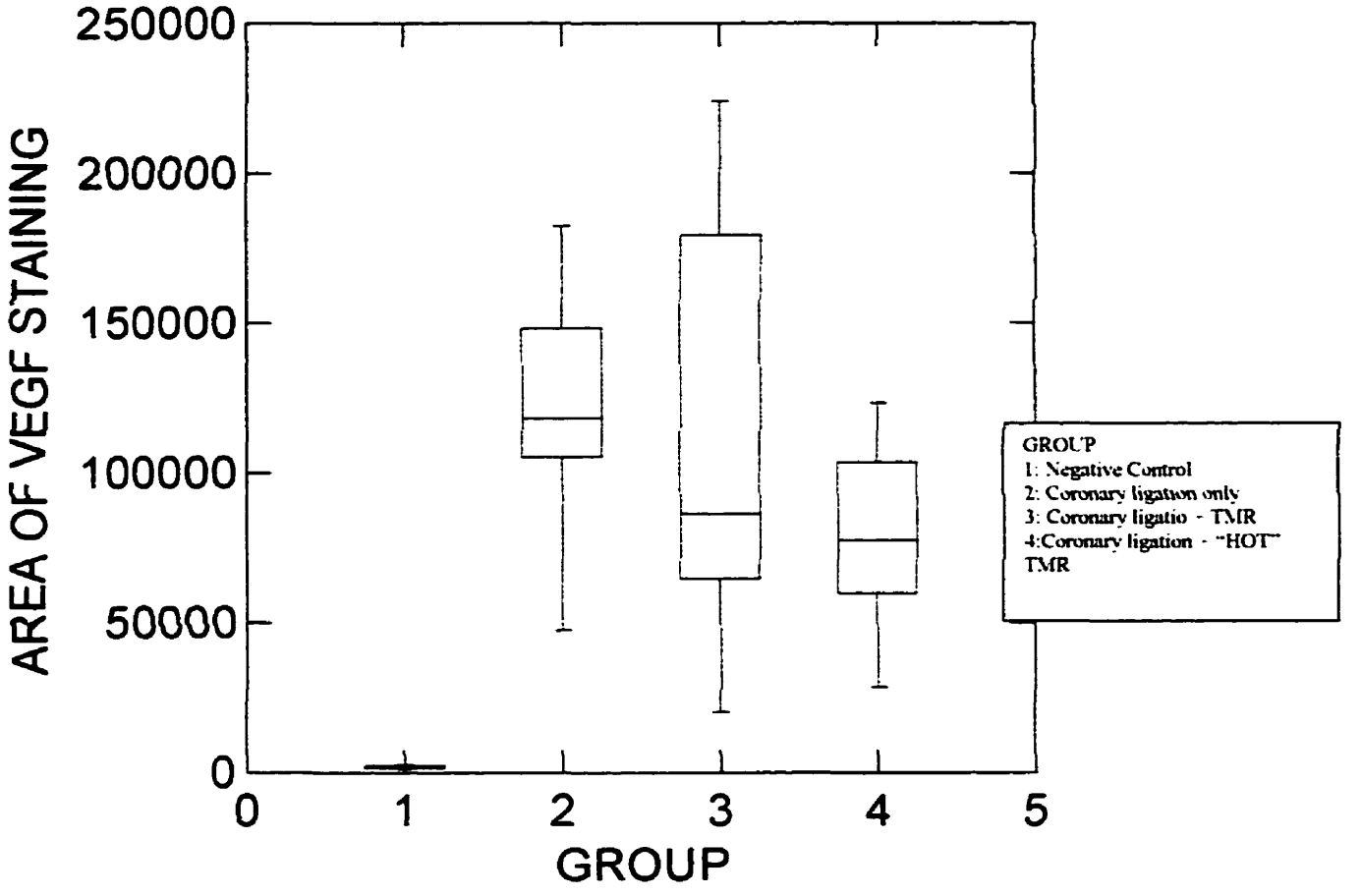
Representative myocardial sections of the four groups stained with anti-Factor VIII antibody. (A) Negative control.(β) Coronary ligation only, (C) Coronary ligation + TMR. (D) Coronary ligation +"HOT"TMR. (400 X magnification)

5.2 VEGF Expression

The expression of VEGF was also observed in groups 2, 3, and 4, however the degree of VEGF immunostaining in the negative control group was so minimal that it may have been just background staining inherent with immunohistochemistry (see figure 5.4). The elevated VEGF expression found in groups 2, 3, and 4 all reached statistical significance over the expression in the negative control group ($p<0.05$, see figure 5.5). Interestingly Group 2 showed a statistically significant increase in expression over Group 4 (121,568 ±41,284 P.P.I. VS. 79,211 ± 31,728 P.P.I., $p=0.038$). Group 3, on the other hand resulted in 138,918 ± 63,759 P.P.I., which was not statistically different from either group 2 or 4. The lack of statistical significance may be the result of its large variance.

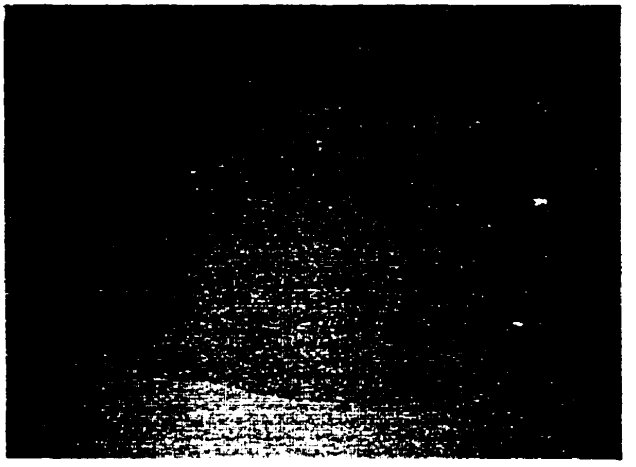
Figure 5.4

VEGF IMMUNOSTAINING

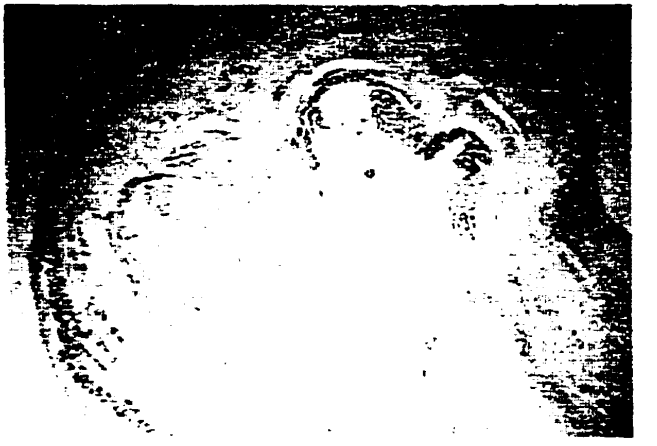


This graph demonstrates the degree of VEGF staining in the various groups. Groups 2,3 & 4 all showed significantly higher VEGF expression ($p<0.01$). Group 2 showed significantly higher VEGF expression than Group 4 ($p=0.002$). However Group 3 showed no significant difference from either Group 2 ($p=0816$) or Group 4 ($p=0.123$). The boxes represent the 2nd and 3rd quartiles of data separated by the median. While the lines extending from the boxes represent the 1st and 4th quartiles of *data*.

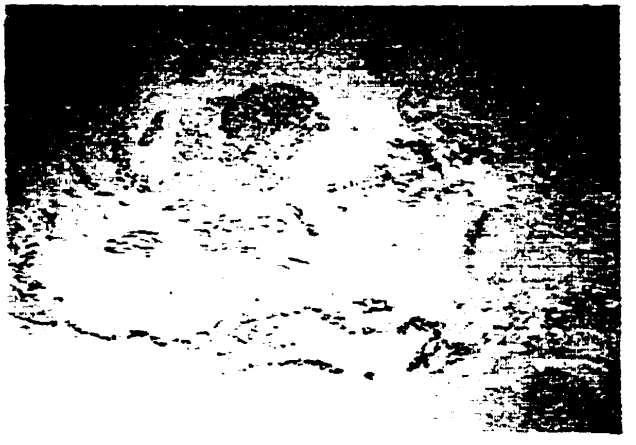
Figure 5.5



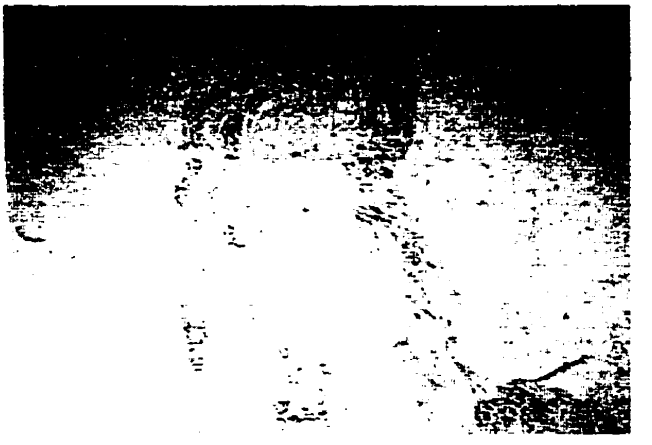
A



B



C



D

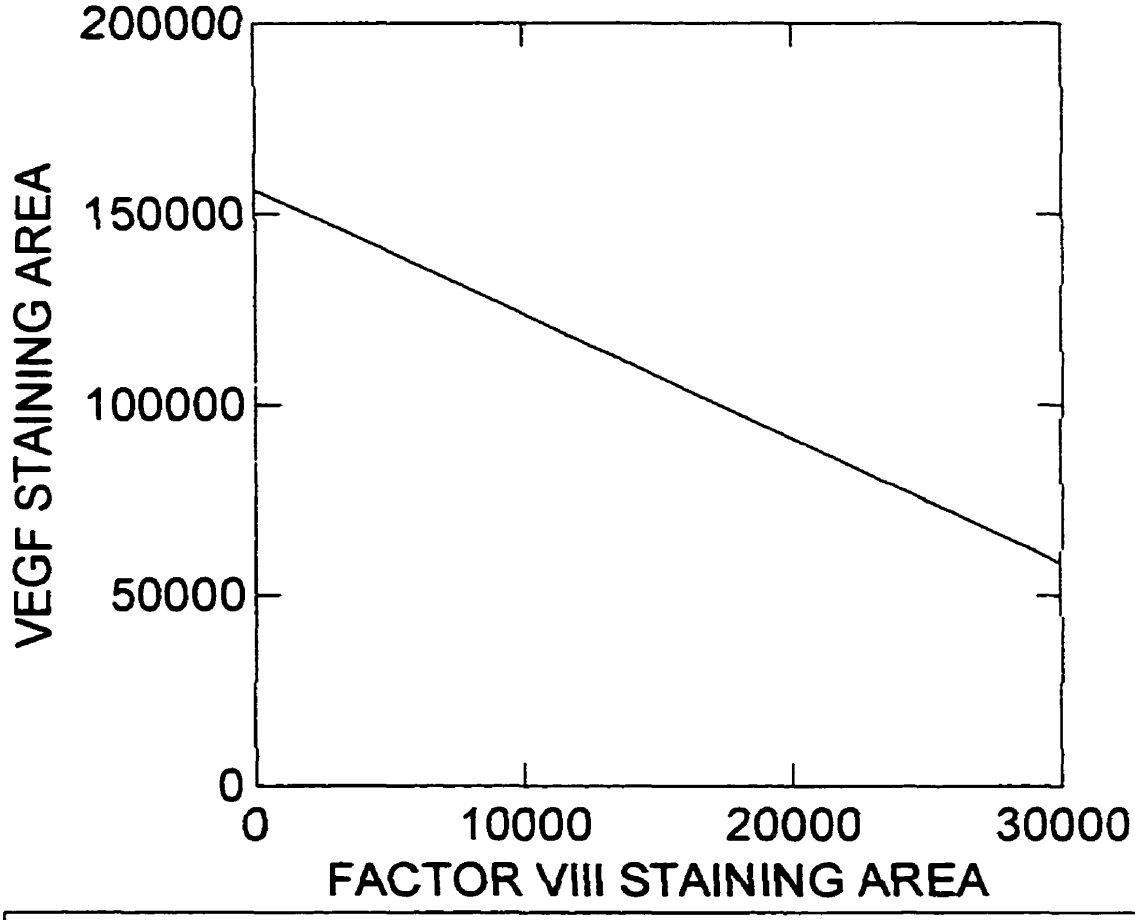
Representative myocardial sections of the four groups stained with an anti-VEGF antibody.(A) negative control.(β) Coronary ligation only. (C) coronary ligation + TMR. (D) Coronary ligation +"HOT"TMR. (40X magnification).

5.3 The VEGF-Factor VIII relationship

Since Group 4 showed significantly higher levels of Factor VIII expression compared to Group 2, yet Group 2 showed significantly higher levels of VEGF expression, a pair wise correlation test was conducted between groups 2 and 4 to determine if a statistically significant relationship existed. In fact, it was found that there was an inverse correlation between the degrees of Factor VIII staining and the degree of VEGF staining between the two groups and this gave a Pearson correlation coefficient of –0.56 ($p=0.036$, see figure 5.6).

Figure 5.6

RELATIONSHIP BETWEEN NEOVASCULARIZATION AND VEGF EXPRESSION AT 1 WEEK

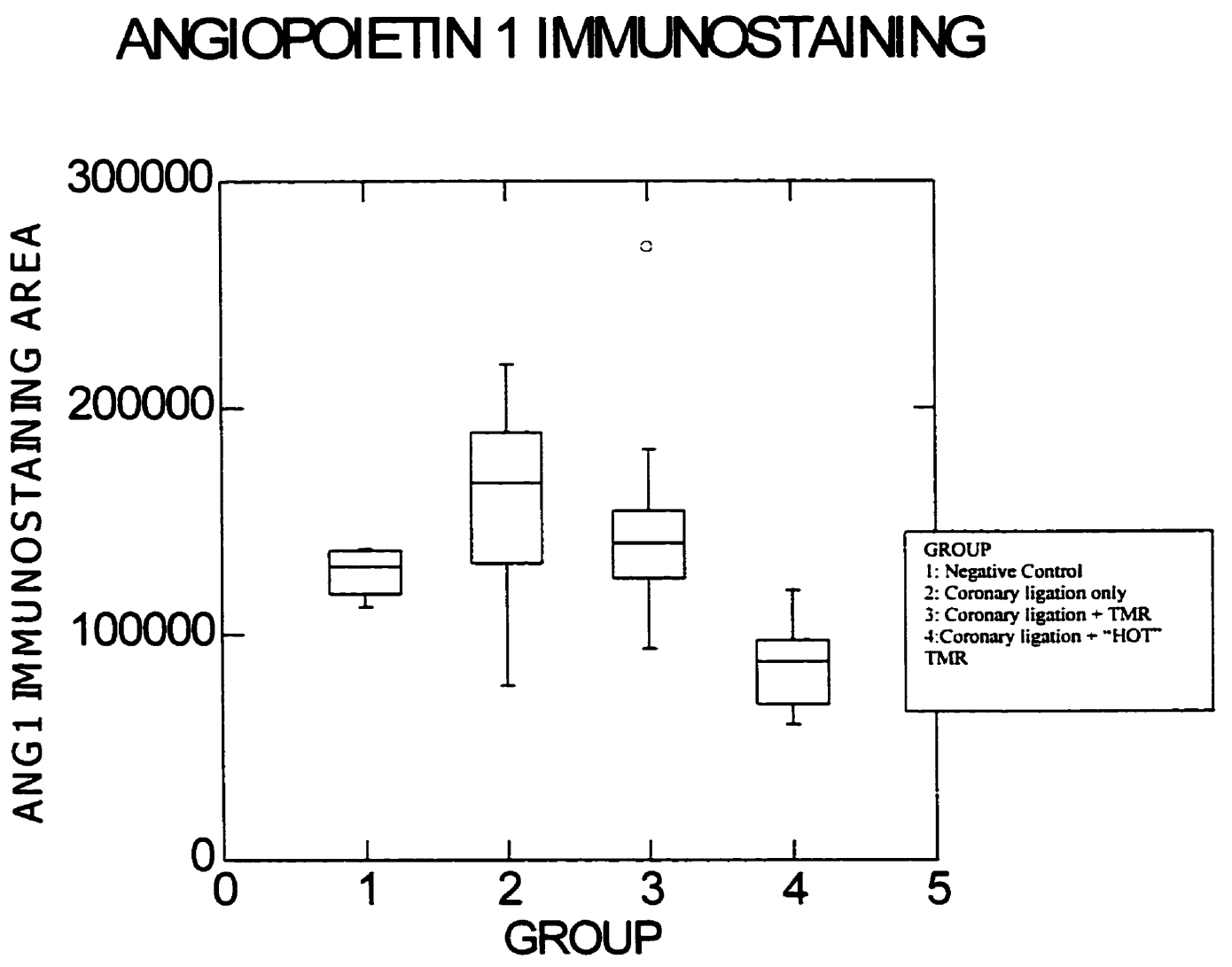


This graph demonstrates a significant inverse correlation between the degree of neovascularization (quantified by Factor VIII staining) and the level of VEGF expression in Groups 2 & 4. (Pearson correlation value:-0.526; p=0.036).

5.4 Angiopoietin Expression

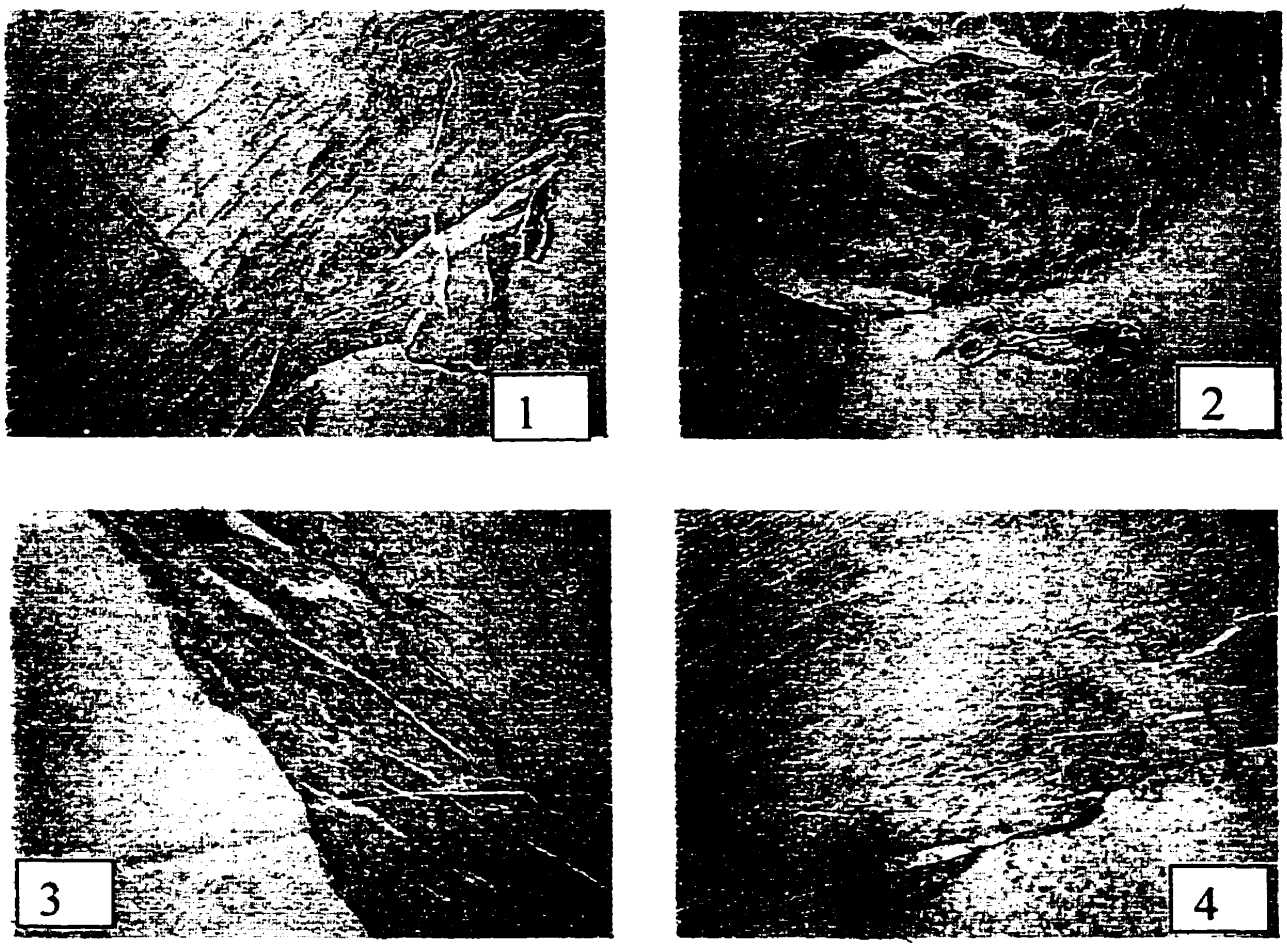
Angiopoietin 1 is constitutively expressed, as there were comparable levels of expression in the negative control group compared to the treatment groups. In fact the only significant difference in expression was observed in Group 4, which showed less expression than either Group1, Group 2 or Group 3 ($p=0.017$, $p=0.007$, and $p=0.019$ respectively; see figure 5.7). Qualitatively, most of the Ang 1 expression was observed in the sub-endocardium (see figure 5.8). Ang 1 was also expressed in the infarcted region, but only by surviving myocytes. Results for each group are presented in table 5.1.

Figure 5.7



This graph demonstrates the differential Angiopoietin 1 immunostaining among the groups. It shows that there is significantly less Ang 1 staining in Group 4 compared to Group1, Group 2 and Group 3 ($p=0.017$, $p=0.007$, and $p=0.019$, respectively). There are no other significant differences. The boxes represent the 2nd and 3rd quartiles of data separated by the median. While the lines extending from the boxes represent the 1st and 4th quartiles of data.

Figure 5.8



These are representative pictures of the four groups stained with Anti-Ang-1 antibody.
1: Negative Control; 2: Coronary ligation only; 3: Coronary ligation + TMR; 4:Coronary ligation + "HOT" TMR

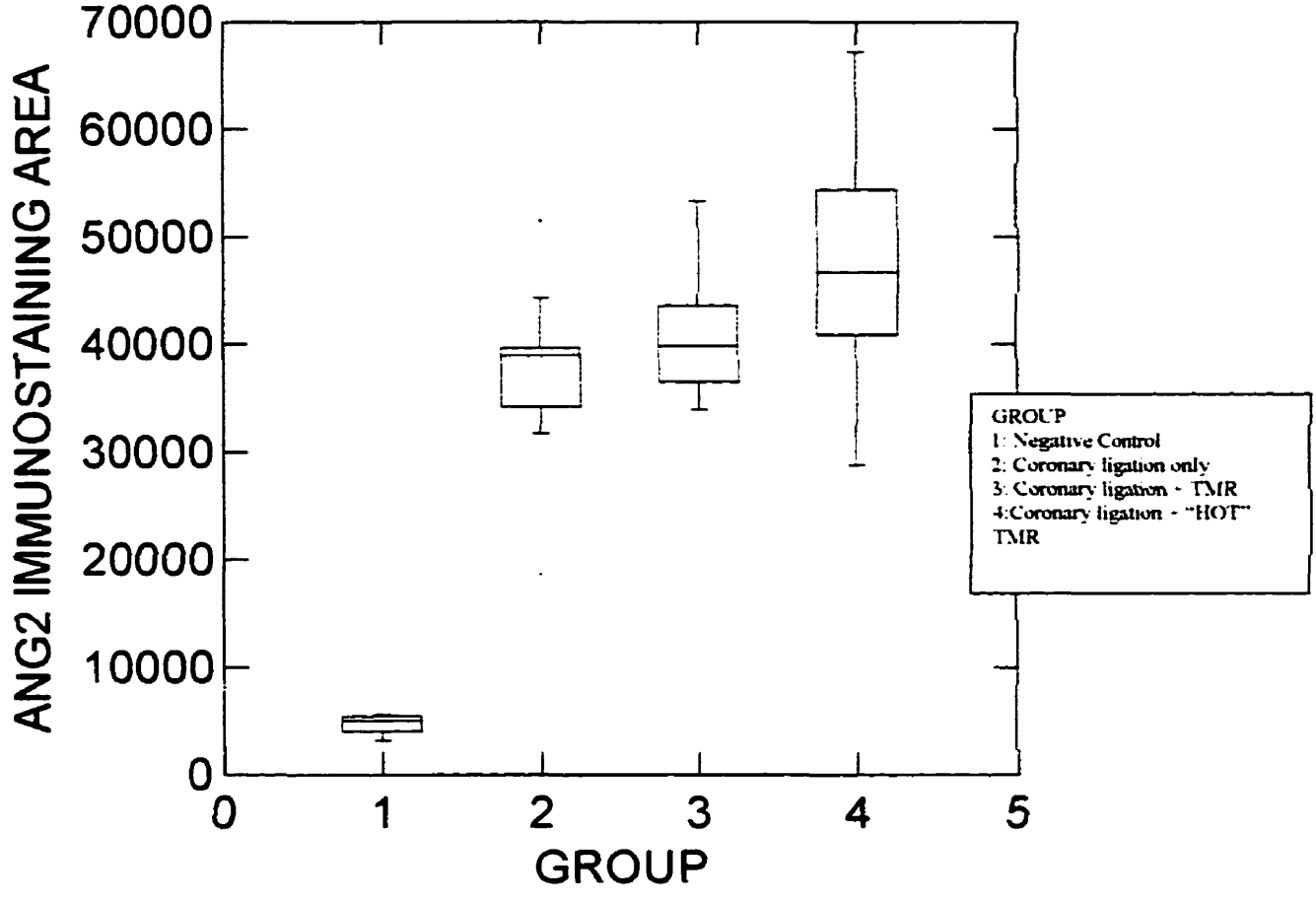
Angiopoietin 2 on the other hand was not highly expressed in the normal myocardium but was highly induced by ischemic damage. In fact all ischemic groups showed significantly elevated Ang 2 expression ($p<0.05$, see figure 5.9). However there was no significant difference in Ang 2 expression between any of the ischemic groups. Qualitatively it was found that most expression occurred in the infarcted region (see figure 5.10). However, there was also expression of Ang 2 in the sub-endocardium, though not as intense as for Ang-1. Interestingly, a significant correlation was observed between the degree of Factor VIII staining and the level of Ang-2 expression (Pearson correlation coefficient: 0.62, $p<0.001$, see figure 5.11). Results for each group are presented in table 5.1.

Table 5.1	Group I	Group II	Group III	Group IV
Factor VIII	1513 ± 297 P.P.I	12129 ± 3516 P.P.I	15172 ± 3594 P.P.I	21983 ± 5350 P.P.I
VEGF	1914 ± 620 P.P.I	121568 ±41284 P.P.I	113002 ± 75463 P.P.I	79211 ± 31728 P.P.I
Ang-1	127377 ± 11993 P.P.I	158906 ± 44838 P.P.I	149436 ± 52156 P.P.I	86648 ± 23481 P.P.I
Ang-2	4732 ± 1054 P.P.I	37330 ± 9039 P.P.I	40888 ± 6474 P.P.I	47612 ± 12768 P.P.I

P.P.I.: Positive Pixels per Image

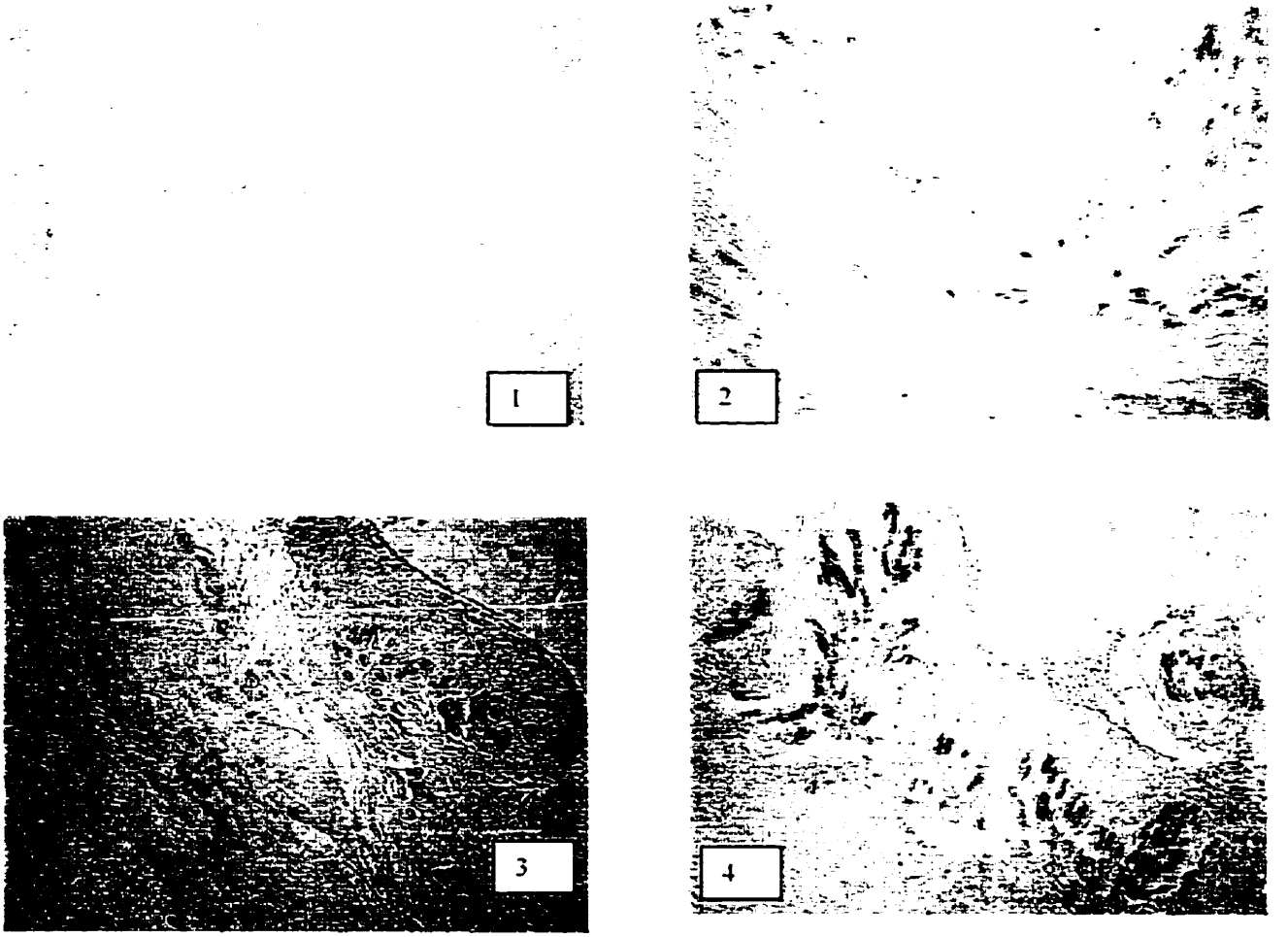
Figure 5.9

ANGIOPOIETIN 2 IMMUNOSTAINING



This graph demonstrates the differential expression in Angiopoietin 2 expression among the groups. Groups 2, 3, and 4 are all significantly higher in Ang 2 expression than Group 1, the negative control ($p<0.001$). There are no other significant differences. The boxes represent the 2nd and 3rd quartiles of data separated by the median. While the lines extending from the boxes represent the 1st and 4th quartiles of data.

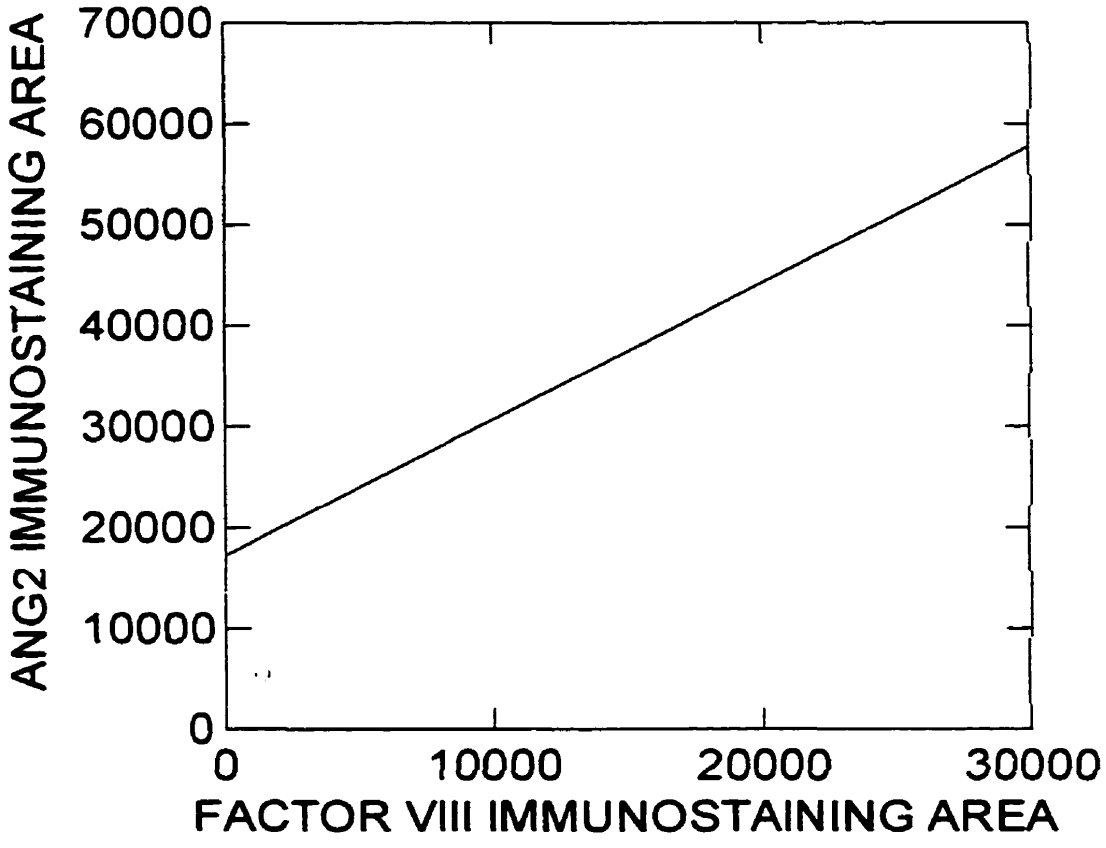
Figure 5.10



These are representative pictures of the four groups stained with Anti-Ang-2 antibody.
1: Negative Control: 2: Coronary ligation only: 3: Coronary ligation – TMR: 4:Coronary ligation – "HOT" TMR

RELATIONSHIP BETWEEN NEOVASCULARIZATION

AND ANG2 EXPRESSION



This graph demonstrates the direct correlation between Factor VIII expression and Ang2 expression. (Pearson correlation coefficient: 0.62, $p<0.001$).

6.0 DISCUSSION

6.1 Neovascularization

Increased perfusion after TMR has been observed in animal studies, but often not in clinical studies [31]. Also symptomatic improvement has been observed but long-term effects have been questioned. For example, in one study conducted by De Carlo et al. it was found that there was significant recurrence of angina at the second follow-up in post-TMR patients [33]. Another study by Aaberge et al. demonstrated that TMR led to significant symptomatic improvement but did not increase exercise tolerance [34]. In a study evaluating the clinical efficacy of combining TMR with conventional revascularization in patients not amendable to complete revascularization it was found that there was no additional symptomatic improvement with the added TMR [94].

The controversial data regarding clinical efficacy of TMR prompted us to determine if TMR therapy could be improved upon. It is known that TMR does result in angiogenesis, and it is now widely believed that this angiogenesis is the result of the wound healing effect mediated via the inflammatory response [27,26]. Hence the degree of angiogenesis is most probably related to the degree of tissue damage and inflammation. In agreement with this, Chu et al. stated that although laser mediated TMR induces a greater angiogenic effect per channel formed, ultimately the same degree of angiogenesis can be achieved using a needle simply by making more needle punctures [20].

Hence it was hypothesized that increasing the degree of tissue damage would lead to an increased inflammatory response and hence an increased angiogenic stimulus. Therefore, this study looked at the potential for angiogenesis using a heated needle for

mechanical TMR, which would lead to a thermal injury in addition to a mechanical injury.

Using immunohistochemistry it was possible to label the vascular endothelium and as such quantify the degree of neovascularization. From these results it was found that the hypoxic stimulus alone (created by the ligation of the left coronary artery) was sufficient to induce angiogenesis. Moreover, it was found that the new "HOT" TMR technique also induced angiogenesis, however this angiogenic effect was increased over and above that produced by either hypoxia alone or by regular TMR. The increased angiogenesis can therefore be attributed to the thermal injury induced by heating of the needle.

However a high mortality rate (44%) was associated with this procedure in comparison with the mortality observed in the regular TMR group (8.3%). Specifically there were two operative deaths and six more within the 24-hour post-operative period. It was found that the "HOT" TMR technique led to excessive bleeding which is attributable to the fact that the channels created by the novel technique would remain patent for 2-3 minutes even with lightly applied pressure. It is likely that that the deaths were due to excessive blood loss and hypovolemic shock. Blood loss may have continued post-operatively even though hemostasis was achieved prior to chest closure. This is supported by the observation of a hemothorax in one animal that died 24 hours post-operatively.

Excessive blood loss is not surprising considering it has been observed in some clinical cases of TMR where it led to pericardial tamponade [100]. Hence future studies should focus on maintaining the increased angiogenesis using the "HOT" TMR technique

while reducing concomitant blood loss. A reduction in blood loss could be achieved by using hemostatic agents such as fibrin glue, gelatin foam, and vicryl patches as adjunctive therapies. Thus by applying a hemostatic agent mortality would be expected to decrease while angiogenesis should remain high. Other considerations include reducing blood loss by using either a smaller needle or by reducing thermal damage. However it is likely this would also lead to a reduced angiogenic stimulus as well. Finally, perhaps other sources of thermal injury that do not lead to excessive bleeding could be considered as adjuncts.

Interestingly it was found that regular TMR did not lead to a statistically significant increase in angiogenesis compared with the hypoxic stimulus created by coronary ligation alone. The neovascularization was increased in this group (i.e. group 3), but it did not reach statistical significance. This could be due to several different factors including the variability of infarct size, which is an inherent problem with ligation of the coronary artery. This is so because of the difficulty associated with ligating such small vessels, which are at times not even visible. Also the coronary vasculature is variable and as such it is not possible to predict the presence of co-laterals that would maintain perfusion distal to the coronary occlusion. To circumvent this variable some researchers such as Li et al. are inducing myocardial necrosis by cryo-injury [93]. This method can produce consistent sized myocardial scars however it is not a hypoxic injury and as such may not be a clinically relevant model for the study of ischemic heart disease.

The lack of a statistically significant angiogenic response may very likely be associated with the level of tissue damage resulting from the needle punctures. In the present study a 28.5 gauge needle was used to make 12 puncture wounds in the ischemic

region. Perhaps more punctures or the use of a larger needle such as a 25-gauge needle would have induced a more significant angiogenic effect.

6.2 VEGF Expression

Immunohistochemical analyses of the sections stained with anti-VEGF antibody showed substantial expression in infarcted regions, however non-ischemic regions showed very little, if any staining. This is in accordance with the fact that hypoxia induces VEGF expression. Interestingly, it was found that group 2 had significantly less angiogenesis, but significantly higher VEGF expression than group 4, the "HOT" TMR group. It is known that VEGF is unstable in normoxic conditions but is stabilized in hypoxic tissue [62]. It is possible that the chronic ischemia experienced by group 2 animals maintained the expression of VEGF. On the other hand, perhaps group 4 animals had increased perfusion due to the greater degree of neovascularization. This increased perfusion may have led to a decrease in tissue hypoxia, thus destabilizing VEGF transcripts. Interestingly, Pelletier et al., who used an identical model failed to demonstrate an increased VEGF expression even with increased angiogenesis [18].

6.3 Angiopoietin Expression

Angiopoietin-1 is a constitutively expressed protein that acts as a vessel stabilizer. In agreement with this it was found that there was substantial Ang-1 expression in the normal myocardium (group 1, negative control.). Interestingly though the expression of Ang-1 in the experimental "HOT" TMR group was found to be significantly decreased. This leads to the speculation that neovascularization induces a decrease in Ang-1 expression. There is evidence to support this idea in studies on tumors. For example one study showed that the normal lung expressed high levels of Ang-1 while non-small cell

lung carcinomas which were undergoing vast angiogenesis showed significant decreases in Ang-1 expression [97]. Another study on a breast cancer cell line showed that overexpression of Ang-1 led to a three-fold reduction in tumor size suggesting it had anti-angiogenic effects in this model [101].

On the other hand, Ang-1 expression has been associated with increased angiogenesis, although only when co-expressed with other angiogenic factors. For example Shyu et al. demonstrated that Ang-1 encoded plasmid DNA induced angiogenesis when injected into an ischemic hindlimb [7]. However, this experiment used an ischemic hindlimb as the model, in which there were already other ischemically induced angiogenic factors present. Also McClain et al. showed that transgenic mice overexpressing Ang-1 resulted in increased vessel number, size, and branching pattern [46]. However this study used a developmental model in which the mice were exposed to the increased Ang-1 levels since embryogenesis and as such the Ang-1 overexpression again occurred during expression of other angiogenic factors that are very prominent during vasculogenesis.

Thus, perhaps Ang-1 potentiated the effects of the other angiogenic factors but it did not necessarily cause the increased angiogenesis. In agreement with this, a study by Asahara et al. found that Ang-1 alone did not induce angiogenesis in a non-ischemic corneal pocket assay, but when it was co-administered with VEGF (via sustained release pellets) there was an angiogenic response larger than that observed by VEGF alone [8].

Alternatively the apparent contrasting effects of Ang-1 could be related to differences in temporal expression of the factor. One study, which investigated the temporal expression of different angiogenic factors in cerebral ischemia, demonstrated

that Ang-1 was significantly increased only two weeks after the ischemic insult [98]. This suggests that the role of Ang-1 may only commence in later stages of angiogenesis, such as after the formation of the primary capillary plexus.

Therefore there is no doubt that Ang-1 is a major factor involved in angiogenesis but its effects must be kept in context both spatially and temporally. Finally, Ang-1 has been implicated as an anti-inflammatory agent as it prevents leakage of plasma and leukocytes from vessels [99]. Thus perhaps there is an association between the increased inflammation and the reduced Ang-1 expression in the "HOT" TMR group. Whether the increased inflammation resulted in an Ang-1 down-regulation and whether or not this had an impact on the angiogenesis remains to be elucidated.

It is known that hypoxia is an inducing stimulus for Ang-2 expression [37]. In agreement with this, groups 2, 3, and 4, which all received ischemic insults showed significant elevations in Ang-2 expression. Although Ang-2 expression did not differ significantly among any of the ischemic groups (i.e. Groups 2-4). There was a tendency for higher Ang-2 expression in the "HOT" TMR group but this did not reach statistical significance. However a direct correlation was observed between Ang-2 expression and neovascularization ($p<0.001$) which demonstrates the direct influence of Ang-2 expression on angiogenesis.

6.4 Conclusion

Thus to summarize it was found that the "HOT" TMR technique led to increased neovascularization (as determined by Factor VIII expression) compared to ischemia alone or regular TMR. This increased angiogenesis correlated inversely with VEGF expression

at 1 week suggesting the possibility of VEGF de-stabilization upon the return to normoxia in newly vascularized tissue. The "HOT" TMR group also showed reduced Ang-1 expression suggesting a necessity for Ang-1 down regulation early in the angiogenic process. Finally Ang-2 was significantly elevated in all ischemic groups representing its induction by hypoxia. A definite association was found between Ang-2 and neovascularization as there was a significant direct correlation between the level of Ang-2 and Factor VIII expression.

6.5 Future studies

Now that there is evidence that the increased tissue injury induced by "HOT" TMR led to increased neovascularization, future studies should focus on reducing the excessive blood loss associated with this procedure. Such adjunctive therapies could include fibrin glue, gelatin foam, and/or vicryl patches. The most appropriate hemostatic agent will need to be determined. Once blood loss has been controlled the focus of study should turn to whether or not this increased angiogenesis actually leads to increased perfusion and improved ventricular function. Alternatively another means of thermal injury could be researched. The observed increase in angiogenesis in this study is however unlikely to be specifically due to the thermal injury. Hence research aimed at inducing a maximal degree of inflammation with minimal irreversible tissue damage has immense potential.

Also studies evaluating the temporal expression of VEGF in the ischemic myocardium may lead to a better understanding of its role in the angiogenic mechanism, which may lead to more effective use of VEGF in therapeutic angiogenesis. Also, the exact role of Angpoietin-1 in the angiogenic process should be further elucidated before

considering it for therapeutic angiogenesis. Finally the old saying remains true "what wont kill you will make you stronger".

REFERENCES

1. Beck CS.
   The development of a new blood supply to the heart by operation.
   Ann Surg 1935;102:801-13

2. Vineberg AM. Development of an anastamosis between the coronary vessels and a transplanted internal mammery artery.
   Can Med Assoc J 1946;55:117-9

3. Sen PK, Udwadia TE, Kinaire SG, Parulkar GB.
   Transmyocardial acupuncture.
   J Thorac Cardiovasc Surg 1950; 50:181-9.

4. Symes JF, Losordo DW, Vale PR, Lathi KG, Esakof DD, Mayskiy M, Isner JM.
   Gene therapy with vascular endothelial growth factor for inoperable coronary artery disease.
   Ann Thorac Surg. 1999 Sep;68(3):830-6; discussion 836-7.

5. Kornowski R, Fuchs S, Leon MB, Epstein SE.
   Delivery strategies to achieve therapeutic myocardial angiogenesis.
   Circulation. 2000 Feb 1:101(4):454-8. Review.

6. Lee LY, Patel SR, Hackett NR, Mack CA, Polce DR, El-Sawy T, Hachamovitch R, Zanzonico P, Sanborn TA, Parikh M, Isom OW, Crystal RG, Rosengart TK.
   Focal angiogen therapy using intramyocardial delivery of an adenovirus vector coding for vascular endothelial growth factor 121.
   Ann Thorac Surg. 2000 Jan;69(1):14-23; discussion 23-4.

7. Shyu KG, Manor O, Magner M, Yancopoulos GD, Isner JM.
   Direct intramuscular injection of plasmid DNA encoding angiopoietin-1 but not angiopoietin-2 augments revascularization in the rabbit ischemic hindlimb.
   Circulation. 1998 Nov 10:98(19):2081-7.

8. Asahara T, Chen D, Takahashi T, Fujikawa K, Kearney M, Magner M, Yancopoulos GD, Isner JM.
   Tie2 receptor ligands, angiopoietin-1 and angiopoietin-2, modulate VEGF-induced postnatal neovascularization.
   Circ Res. 1998 Aug 10;83(3):233-40.

9. Peters KG.
   Vascular endothelial growth factor and the angiopoietins: working together to build a better blood vessel.
   Circ Res. 1998 Aug 10;83(3):342-3.

10. Kawasuji M, Nagamine H, Ikeda M, Sakakibara N, Takemura H, Fujii S, Watanabe Y.
herapeutic angiogenesis with intramyocardial administration of basic fibroblast growth factor.
Ann Thorac Surg. 2000 Apr;69(4):1155-61.

11. Laham RJ, Rezaee M, Post M, Novicki D, Sellke FW, Pearlman JD, Simons M, Hung D.
Intrapericardial delivery of fibroblast growth factor-2 induces neovascularization in a porcine model of chronic myocardial ischemia.
J Pharmacol Exp Ther. 2000 Feb;292(2):795-802.

12. Gowdak LH, Poliakova L, Wang X, Kovesdi I, Fishbein KW, Zacheo A, Palumbo R, Straino S, Emanueli C, Marrocco-Trischitta M, Lakatta EG, Anversa P, Spencer RG, Talan M, Capogrossi MC.
Adenovirus-mediated VEGF(121) gene transfer stimulates angiogenesis in normoperfused skeletal muscle and preserves tissue perfusion after induction of ischemia.
Circulation. 2000 Aug 1;102(5):565-71.

13. Hendel RC, Henry TD, Rocha-Singh K, Isner JM, Kereiakes DJ, Giordano FJ, Simons M, Bonow RO.
Effect of intracoronary recombinant human vascular endothelial growth factor on myocardial perfusion: evidence for a dose-dependent effect.
Circulation. 2000 Jan 18;101(2):118-21.

14. Laham RJ, Sellke FW, Edelman ER, Pearlman JD, Ware JA, Brown DL, Gold JP, Simons M.
Local perivascular delivery of basic fibroblast growth factor in patients undergoing coronary bypass surgery: results of a phase I randomized, double-blind, placebo-controlled trial.
Circulation. 1999 Nov 2;100(18):1865-71.

15. Moussa I, Moses JW.
Angiogenesis for treatment of ischemic heart disease: should we worry about progression of atherosclerosis?
Circulation. 1999 Nov 30;100(22):e109.

16. SoRelle R.
Dilemma of angiogenesis.
Circulation. 2000 Jan 18;101(2):E23-4.

17. Whittaker P
Transmyocardial Revascularization: The fate of myocardial channels

Ann Thorac Surg 1999 68(6):2376-82

18. Pelletier MP, Giaid A, Sivaraman S, Dorfman J, Li CM, Philip A, Chiu RC.
Angiogenesis and growth factor expression in a model of transmyocardial revascularization.
Ann Thorac Surg. 1998 Jul;66(1):12-8.

19. Hughes GC, Lowe JE, Kypson AP, St Louis JD, Pippen AM, Peters KG, Coleman RE, DeGrado TR, Donovan CL, Annex BH,
Landolfo KP.
Neovascularization after transmyocardial laser revascularization in a model of chronic ischemia.
Ann Thorac Surg. 1998 Dec;66(6):2029-36.

20. Chu V, Kuang Jq, McGinn A, Giaid A, Korkola S, Chiu RC.
Angiogenic response induced by mechanical transmyocardial revascularization.
J Thorac Cardiovasc Surg. 1999 Nov;118(5):849-56.

21. Horvath KA, Chiu E, Maun DC, Lomasney JW, Greene R, Pearce WH, Fullerton DA.
Up-regulation of vascular endothelial growth factor mRNA and angiogenesis after transmyocardial laser revascularization.
Ann Thorac Surg. 1999 Sep;68(3):825-9.

22. Yamamoto N, Kohmoto T, Roethy W, Gu A, DeRosa C, Rabbani LE, Smith CR, Burkhoff D
Histologic evidence that basic fibroblast growth factor enhances the angiogenic effects of transmyocardial laser revascularization.
Basic Res Cardiol 2000 Feb;95(1):55-63

23. Yamamoto N, Kohmoto T, Gu A, DeRosa C, Smith CR, Burkhoff D
Angiogenesis is enhanced in ischemic canine myocardium by transmyocardial laser revascularization.
J Am Coll Cardiol 1998 May;31(6):1426-33

24. Hirsch GM, Thompson GW, Arora RC, Hirsch KJ, Sullivan JA, Armour JA.
Transmyocardial laser revascularization does not denervate the canine heart.
Ann Thorac Surg. 1999 Aug;68(2):460-8; discussion 468-9.

25. Lansing AM
Transmyocardial Revascularization—late results and mechanism of action
J KY Med Assoc 2000 Sep;98(9):406-12

26. Bridges CR
Myocardial laser revascularization : the controversy and the data.
Ann Thorac Surg 2000 Feb ; 69(2):655-62

27. Bortone AS, D'Agostino D, Schena S, Rubini G, Brindicci P, Sardaro V, D'Addabbo A, de Luca Tupputi Schinosa L.
Inflammatory response and angiogenesis after percutaneous transmyocardial laser revascularization.
Ann Thorac Surg. 2000 Sep;70(3):1134-8.

28. Bortone AS, D'Agostino D, Schena S, Rubini G, Viecca M, Sardaro V, Tucci A, de Luca Tupputi Schinosa L.
Instrumental validation of percutaneous transmyocardial revascularization: follow-up data at one year.
Ann Thorac Surg. 2000 Sep:70(3):1115-8.

29. Frazier OH, March RJ, Horvath KA
Transmyocardial revascularization with a carbon dioxide laser in patients with end-stage coronary artery disease.
N Engl J Med 1999 Sep 30;341(14):1021-8

30. Allen KB, Dowling RD, Fudge TL, Schoettle GP, Selinger SL, Gangahar DM, Angell WW, Petracek MR, Shaar CJ, O'Neill WW
Comparison of transmyocardial revascularization with medical therapy in patients with refractory angina.
N Engl J Med 1999 Sep 30;341(14):1029-36

31. Lutter G, Sarai K, Nitzsche E, Saurbier B, Frey M, Hoegerle S, Martin J, Zipfel M, Spillner G, Beyersdorf F.
Evaluation of transmyocardial laser revascularization by following objective parameters of perfusion and ventricular function.
Thorac Cardiovasc Surg. 2000 Apr;48(2):79-85.

32. Lee LY, O'Hara MF, Finnin EB, Hachamovitch R, Szulc M, Kligfield PD, Okin PM, Isom OW, Rosengart TK.
Transmyocardial laser revascularization with excimer laser: clinical results at 1 year.
Ann Thorac Surg. 2000 Aug;70(2):498-503.

33. De Carlo M, Milano AD, Pratali S, Levantino M, Mariotti R, Bortolotti U.
Symptomatic improvement after transmyocardial laser revascularization: how long does it last?
Ann Thorac Surg. 2000 Sep;70(3):1130-3.

34. Aaberge L, Nordstrand K, Dragsund M, Saatvedt K, Endresen K, Golf S, Geiran O, Abdelnoor M, Forfang K.
Transmyocardial revascularization with CO2 laser in patients with refractory angina pectoris. Clinical results from the Norwegian randomized trial.
J Am Coll Cardiol. 2000 Apr;35(5):1170-7.

35. Weihrauch D, Arras M, Zimmermann R, Schaper J
Importance of monocytes/macrophages and fibroblasts for healing of micronecroses in porcine myocardium.
Mol Cell Biochem 1995 Jun 7-21;147(1-2):13-9

36. Kluge A, Zimmermann R, Munkel B, Mohri M, Sack S, Schaper J, Schaper W
Insulin-like growth factor I is involved in inflammation linked angiogenic processes after microembolisation in porcine heart.
Cardiovasc Res 1995 Mar;29(3):407-15

37. Oh H, Takagi H, Suzuma K, Otani A, Matsumura M, Honda Y.
Hypoxia and vascular endothelial growth factor selectively up-regulate angiopoietin-2 in bovine microvascular endothelial cells.
J Biol Chem. 1999 May 28;274(22):15732-9.

38. Li J, Post M, Volk R, Gao Y, Li M, Metais C, Sato K, Tsai J, Aird W, Rosenberg RD, Hampton TG, Sellke F, Carmeliet P, Simons M.
PR39, a peptide regulator of angiogenesis.
Nat Med. 2000 Jan;6(1):49-55.

39. Michiels C, Arnould T, Remacle J
Endothelial cell responses to hypoxia: initiation of a cascade of cellular interactions.
Biochim Biophys Acta 2000 Jun 2;1497(1):1-10

40. Helisch A, Ware JA.
Therapeutic angiogenesis in ischemic heart disease.
Thromb Haemost. 1999 Aug;82(2):772-80. Review.

41. McCourt M, Wang JH, Sookhai S, Redmond HP
Proinflammatory mediators stimulate neutrophil-directed angiogenesis.
Arch Surg 1999 Dec;134(12):1325-31; discussion 1331-2

42. Yasuda M, Shimizu S, Tokuyama S, Watanabe T, Kiuchi Y, Yamamoto T
A novel effect of polymorphonuclear leukocytes in the facilitation of angiogenesis.
Life Sci 2000 Apr 14;66(21):2113-21

43. Sunderkotter C, Steinbrink K, Goebeler M, Bhardwaj R, Sorg C
Macrophages and angiogenesis.
J Leukoc Biol 1994 Mar;55(3):410-22

44. Torisu H, Ono M, Kiryu H, Furue M, Ohmoto Y, Nakayama J, Nishioka Y, Sone S, KuwanoM
Macrophage infiltration correlates with tumor stage and angiogenesis in human malignant melanoma:
possible involvement of TNFalpha and IL-1alpha.
Int J Cancer 2000 Jan 15;85(2):182-8

45. Steinbrech DS, Longaker MT, Mehrara BJ, Saadeh PB, Chin GS, Gerrets RP, Chau DC, Rowe NM, Gittes GK
Fibroblast response to hypoxia: the relationship between angiogenesis and matrix regulation.
J Surg Res 1999 Jun 15;84(2):127-33

46. Lauren J, Gunji Y, Alitalo K.
Is angiopoietin-2 necessary for the initiation of tumor angiogenesis?
Am J Pathol. 1998 Nov;153(5):1333-9. Review.

47. Nehls V, Denzer K, Drenckhahn D.
Pericyte involvement in capillary sprouting during angiogenesis in situ.
Cell Tissue Res. 1992 Dec;270(3):469-74.

48. Folkman J, D'Amore PA.
Blood vessel formation: what is its molecular basis?
Cell. 1996 Dec 27;87(7):1153-5. Review.

49. Stratmann A, Risau W, Plate KH.
Cell type-specific expression of angiopoietin-1 and angiopoietin-2 suggests a role in glioblastoma angiogenesis.
Am J Pathol. 1998 Nov;153(5):1459-66.

50. Nehls V, Schuchardt E, Drenckhahn D.
The effect of fibroblasts, vascular smooth muscle cells, and pericytes on sprout formation of endothelial cells in a fibrin gel angiogenesis system.
Microvasc Res. 1994 Nov;48(3):349-63.

51. Nicosia RF, Villaschi S.
Rat aortic smooth muscle cells become pericytes during angiogenesis in vitro.
Lab Invest. 1995 Nov;73(5):658-66.

52. Doherty MJ, Canfield AE.
Gene expression during vascular pericyte differentiation.

Crit Rev Eukaryot Gene Expr. 1999;9(1):1-17. Review.

53. Hirschi KK, D'Amore PA.
Pericytes in the microvasculature.
Cardiovasc Res. 1996 Oct;32(4):687-98. Review

54. Grosskreutz CL, Anand-Apte B, Duplaa C, Quinn TP, Terman BI, Zetter B, D'Amore PA.
Vascular endothelial growth factor-induced migration of vascular smooth muscle cells in vitro.
Microvasc Res. 1999 Sep;58(2):128-36.

55. Verbeek MM, Otte-Holler I, Wesseling P, Ruiter DJ, de Waal RM.
Induction of alpha-smooth muscle actin expression in cultured human brain pericytes by transforming growth factor-beta 1.
Am J Pathol. 1994 Feb;144(2):372-82.

56. Carmeliet P.
Mechanisms of angiogenesis and arteriogenesis.
Nat Med. 2000 Apr;6(4):389-95. Review.

57. Helisch A, Ware JA.
Therapeutic angiogenesis in ischemic heart disease.
Thromb Haemost. 1999 Aug;82(2):772-80. Review.

58. Breier G, Damert A, Plate KH, Risau W.
Angiogenesis in embryos and ischemic diseases.
Thromb Haemost. 1997 Jul;78(1):678-83. Review.

59. Gale NW, Yancopoulos GD.
Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular
development.
Genes Dev. 1999 May 1;13(9):1055-66. Review.

60. Ziche M, Morbidelli L, Choudhuri R, Zhang HT, Donnini S, Granger HJ, Bicknell R.
Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis.
Clin Invest. 1997 Jun 1;99(11):2625-34.

61. Hojo Y, Ikeda U, Zhu Y, Okada M, Ueno S, Arakawa H, Fujikawa H, Katsuki T, Shimada K.

Expression of vascular endothelial growth factor in patients with acute myocardial infarction.
J Am Coll Cardiol. 2000 Mar 15;35(4):968-73.

62. Miraliakbari R, Francalancia NA, Lust RM, Gerardo JA, Ng PC, Sun YS, Chitwood WR Jr.
Differences in myocardial and peripheral VEGF and KDR levels after acute ischemia.
Ann Thorac Surg. 2000 Jun;69(6):1750-3; discussion 1754.

63. Kumamoto H, Okamoto H, Watanabe M, Onozuka H, Yoneya K, Nakagawa I, Chiba S, Watanabe S, Mikami T, Abe K, Kitabatake A.
Beneficial effect of myocardial angiogenesis on cardiac remodeling process by amlodipine and MCI-154.
Am J Physiol. 1999 Apr;276(4 Pt 2):H1117-23.

64. Starnes SL, Duncan BW, Kneebone JM, Rosenthal GL, Jones TK, Grifka RG, Cecchin F, Owens DJ, Fearneyhough C, Lupinetti FM.
Vascular endothelial growth factor and basic fibroblast growth factor in children with cyanotic congenital heart disease.
J Thorac Cardiovasc Surg. 2000 Mar;119(3):534-9.

65. Mandriota SJ, Pepper MS.
Regulation of angiopoietin-2 mRNA levels in bovine microvascular endothelial cells by cytokines and hypoxia.
Circ Res. 1998 Oct 19;83(8):852-9.

66. Thurston G, Suri C, Smith K, McClain J, Sato TN, Yancopoulos GD, McDonald DM.
Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1.
Science. 1999 Dec 24;286(5449):2511-4.

67. Thakker GD, Hajjar DP, Muller WA, Rosengart TK.
The role of phosphatidylinositol 3-kinase in vascular endothelial growth factor signaling.
J Biol Chem. 1999 Apr 9;274(15):10002-7.

68. Guo DQ, Wu LW, Dunbar JD, Ozes ON, Mayo LD, Kessler KM, Gustin JA, Baerwald MR, Jaffe EA, Warren RS, Donner DB.
Tumor necrosis factor employs a protein-tyrosine phosphatase to inhibit activation of KDR and vascular endothelial cell growth factor-induced endothelial cell proliferation.
J Biol Chem. 2000 Apr 14;275(15):11216-21.

69. Wu LW, Mayo LD, Dunbar JD, Kessler KM, Ozes ON, Warren RS, Donner DB.
VRAP is an adaptor protein that binds KDR, a receptor for vascular endothelial cell growth factor.

Biol Chem. 2000 Mar 3;275(9):6059-62.

70. Stefanec T.
Endothelial apoptosis: could it have a role in the pathogenesis and treatment of disease?
Chest. 2000 Mar;117(3):841-54. Review.

71. Huang L, Sankar S, Lin C, Kontos CD, Schroff AD, Cha EH, Feng SM, Li SF, Yu Z, Van Etten RL, Blanar MA, Peters KG.
HCPTPA, a protein tyrosine phosphatase that regulates vascular endothelial growth factor receptor-mediated signal transduction and biological activity.
J Biol Chem. 1999 Dec 31;274(53):38183-8.

72. Lee RJ, Springer ML, Blanco-Bose WE, Shaw R, Ursell PC, Blau HM.
VEGF gene delivery to myocardium: deleterious effects of unregulated expression.
Circulation. 2000 Aug 22;102(8):898-901.

73. Kwak HJ, So JN, Lee SJ, Kim I, Koh GY.
Angiopoietin-1 is an apoptosis survival factor for endothelial cells.
FEBS Lett. 1999 Apr 9;448(2-3):249-53.

74. Valenzuela DM, Griffiths JA, Rojas J, Aldrich TH, Jones PF, Zhou H, McClain J, Copeland NG, Gilbert DJ,
Jenkins NA, Huang T, Papadopoulos N, Maisonpierre PC, Davis S, Yancopoulos GD.
Angiopoietins 3 and 4: diverging gene counterparts in mice and humans.
Proc Natl Acad Sci U S A. 1999 Mar 2;96(5):1904-9.

75. Witzenbichler B, Maisonpierre PC, Jones P, Yancopoulos GD, Isner JM.
Chemotactic properties of angiopoietin-1 and -2, ligands for the endothelial-specific receptor tyrosine kinase Tie2.
J Biol Chem. 1998 Jul 17;273(29):18514-21.

76. Holash J, Maisonpierre PC, Compton D, Boland P, Alexander CR, Zagzag D, Yancopoulos GD, Wiegand SJ.
Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF.
Science. 1999 Jun 18;284(5422):1994-8.

77. Papapetropoulos A, Fulton D, Mahboubi K, Kalb RG, O'Connor DS, Li F, Altieri DC, Sessa WC.
Angiopoietin-1 inhibits endothelial cell apoptosis via the Akt/survivin pathway.
J Biol Chem. 2000 Mar 31;275(13):9102-5.

78. Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, Sato TN, Yancopoulos GD.
Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis.
Cell. 1996 Dec 27;87(7):1171-80.

79. Suri C, McClain J, Thurston G, McDonald DM, Zhou H, Oldmixon EH, Sato TN, Yancopoulos GD.
Increased vascularization in mice overexpressing angiopoietin-1.
Science. 1998 Oct 16;282(5388):468-71.

80. Holash J, Wiegand SJ, Yancopoulos GD.
New model of tumor angiogenesis: dynamic balance between vessel regression and growth mediated by angiopoietins and VEGF.
Oncogene. 1999 Sep 20;18(38):5356-62. Review.

81. Kim I, Moon SO, Koh KN, Kim H, Uhm CS, Kwak HJ, Kim NG, Koh GY.
Molecular cloning, expression, and characterization of angiopoietin-related protein. angiopoietin-related protein induces endothelial cell sprouting.
J Biol Chem. 1999 Sep 10;274(37):26523-8.

82. Davis S, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, Ryan TE, Bruno J, Radziejewski C, Maisonpierre PC, Yancopoulos GD.
Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning.
Cell. 1996 Dec 27;87(7):1161-9.

83. Jones N, Master Z, Jones J, Bouchard D, Gunji Y, Sasaki H, Daly R, Alitalo K, Dumont DJ.
Identification of Tek/Tie2 binding partners. Binding to a multifunctional docking site mediates cell survival and migration.
J Biol Chem. 1999 Oct 22;274(43):30896-905.

84. Willam C, Koehne P, Jurgensen JS, Grafe M, Wagner KD, Bachmann S, Frei U, Eckardt KU.
Tie2 receptor expression is stimulated by hypoxia and proinflammatory cytokines in human endothelial cells.
Circ Res. 2000 Sep 1;87(5):370-7.

85. Fachinger G, Deutsch U, Risau W.

Functional interaction of vascular endothelial-protein-tyrosine phosphatase with the angiopoietin receptor Tie-2.
Oncogene. 1999 Oct 21;18(43):5948-53.

86. Partanen TA, Makinen T, Arola J, Suda T, Weich HA, Alitalo K.
Endothelial growth factor receptors in human fetal heart.
Circulation. 1999 Aug 10;100(6):583-6.

87. Hirschi KK, Rohovsky SA, Beck LH, Smith SR, D'Amore PA.
Endothelial cells modulate the proliferation of mural cell precursors via platelet-derived growth factor-BB and heterotypic cell contact.
Circ Res. 1999 Feb 19;84(3):298-305.

88. Sun Y, Weber KT.
Cardiac remodelling by fibrous tissue: role of local factors and circulating hormones.
Ann Med. 1998 Aug;30 Suppl 1:3-8. Review.

89. Hu GF.
Copper stimulates proliferation of human endothelial cells under culture.
J Cell Biochem. 1998 Jun 1;69(3):326-35.

90. Brewer GJ, Dick RD, Grover DK, LeClaire V, Tseng M, Wicha M, Pienta K, Redman BG, Jahan T, Sondak VK, Strawderman M, LeCarpentier G, Merajver SD.
Treatment of metastatic cancer with tetrathiomolybdate, an anticopper, antiangiogenic agent: Phase I study.
Clin Cancer Res. 2000 Jan;6(1):1-10.

91. Bouloumie A, Drexler HC, Lafontan M, Busse R.
Leptin, the product of Ob gene, promotes angiogenesis.
Circ Res. 1998 Nov 16;83(10):1059-66.

92. Fabre JE, Rivard A, Magner M, Silver M, Isner JM.
Tissue inhibition of angiotensin-converting enzyme activity stimulates angiogenesis in vivo.
Circulation. 1999 Jun 15;99(23):3043-9.

93. Li RK, Jia ZQ, Weisel RD, Merante F, Mickle DA
Smooth muscle cell transplantation into myocardial scar tissue improves heart function.
J Mol Cell Cardiol 1999 Mar;31(3):513-22

94. Allen KB, Dowling RD, DelRossi AJ, Realyvasques F, Lefrak EA, Pfeffer TA, Fudge TL, Mostovych M, Schuch D, Szentpetery S, Shaar CJ.
Transmyocardial laser revascularization combined with coronary artery bypass grafting: a multicenter, blinded, prospective, randomized, controlled trial.

J Thorac Cardiovasc Surg. 2000 Mar;119(3):540-9.

95. Lee SH, Wolf PL, Escudero R, Deutsch R, Jamieson SW, Thistlethwaite PA
Early expression of angiogenesis factors in acute myocardial ischemia and infarction.
N Engl J Med 2000 Mar 2;342(9):626-33

96. Levy AP
A cellular paradigm for the failure to increase vascular endothelial growth factor in chronically hypoxic states.
Coron Artery Dis 1999 Sep;10(6):427-30

97. Wong MP, Chan SY, Fu KH, Leung SY, Cheung N, Yuen ST, Chung LP
The angiopoietins, tie2 and vascular endothelial growth factor are differentially expressed in the transformation of normal lung to non-small cell lung carcinomas.
Lung Cancer 2000 Jul;29(1):11-22

98. Lin TN, Wang CK, Cheung WM, Hsu CY
Induction of angiopoietin and Tie receptor mRNA expression after cerebral ischemia-reperfusion.
J Cereb Blood Flow Metab 2000 Feb;20(2):387-95

99. Gamble JR, Drew J, Trezise L, Underwood A, Parsons M, Kasminkas L, Rudge J, Yancopoulos G, Vadas MA
Angiopoietin-1 is an antipermeability and anti-inflammatory agent In vitro and targets cell junctions.
Circ Res 2000 Sep 29;87(7):603-7

100. Kaul U, Shawl F, Singh B, Sudan D, Sapra R, Ghose T, Dixit NS.
Percutaneous transluminal myocardial revascularization with a holmium laser system: procedural results and early clinical outcome.
Catheter Cardiovasc Interv. 1999 Jul;47(3):287-91.

101. Hayes AJ, Huang WQ, Yu J, Maisonpierre PC, Liu A, Kern FG, Lippman ME, McLeskey SW, Li LY
Expression and function of angiopoietin-1 in breast cancer.
Br J Cancer 2000 Nov;83(9):1154-60