Ph.D. Thesis

EVALUATION OF ANGIOGENIC STIMULATION FOR REVASCULARIZATION OF ISCHEMIC LIMBS

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PREFACE

The experimental study presented in this thesis was performed while the author was pursuing his surgical research training in the Department of Surgery, McGill University, Montreal, Quebec, Canada. The author was fortunately enrolled in the program at McGill University leading to a Ph.D. degree in experimental surgery. This thesis is primarily composed of 7 chapters. Chapter 1 includes a general introduction and an extensive literature review pertinent to the research project. The hypotheses and purposes of the present study are presented in chapter 2. The overall conclusions and contributions to original knowledge are presented in chapter 7. Except for chapters 1, 2, and 7, each chapter is written as a separate paper, as they have been submitted for publications. The author does not include connecting texts between chapter 3 to 6 because each chapter, by order, represents a continuing step–by–step approach for the study of angiogenic stimulation in revascularization of ischemic limbs. The literary style of the references in this thesis is followed by the one published in the Journal of Vascular Surgery. All findings contained in this thesis are that of the author, who is responsible for all the research work and who lays claim to the originality.

ABSTRACT

Limb salvage in patients with advanced peripheral vascular disease or effective nonoperative therapy for patients with limited disease still remains a challenge in modern vascular surgery. Based on recent advances in the area of angiogenesis as well as our previous studies of alternative approaches for limb ischemia, we have presented herein the step–by–step experimental investigations that are directed toward the development of a novel therapy, angiogenic stimulation, for the revascularization of ischemic limbs. An animal ischemic hindlimb model has been developed suitable for testing our hypothesis and further understanding of this potential therapy. We have demonstrated that an angiogenic factor, ECGF, when administered intramuscularly into the ischemic limb, markedly enhances revascularization of the limb in the models of unilateral as well as bilateral hindlimb ischemia. In addition, we have also demonstrated that the angiogenic effect of ECGF is dose dependent and is demonstrable only when it is administered directly into the limb in the presence of ischemia. Therefore, we conclude that angiogenic therapy may have the potential for the treatment of patients with chronic limb ischemia.

ABRÉGÉ

Les possibilités de sauver des membres chez les patients atteints d'une maladie vasculaire périphérique avancée, ou de suivre une thérapie non opératoire pour les patients souffrant d'une maladie limité, représentent toujours un défi dans le domaine de la chirurgie vasculaire moderne. En nous basant sur les récents progrès réalisés dans le secteur de l'angiogenèse et sur nos études antérieures en ce qui a trait aux approches touchant l'ischémie des membres, nous vous présentons ici les développement d'une nouvelle thérapie, la stimulation angiogénique, pour la revascularisation des membres ischémiques. En utilisant un modèle de membre postérieur ischémique d'un animal, nous avons été en mesure de tester notre hypothèse et de mieux connaître cette thérapie potentielle. Nous avons démontré qu'un facteur angiogénique, soit le facteur ECGF, lorsqu'il est administré de façon intramusculaire dans le membre ischémique, améliore de façon marquée la revascularisation du membre chez les modèles de membres postérieurs présentant une ischémie unilatérale et/ou bilatérale. De plus, nous avons également démontré que l'effet angiogénique du ECGF dépend de la dose et est démontrable seulement lorsqu'il est administré directement dans le membre en présence d'une ischémie. Par conséquent, nous en arrivons à la conclusion que la thérapie angiogénique peut avoir un certain potentiel en ce qui a trait au traitement de patients souffrant d'une ischémie chronique d'un membre.

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ABBREVIATIONS USED IN THE TEXT

ABI	Ankle–brachial index
aFGF	Acidic FGF
AVF	Arteriovenous fistula
AVR	Arteriovenous reversal
bFGF	Basic FGF
CP	Phosphocreatine
EC or ECs	Endothelial cell or Endothelial cells
ECGF	Endothelial cell growth factor
EFG	Epidermal growth factor
ESES	Epidural spinal cord electrical stimulation
FGF	Fibroblast growth factor
HDL	High density lipoproteins
H_2	Histamine–2
IMA	Internal mammary artery
LAD	Left anterior descending coronary artery
LCX	Left circumflex artery
LDL	Low density lipoproteins
LDV	Laser Doppler velocimetry
L/R	Left versus right
MCi	Millicurie
NMR	Nuclear magnetic resonance
NZW	New Zealand white
PD–ECGF	Platelet–derived endothelial cell growth factor
PDGF	Platelet–derived growth factor
PDWHF	Platelet–derived wound healing factors
PGE	Prostaglandins E
PTA	Percutaneous balloon angioplasty
PTFE or ePTFE	Polytetrafluoroethylene or expanded PTFE
PVR	Pulse volume recordings
SEM	Standard error of mean
SFA	Superficial femoral artery
^{99m}Tc	^{99m}Technetium
$TcPO_2$	Transcutaneous partial pressure of oxygen
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TTC	Triphenyltetrazolium chloride
VEGF	Vascular endothelial growth factor
VPF	Vascular permeability factor

CHAPTER I

GENERAL INTRODUCTION

1. PERIPHERAL VASCULAR DISEASE

Peripheral vascular disease is commonly thought to be the consequence of the extensive atherosclerotic lesions that narrow or occlude various arteries of the body. The lesions are more common in the femoropopliteal as well as in the aortoiliac or tibioperoneal arterial systems of patients who present with chronic lower extremity ischemia. The disease may also coexist with coronary and/or cerebrovascular disease and contributes significantly to the morbidity and mortality of the elderly population in modern western societies. In the first part of this chapter, I will briefly review the current knowledge of peripheral vascular disease including its epidemiology, etiology and risk factors, natural history, diagnosis, and treatment as well as the persisting or unresolved issues for patients with the disease. It represents the current understanding of peripheral vascular disease in the field.

1.1 Epidemiology

Endemic in the most developed countries, peripheral vascular disease is increasing in its prevalence and incidence. Age, cigarette smoking, dietary excess, diabetes mellitus, high blood pressure, hypercholesterolemia, and genetic predisposition, which play leading roles in the development of atherosclerosis, may contribute to this increase.[1] The number of patients discharged from the hospitals in the United States with the first–listed diagnosis of lower extremity atherosclerosis has increased steadily by 1% per year in the last decade.[2] However, despite decades of epidemiologic studies, we still do not know

with certainty the incidence and prevalence of peripheral vascular disease in the general population. Of note, the summary of the results of selected epidemiologic surveys from the past several decades have similar overall results despite varying methods for diagnosing the disease. In the general adult (age 20 and above) population, the annual incidence and prevalence of clinically significant peripheral vascular disease presenting with chronic lower–extremity ischemia are between 0.5% to 2% with the highest incidence and prevalence occurring in the age group of 65 to 74 years. The incidence of the disease is found to be highly age–dependent.[1]

1.2 Etiology and Risk Factors of Atherosclerosis

Despite recent advances in atherosclerotic research, the etiology of atherosclerosis still remains unclear.[3] The lesions of atherosclerosis are characterized by subintimal proliferation of smooth muscle cells, invasion of the damaged intimal layer by macrophages, and accumulation of large amounts of connective tissue matrix and lipid. Early lesions are known as fatty streaks. Some of these fatty streak lesion will disappear, while others will progress to fibromuscular lesions and, eventually, to complex lesions.[4] In an attempt to explain the pathogenesis of atherosclerosis, several hypotheses have been suggested. These hypotheses take the pathologic lesions into account as well as explain the effects of the associated risk factors on the incidence and clinical sequelae of atherosclerosis. The present hypotheses of atherosclerosis include the lipid hypothesis, the response–to–injury hypothesis, the monoclonal hypothesis and the clonal–senescence

hypothesis. [3,4]

Risk factors of atherosclerosis have been defined as habits and abnormalities which lead to an increased susceptibility to atherosclerosis.[5] A risk factor may be a causative agent, a secondary manifestation of an underlying metabolic abnormality, or an early symptom of atherosclerosis.[6] The epidemiologic studies by the Framingham study relate quantitatively the intensity and duration of these risk factors to the frequency of the clinical disorders that signal atherosclerotic disease. The major risk factors for atherosclerosis are:

(1) Age

It is well established that aging is the predominant risk factor for the development of atherosclerosis and has the strongest and most consistent correlation with peripheral vascular disease.[7] Several studies in the analysis of clinical and autopsy data have demonstrated significant positive correlation between age and atherosclerosis.[1,7,8]

(2) Cigarette Smoking

Cigarette smoking remains an important risk factor for cardiovascular disease. It is related to risk of premature atherosclerotic disease which is independent of and in addition to other major risk factors.[9] The exact mechanisms by which cigarette smoking promotes atherosclerosis are not fully understood. Intermittent carbon monoxidemia

possibly predisposes the arterial wall to endothelial injury, leading to increased plasma flux and LDL entry into the arterial wall. Cigarette smoking also causes increased platelet reactivity, promotes peripheral vasoconstriction, and reduces HDL level.

(3) Diabetes Mellitus

It is well-known that diabetes mellitus of the maturity-onset type represents an enhanced risk of premature atherosclerotic disease involving the arterial system. Several studies have shown that diabetics have atherosclerotic disease more often, more distantly, more severely, and more prematurely than do nondiabetics.[10]

(4) Hypertension

Hypertension is a strong risk factor for atherosclerosis and the lesions in major vessels are more extensive and more severe in hypertensive than in normotensive subjects. The data on prospective studies have demonstrated that hypertension is related to the risk of premature atherosclerotic disease independently of other major risk factors.[11] Hypertension probably predisposes to continued hemodynamic injury, thereby accelerating plaque lesions.

(5) Hyperlipidemia

Hyperlipidemia has been associated with an increase in atherosclerosis, a conclusion established in several epidemiological studies.[12] Hyperlipidemias are divided

into two categories, primary and secondary. Primary hyperlipidemia may be associated with a genetic defect or a lipase deficiency, which is usually exaggerated by dietary intake. Defective lipid metabolism is due either to overproduction of lipoprotein or to insufficient clearance of lipoprotein from the plasma. Secondary hyperlipidemia is associated with hypothyroidism, nephrotic syndrome, diabetes mellitus, and biliary obstruction, etc.

(6) Other Factors

Genetic influences are thought to play a significant role in the development of atherosclerosis. However, little is known about the underlying biochemical, physiologic, and hemodynamic factors which identify the genetic characteristics of an individual prone to develop the disease. Gender and race have also been evaluated for their effects on atherosclerotic disease, but the relative importance of each in different clinical settings is widely debated.

1.3 Natural History

Patients with peripheral vascular disease traditionally have been grouped into two distinct categories, each with a distinct natural history. The group with intermittent claudication has a relatively good prognosis, while those with limb–threatening ischemia invariably deteriorate. The patients with peripheral vascular disease also exhibit a high incidence and prevalence of coronary artery disease and cerebrovascular disease. While

the overall prognosis of peripheral vascular disease is favorable, patients with severe claudication have a significant higher incidence of tissue loss. The severity of disease is also a marker for increased mortality from all causes. Overall, 30% of patients with clinically significant chronic lower extremity ischemia die within 5 years.[1]

(1) Intermittent Claudication

Intermittent claudication is a clinical condition where lower extremity muscle pain appears during exercise and promptly (within 1 to 2 minutes) resolves with rest.[13] Fixed atherosclerotic obstructions proximal to the affected muscle prevent the normal increase in blood flow necessary to meet the exercise–induced metabolic demand. Many large studies investigating the natural history of intermittent claudication support a relatively benign course for this condition. Although the symptoms in the majority of patients may remain stable or improve over several years, long–term amputation rates in these patients with or without surgical intervention range from 4% to 12%.[1] Patients with intermittent claudication often present with certain degrees of disability in their daily activities depending on the level of exercise and the perception of discomfort by each individual. The quality of life among these patients is therefore impaired during their shortened life expectancy. Cigarette smoking and diabetes mellitus, as well as the level of disease, have been identified as the major factors associated with a higher likelihood of progression to severe ischemia in these patients.[13]

(2) Limb–threatening Ischemia

Limb–threatening ischemia occurs when blood flow is insufficient to meet the maintenance metabolic requirements for resting tissue.[13] Its clinical manifestations include rest pain and ischemic ulceration or gangrene. It is generally considered a pregangrenous condition heralding tissue loss unless urgent surgical intervention is accomplished. The development of collateral circulation which is sufficient to permit clinical improvement is rare and unpredictable and most surgeons prefer to revascularize these ischemic limbs. Some studies, however, have reported that the long–term improvement of ischemic rest pain or ulceration in ischemic limbs can be ranging from 25% to 50% with only minimal or placebo therapy,[14,15] but most of these studies have relied on the presence of symptoms or ulceration without accepted objective confirmation of the ischemic state.

1.4 Diagnosis

(1) History and Physical Examination

The clinical manifestations of peripheral vascular disease are divided into three major groups of increasing severity: (1) intermittent claudication, (2) ischemic rest pain, and (3) ischemic ulcers and gangrene.[16] The clinical history continues to be of central importance in the evaluation of patients with the disease including questioning for atherosclerosic risk factors as well as the history of other associated vascular diseases. Almost all patients with intermittent claudication have diminished or absent palpable pulses of the lower extremity . In addition to an absence of lower extremity pulses,

patients with rest pain will frequently have thin, atrophic skin of the foot, and may have dependent rubor as well as areas of cutaneous gangrene and ulceration. For most patients, the clinical history coupled with the physical examination is necessary to firmly establish the diagnosis of intermittent claudication or ischemic rest pain.

(2) Noninvasive Vascular Laboratory Tests

The objectives of modern noninvasive vascular laboratory tests for the patients with peripheral vascular disease are to confirm the presence of arterial ischemia; to provide quantitative, reproducible, and physiologic data concerning its severity; and finally, to document the location and hemodynamic importance of individual arterial lesions. Two broad categories of noninvasive techniques are used to evaluate lower extremity arterial occlusive disease: plethysmography and variations of Doppler ultrasound. Each technique, however, has its own diagnostic criteria, advantages, disadvantages, and limitations. Of the noninvasive modalities, Doppler–determined segmental blood pressures and pulse volume recordings (PVR) are generally the most useful means for evaluating lower–extremity ischemia.[16]

Plethysmography:

Plethysmography is based on the detection of volume changes in the limb in response to arterial inflow. This technique can be modified to measure calf or foot blood flow, pulse volume waveforms (Pulse volume recordings), and digital pressures and

waveforms.

Doppler Ultrasound:

Ultrasound has proved to be the single most important modality in the noninvasive evaluation of lower–extremity ischemia. Ultrasound techniques are based on the principle that sound waves emitted from a transducer are reflected at the interface of two surfaces that can be processed into an audible signal as well as a picture or a velocity waveform. Clinically, this technique can be used to determine the ankle–brachial systolic blood pressure index (ABI) and the segmental limb pressures. Both measurements can be combined with treadmill exercise testing, assuming the patient dose not have a medical contraindication to exercise. This technique can also be used to obtain Doppler analog waveforms and images of the peripheral arteries, called Duplex scanning.

Duplex scanning of the peripheral arteries is the newest development in the noninvasive assessment of peripheral vascular disease. This technique has been used for many years in the examination of the carotid artery but recent engineering improvements have made the development of new transducers that permit examination of the peripheral arteries. A prospective, blinded study indicates that the Duplex scanning has an overall sensitivity of 82%, a specificity of 92%, a positive predictive value of 80%, and a negative predictive value of 93% in the determination of a hemodynamically significant stenosis in the peripheral arteries.[17] The principal advantage of Duplex scanning over

other noninvasive methods in the assessment of peripheral vascular disease is that it allows the direct and precise mapping of hemodynamically significant lesions. In addition, the technique allows discrimination between stenotic and occlusive lesions. The development of color–flow imaging combined with Doppler permits rapid and accurate mapping of the length of arterial occlusion.[18]

(3) Angiography

Angiographic visualization of the entire lower–extremity vascular system including the pelvic arteries, as well as the small vessels in the distal part of the leg and foot, is mandatory for the planning and performance of arterial reconstructive procedures.[19] Angiography is essential to make the most accurate diagnosis of arterial occlusive disease and provide information not only about the lesions of the arteries in the lower–extremity but also about the state of the inflow and outflow tracts.[16,20] This method can be achieved by aortoarteriography, performed preferably by a transfemoral route with local anesthesia.

1.5 Treatment

(1) Medical Therapy

Medical therapy of peripheral vascular disease includes modification of risk factors known to contribute to severity symptoms and progression of disease, modification of life habits, and drug treatment.[21,22] Although it is palliative, this therapy can still be utilized safely to manage a majority of claudication patients, especially for those with their ankle–

brachial indexes (ABI) which are greater than or equal to 0.55. The types of current medical therapy include smoking cessation, supervised exercise, and drug therapy which include vasodilators, antiplatelet drugs, and metabolic enhancing drugs, etc. One drug, pentoxifylline, has been shown to reliably improve claudicating distances to a modest extent and improve the quality of life in some patients. Several other drugs available only in Europe such as ticlopidine, naftidrofuryl, and carnitine show some promise.[23] Overview of drug therapy indicates that many of these trials were flawed due to absence of controls, small sample size, and lack of objective evaluation. To update, the hallmark of medical therapy is smoking cessation, exercise, and drug therapy. Although it is of minimal value for the treatment of claudication patients, medical therapy still remains the initial or primary treatment of choice in a large majority of patients with intermittent claudication alone.[23,24]

(2) Endovascular Procedures

The term "endovascular procedures" has been used in recent years to encompass the new therapeutic transluminal techniques, including percutaneous balloon angioplasty (PTA), laser angioplasty, mechanical atherectomy, and intravascular stents. With improvement of technology, these techniques have gained increasing acceptance for the treatment of peripheral vascular disease. Despite widespread clinical application, however, clear documentation of the safety, effectiveness, and long–term benefit of endovascular procedures is not yet available due to a remarkable lack of either uniform

or conventional reporting standards for patency, morbidity, and mortality.

PTA has a particular role in the treatment of iliac arterial disease, either as treatment of choice in isolated short–segment common iliac artery stenosis, or to provide an improved inflow to support a distal vascular bypass procedure.[25,26] A recent review documented the efficacy and safety of iliac angioplasty. However, external iliac, femoral, and tibial angioplasty should still be considered as unproven alternatives compared to well–performed surgical bypass.[27] Initiating a healing process in the arterial wall following PTA has been thought to be a mechanism of restenosis in the dilated artery or even worse, accelerating the occlusive process of the vessel, and both will ultimately lead to late failures of the procedures.[20]

Recently, laser energy has been applied to atherosclerotic vessels as an adjunct to angioplasty in an effort to fuse the plaque elements and hopefully limit the stimulus for neointimal hyperplasia. Initial clinical experience with direct use of laser energy to atherosclerotic plaque through fiberoptic catheters yielded a high incidence of vessel perforation. Therefore, the ideal, controlled application of laser energy to the plaque remains an unrealized research objective.[28] Investigational trials of Simpson catheter atherectomy as well as insertion of intravascular stents after endovascular procedures are currently continuing and the preliminary results suggest that restenosis commonly occurs after atherectomy and significant neointimal hyperplasia after implantation of the

stents.[29,30]

(3) Direct Surgical Revascularization

The lower extremity arterial bypass procedure has become one of the most frequently performed operations in modern vascular surgery. Direct surgical revascularization is the only effective means of limb salvage for severe chronic extremity ischemia, but it has no impact on the underlying cause of the disease. Generally, the indications for bypass surgery are considered if patients have severe intermittent claudication, rest pain, nonhealing ulcers, or gangrene. There are several arterial bypass procedures for the treatment of patients with peripheral vascular disease. These procedures may also combined with PTA or endarterectomy. The treatment of choice usually depends on the extent and distribution the atherosclerotic occlusive lesions, but may also depend on the general condition of the patient and experience of the surgeon as well. Several investigators are still trying to develop more effective and durable revascularization procedures to salvage severe ischemic limbs.

Aortoiliac Disease:

The most preferred procedure for the treatment of limb ischemia due to aortoiliac occlusive disease is an aorto-bifemoral bypass using a synthetic graft. Initial graft patency rates now approach 100 per cent, and 5-year patency is about 85 to 90 per cent. Long-term patency has also improved to 70 to 75 per cent at 10 years.[31] In patients with

high risk for major surgery under general anesthesia, aortoiliac graft occlusion or infection, or multiple prior abdominal procedures, an extra–anatomic axillofemoral bypass can be performed to treat aortoiliac occlusions. In patients whose occlusive disease is confined to one iliac artery and whose aorta and contralateral iliac system is free of significant lesions, a femorofemoral bypass can be employed with quite satisfactory long–term results.[32]

Femoropopliteal Disease:

In patients with femoropopliteal occlusive disease, the treatment of choice is to bypass the obstruction of lesions, frequently with autogenous saphenous vein graft. Femoropopliteal bypass can be performed either above or below the knee and the vein graft can be placed either reversely or in situ. In most studies, there is no significant difference between patency of above–knee compared to below–knee vein bypass and both reversed and in situ vein bypass have been demonstrated equivalent patency and limb salvage rates.[33,34] A variety of nonautogenous prostheses (such as PTFE) have also been used for femoropopliteal bypass when autogenous vein is not available, but patency results are clearly inferior to results of autogenous vein graft and such bypass should be placed above the knee joint. The 5–year patency of autogenous saphenous vein graft is about 75% to 80% for both reverse and in situ methods and that of PTFE graft is about 25% to 55% depending on operative indications and location of distal anastomosis.[35] Endarterectomy combined with profundaplasty can also be used to treat localized common

femoral or profunda femoris occlusive disease. A composite (proximal PTFE graft with a distal autogenous vein) or sequential graft (a vein or PTFE graft with multiple anastomoses) to the infrapopliteal vessels can be performed for limb salvage when patients do not have adequate length of autogenous vein.

Distal Small Vessel Disease:

In the last decade several developments have occurred that have made distal bypasses possible to even diseased tibial and peroneal arteries in the leg or foot, the so-called "distal" or "small artery bypass". The success of distal or small artery bypass has been a major factor in reducing the proportion of patients whose arterial disease was so distal that they were "unsuitable for an attempt for limb salvage" or inoperable in the past.[35,36] A recent large randomized prospective study demonstrated that small-artery bypass patency at 4 years with vein grafts was about 40% to 60%, whereas with PTFE grafts it was less than 20%. Clearly, autogenous vein is again the best graft material with which to perform a short reversed vein bypass to the distal small vessels.[37] Distal bypasses to small infrapopliteal arteries, therefore, represent an evolution in vascular surgery.

(4) Other Surgical Treatments

Sympathectomy:

Although lumber sympathectomy was introduced many years ago as a method of

treatment for ischemic and painful disorders of the lower extremity, much controversy still persists over its physiologic effects, clinical indications, and long–term results. The procedure has only limited value in the management of peripheral vascular disease. However, when reconstructive arterial procedures are contraindicated or unsuitable, lumber sympathectomy may be considered as an option, since its objective is usually thought to promote development of collateral circulation in the ischemic limb.[38]

Amputation:

Despite numerous advances in distal revascularization in recent years, amputation of the lower extremity still remains as a treatment of choice for patients with end–stage peripheral vascular disease.[39] The objectives of amputation are to achieve primary healing, relief of pain, and restoration of ambulatory ability of patients. Currently, there has been a trend towards trying to amputate at the most distal possible level even after multiple failed bypasses, if the risk of a nonhealing wound can be avoided.[40,41] The primary healing rates exceed 70% for forefoot amputations and 90% in major lower–extremity amputations. The mortality rate in modern series should not be greater than 5% to 7%.[41]

1.5 Persisting or Unresolved Issues

Atherosclerosis usually presents as a segmental disease in proximal arteries. Although current direct revascularization techniques for treatment of limb–threatening

ischemia are quite successful in patients with hemodynamically significant lesions of large vessels within an otherwise normal arterial tree, it has become apparent that many patients with limb–threatening ischemia have a different pattern of arterial disease which is characterized by diffuse and multilevel arterial involvement. The disease may be present entirely or predominantly below the lower half of the thigh or entirely below the knee.[40] Many of these patients are inoperable because their disease is too distal and the arteries involved are too small. Even after direct revascularization for patients with limb–threatening ischemia, failure of the procedure may still occur due to an inadequate conduit for arterial reconstruction, progression of the atherosclerotic disease, or thrombosis of the bypass graft. Therefore, there remains approximately 20% of patients with limb–threatening ischemia where direct revascularization is either technically impossible or not successful.[42] It is obvious that these patients will eventually face a major amputation for rest pain, ischemic ulcers, and gangrene with significant morbidity and mortality. It is particularly crucial in elderly patients because they always present with problems in terms of higher surgical risk and the operative mortality of amputation is even higher than that of the vascular procedure.[43] In addition, a far larger population of patients with stable but disabling claudication have no choice but "live with" their symptoms for many years because no medical alternative has been proved effective.

Although the trends in the treatment of peripheral vascular disease at a few institutions have reported decreased rates of amputation in association with an increase

in the use of direct lower–extremity revascularization,[40,44] population–based studies have not confirmed this association.[39,45] Increasing the number of revascularization procedures have failed to decrease the rates of amputation in a recent Maryland study.[39] This could be an example of an increasingly aggressive but ineffective approach to the disease that may be too severe and advanced to be associated with an increase in limb salvage. With increasing life expectancy, an enlarging group of patients will seek treatment for ischemic limbs in whom significant large vessel occlusive disease is combined with small vessel disease. These limbs may fall into the nonreconstructible category. Are there any new approaches that can be offered to these patients for limb salvage either surgically or medically? In addition, at least 10% of persons over the age of 70 have intermittent claudication and the majority of them are currently being treated medically or are receiving no treatment at all.[23] Are there any effective nonoperative treatments that will be beneficial to them and will significantly improve the quality of life in these patients? Both questions still remain a challenge in modern medicine.

2. ALTERNATIVE APPROACHES FOR LIMB-THREATENING ISCHEMIA

Current treatment for severe chronic limb ischemia is limited by direct surgical revascularization or endovascular procedures. Frequently however, in many patients with limb–threatening ischemia, the direct conventional therapies may be technically impossible or unsuccessful because of the extent of the disease. In the past decade, several new alternative approaches have been reported experimentally for the treatment of limb–threatening ischemia along with a few clinical reports. Most of these alternative approaches are indirect revascularization procedures and thus their effectiveness and practicality have yet to be clearly established.

2.1 Staged Arteriovenous Reversal

The fact that the venous system is not affected by even the most severe atherosclerotic process had led several investigators over the years to explore the possibility of using this nondiseased pathway to revascularize ischemic limb by the creation of an arteriovenous fistula. However, most of these attempts have been unsuccessful due to venous congestion or inadequate tissue perfusion.[46] A report by Johansen and Bernstein[47] has experimentally demonstrated that a staged approach, in the form of an end–to–side arteriovenous fistula and the subsequent conversion of this to an arteriovenous reversal (AVR) preparation by ligation of the proximal vein a week later, could salvage an otherwise irreversibly ischemic canine hindlimb without excessive edema formation. In our laboratory, Graham et al[48] also developed and used a canine model of

acutely severe hindlimb ischemia to study the physiological consequences of staged AVR at the popliteal level. The results not only demonstrated successful hindlimb salvage following the procedure but also provided new insight into the mechanism by which nutrient tissue flow was affected by AVR. In a subsequent study, Baffour et al[49] showed that the neovascularization seen on angiograms after AVR developed as a response to severe ischemia in the same canine model. Recently, the study by Graham et al[50] from our laboratory has indicated that the dramatic increase in vascularity observed angiographically following AVR consists primarily of a rapidly expanding capillary network. This was shown by increased tritiated thymidine uptake and capillary density on histology. It was, in fact, this vascular network that provided nutritive flow to the ischemic limb.

Clinically, Symes et al[51] from our institution demonstrated the efficacy of femorotibial arteriovenous revascularization in a patient who had an otherwise irreversibly ischemic limb. As a result of this preliminary success, this procedure was justified to be used in more patients and a longer follow–up carried out. The initial clinical experience in 12 patients also demonstrated that AVR could provide sufficient nutritive flow to relieve rest pain and avoid amputation of some limbs where severe ischemic rest pain and minor tissue necrosis were not amenable to conventional revascularization procedures.[52] In another series, Sun and Zhang[53] successfully treated 33 patients by a staged arteriovenous reversal procedure. These patients were followed for up to fifty–five

months. Except for one, the results of the patients from that series had excellent or good for limb salvage. They also suggest that this alternative approach could be used in the treatment of extensive lower limb arterial occlusive disease. However, one major limitation of this procedure in clinical setting is the time required for improvement which varies from two to eight weeks. Therefore, a means which can accelerate this revascularization process is still warranted.

2.2 Conventional Bypass with Distal Arteriovenous Fistula

The use of a synthetic graft may be the vascular surgeon's only option when an autologous vein of sufficient length or size is not available. However, the use of synthetic grafts in similar limb salvage situations has not been as successful as the use of autologous vein. In a recent multicenter prospective trial with PTFE for infrapopliteal bypass grafting, the 4–year patency was only 12% compared to 80% with autologous vein.[54,55] Methods used to extend the patency of synthetic graft have involved modification of the coagulation status, graft surface, and graft flow volume and velocity characteristics.

Although the creation of a distal anastomotic arteriovenous fistula (AVF) to augment blood flow and velocity through the bypass graft is well known,[56] the concept and use of the distal AVF to promote synthetic graft patency has been surrounded by skepticism and controversy. One of the great concerns is that this adjunctive distal AVF

may create turbulence at the anastomosis and steal blood flow away from the distal artery. In a canine model of PTFE femoro–femoral crossover bypass with a remote distal AVF, Paty et al[57] demonstrated that unlike an AVF created at the distal anastomosis, a remote distal AVF not only increased graft blood flow but also augmented native distal arterial blood flow between the distal anastomosis and fistula. These results were then incorporated into their clinical practice in 16 patients with limb–threatening ischemia who had previously failed multiple reconstructions and who did not have usable autologous veins. Femorotibial bypass graft reconstructions were performed with PTFE followed by the creation of a remote distal side–to–side AVF 5 to 15 cm below the distal anastomosis in the same artery and accompanying veins. The graft patency rate in this series at 1 year was 67% compared with less than 50% in patients with only PTFE bypasses to infra–popliteal arteries[55] and compared with 39% in Dardik's series with umbilical vein bypasses in adjuvant with a distal side–to–side AVF.[56] In a self–controlled experimental study from the same center, Calligaro et al[58] also demonstrated that, in a canine bilateral iliofemoral bypass model using PTFE grafts with or without adjunctive distal AVF, cumulative life–table patency rates were always higher in the AVF bypasses than the control grafts during the 12 months after bypasses. A distal AVF when constructed clinically with a femorotibial PTFE bypass may therefore improve the patency rate of the graft and the procedure may have a potential value in those patients with a history of multiple previous bypass failures and no usable veins.

2.3 Omental Pedicle or Muscle Flap Transfer

The omental pedicle transfer has been reported experimentally to be effective in supplying additional blood to the ischemic heart, brain, or extremity by providing collateral circulation to these tissues.[59-61] For patients with end-stage peripheral vascular disease who were considered clinically to have inoperable ischemic limbs, Goldsmith[61] successfully salvaged an ischemic upper extremity of a patient by transferring his intact omentum into that extremity. Hoshino et al[62] reported the long-term results of omental transplantation for chronic occlusive arterial disease. In their series, twenty-five cases without indication for direct reconstruction were treated by this procedure and were followed for an average of 5 years. They found that this approach was remarkably effective in treating thromboangitis obliterans (or Buerger's disease) but not atherosclerosis obliterans. Their result was confirmed by the later study of Maurya et al[63] in which 12 patients with Buerger's disease were shown to have objective improvement of tissue perfusion in their ischemic lower limbs. Further, Goldsmith et al[64] and Cartier et al[65] have recently suggested that the revascularization property of the omentum is attributable to a specific lipid fraction (so called "omental angiogenic factor") it contains and this fraction has potent angiogenic activity. The lipid fraction from the omentum is abundant in supply and has been shown to increase localized vascular perfusion when administered intramuscularly, as measured by nuclear imaging technique in an animal model.[66]

Muscle flap transfer is another approach to providing arterial inflow to the ischemic muscle for the treatment of the end–stage ischemic limbs. In a rabbit model of hindlimb ischemia, Pevec et al[67] demonstrated angiographically that the arterial connections between a well–vascularized muscle flap and an ischemic limb could develop 17 days after the transfer. In a subsequent study, Pevec et al[68] also demonstrated, by comparing muscle blood flow in the flap group vs the sham group, that the arterial connections between a well–perfused muscle flap and an ischemic limb was hemodynamically significant 7 days after the transfer. In a recent study, DeBaise et al[69] from the same institution aspirated interstitial fluid at the interface between the muscle flap and the ischemic muscle recipient bed and assayed for bFGF using a specific monoantibody Elisa. Surprisingly, they demonstrated the expression of bFGF, a potent angiogenic factor, 14 and 21 days after the transfer and thus they concluded that bFGF might be one factor responsible for neovascularization in this rabbit ischemic hindlimb model. Clinically, they performed a free microsurgical transfer of a muscle flap in a patient with end–stage peripheral vascular disease and ischemic ulceration of lower extremity in whom an obliteration of the distal arterial bed precluded conventional arterial reconstruction.[70] The successful limb salvage of this patient suggests that free muscle flap itself may provide a means of indirect revascularization for patients with nonreconstructible ischemic limbs.

2.4 Epidural Spinal Cord Electrical Stimulation

The most important symptom in patients with limb–threatening ischemia is perhaps ischemic rest pain. These patients suffer from the pain day and night, and it is almost impossible for them to live a normal life. Under certain circumstances, amputation is often the only means of relief for the intractable pain of the patients in whom when reconstructive surgery is impossible or has failed. Epidural spinal cord electrical stimulation (ESES), a pain relief modality in patients with chronic pain or a motor stimulant in patients with partial lesions of the spinal cord, has recently been suggested by a number of investigators from Europe as an alternative treatment for patients with limb–threatening ischemia in whom reconstructive surgery is not possible.[100-102] This therapy has been reported to be able to relieve ischemic rest pain, to improve peripheral circulation of the ischemic limbs, and even to have a possible limb–salvage effect for patients with severe chronic limb ischemia. However, the mechanisms by which ESES exerts its effects are still unclear.

In a clinical trial in Sweden, Augustinsson et al[71] have used ESES in 34 patients with severe limb ischemia. Among those, 26 patients had arteriosclerotic disease, 1 had Buerger's disease, and 7 had severe vasospastic disorders. All patients had severe ischemic rest pain and most had ischemic ulcers, but the arterial reconstruction in these patients was technically impossible. During a mean follow–up period of 16 months, 94% of the patients experienced pain relief, 50% healed their previous nonhealing skin ulcers,

and 70% showed improved skin temperature recordings. Only 38% of the arteriosclerotic patients treated with ESES underwent amputations as compared to 90% in a comparable group of patients during the same follow–up period. In a similar study from the Netherlands, Jacobs et al[72] evaluated the effects of ESES on microcirculatory blood flow in 10 patients with severe limb ischemia due to atherosclerotic disease. All patients revealed clinical improvement after ESES. There was significantly increased capillary density and RBC velocity as assessed noninvasively by intravital capillary microscopy in the foot. However, their systolic ankle/brachial pressure ratios and digital arterial pressures did not significantly increase. These results were confirmed by a larger series with longer follow–up which showed a cumulative foot salvage of 80% and 56% after 1 and 2 years, respectively, in 20 patients with nonoperable ischemic lower limbs. They concluded that ESES could relieve ischemic rest pain, improve skin nutrient flow, and lead to healing of ulcers of moderate size. In addition, there was an increase of the foot salvage with limb–threatening ischemia.[73] However, ESES has not been well accepted as an alternative means in patients with the same clinical situations in North America centers.

3. ANGIOGENESIS AND ITS STIMULATION

3.1 Historical Background

Angiogenesis is the term reserved to describe the formation of new blood vessels. It is an important biological process which occurs in a variety of physiological and pathological conditions such as embryogenesis, tissue development and growth, wound healing, inflammation, ischemia, and tumor growth. It is a complex cellular process which is generally agreed that the process can, at least conceptually, be conveniently divided into several overlapping events or stages which lead to the development of a new vessel. These stages include degradation of capillary basement membrane, migration and proliferation of endothelial cells, and tube formation. The occurrence of angiogenesis in normal adult is infrequent and the period of angiogenesis is relatively brief and tightly regulated. Although normal angiogenesis may occur as part of the body's repair processes, uncontrolled angiogenesis may often be pathological. One example is in diabetic retinopathy where excessive vascular growth eventually occludes the retina.

Reports on the growth of new blood vessels have appeared since the late 18th century and the term "angiogenesis" was first introduced by Hertig in 1935 to describe new blood vessels in the placenta.[74] Although the topic of angiogenesis has long been considered important, our basic knowledge of angiogenesis at the cellular and molecular levels has accumulated only in the past 25 years. Currently, angiogenesis and its

stimulation are predominantly studied by *in vitro* assays (endothelial cell culture) or by one of the two *in vivo* assays (cornea or chick chorioallantoic membrane). Several angiogenic factors have been identified by these assays. Most are found to be mitogenic for endothelial cells and some are responsible only for tube formation. One of the most compelling reasons for the growing interest in the study of angiogenesis is the demonstration by Folkman[7] that solid tumors are angiogenesis–dependent; that is, they require formation of a vascular system derived from host blood vessels to support their growth. From this concept follows the hypothesis that tumors produce certain factors that stimulate angiogenesis.[76]

The pioneering work of Folkman and his associates in this field has resulted not only in the elucidation of the mechanisms of angiogenesis,[77–79] but also provided the first examples of isolated and purified angiogenic factors.[79,80] Their primary objective of research in angiogenesis was to understand the angiogenic process and its role in tumor development with the hope that inhibitors of angiogenesis could be developed and used as a powerful and selective therapy for the treatment of cancer. A significant "secondary" outcome of that work was a much broader understanding of the angiogenic process and its implications for all aspects of tissue repair. Efforts have been made to develop agents which stimulate blood vessel growth in tissues or organs. For example, revascularization of skeletal or cardiac muscle following ischemic injury may improve tissue blood perfusion by means of angiogenesis. Stimulation of angiogenesis may also

increase the rate of tissue repair and wound healing following skin defects, burns, or bony fractures. In general, the development of pharmacologic agents that specifically stimulate or inhibit blood vessel growth would have great clinical potential and would lead eventually to the development of specific and effective therapies for numerous ischemic or angiogenic diseases.

3.2 Collateral Vessel Formation

Collateral vessels can be simply defined as alternative routes of blood supply to an organ or tissue that are not functional or present under normal circumstances. Collateral circulation is, therefore, a response to ischemic injury, and is due to both the opening or enlargement of preexisting vessels and the growth of new vessels in ischemic tissues or organs.[81,82] Preexisting vessels are very important, as some small arteries in the muscle may play a significant role in the formation of collateral circulation to maintain vascularity of the limb muscles when large vessels have been ligated.[83]

Ischemia can itself be a stimulant to neovascularization and this has been demonstrated in the limb,[84] kidney,[81] heart,[82] and retina.[85] Ischemia was also shown to be a major determinant of neovascularization of the canine hindlimb in the presence of an arteriovenous fistula.[49] Ischemia appears to be the common stimulus required for all initiating mechanisms. As noted previously, gradual and intermittent vessel occlusion appears to be the most effective meams of generating collateral growth.[86] In the last 100

years, many investigators have sought to identify the stimuli responsible for the development of collateral vessels in ischemic tissue.[87] Although the precise mechanism for the development of collateral vessels in ischemic tissue still remains a mystery, mechanical stimuli and endogenously released biochemical factors have been implicated.

Four main mechanisms for the development of collateral vessels have been postulated. They are: (1) increase in the pressure gradient around the occluded vessel; (2) increase in blood flow around the occluded vessel; (3) release of vasoconstriction tone in collateral vessels by neuromuscular controls; and (4) the production of biochemical factors in distal hypoxic tissue.[87] Indeed, all four mechanisms may act together to form collateral vessels in ischemic tissue. The mechanical stimulus for the use of preformed vessels that has received greatest acceptance is the pressure gradient.[81] Since the pressure distal to an obstruction is low, the collateral vessels communicating with the distal artery encounter a low pressure outlet into which a higher volume of blood can flow and dilate these channels. Other mechanical stimuli for the use of preformed vessels include increased blood flow and vasodilation.

Biochemical factors from ischemic tissue have been considered as potentially important stimuli for collateral circulation formation. This may be due to the dilation of preexisting vessels or the development of new vessels. Cell hypoxia or injury releases several vasoactive products such as lactate, hydrogen ion, potassium, and adenosine

nucleotides. They are all potent vasodilators that may act on preexisting vessels.[81] A number of tissue extracts from myocardial infarcts,[82] interstitial fluid or tissue of an ischemic kidney,[81] ocular tissue,[88] and omentum[64] have been shown to stimulate angiogenesis in a variety of *in vivo* models. However, only the growth factors have recently received much attention. Most of the growth factors are now considered as angiogenic stimulants as well and are also found to be released from ischemic tissue endogenously.[89] The growth factors have been demonstrated to promote both endothelial cell proliferation *in vitro* and angiogenesis *in vivo*.[80] Although endogenously released biochemical factors (including growth factors) and mechanical stimuli may stimulate collateral vessel formation in ischemic tissues, these collaterals are not always sufficient for preventing clinical manifestations of disease.

3.3 Sequence of Events in Angiogenesis

The pattern of new capillary growth *in vivo* is similar for all tissues.[80] Light and electron microscopic studies of early angiogenic reactions show that endothelial cells migrate away from pre–existing capillaries or venules to form sprouts and loops. These sprouts and loops are directed toward the source of the angiogenic stimulus, regardless of its nature.[90] At least four sequential steps are needed in the development of a new capillary: they are briefly summarized as follows: (1) Endothelial cells from capillaries or small venules closest to the angiogenic stimulus degrade the basement membrane of the parent vessels and start to migrate toward the source of the angiogenic stimulus, (2)

the migratory phase is accompanied by the proliferation of the endothelial cells behind the migratory cells. As a sprout of capillary cells begins to form, the cells at the very tip of the sprout continue to migrate, while the cells posterior to the invasive cells multiply, thus maintaining vascular continuity, (3) individual sprouts join with each other leading to the formation of loops which in turn can initiate new sprouts, and (4) pericytes and fibroblasts migrate to the sites of the capillary loops, blood flow begins, and the newly formed capillaries substantially go through remodelling, regression, and rearrangement.[90,91]

3.4 Factors Affecting New Vessel Growth

A number of factors may contribute to the regulation of new blood vessel growth. Among those, four are now considered to have the strongest influence. They are mechanical stimuli, cell–to–cell interactions, extracellular matrix, and growth factors.[79]

(1) Mechanical Stimuli

The DNA synthesis of endothelial cells varies with their cell shape: the flatter the cells, the higher the rate of DNA synthesis.[92] Round cells, such as confluent endothelial cells (ECs), have a lower rate of DNA synthesis. Therefore, *in vivo* changes in EC shape, such as flattening or stretching of EC induced by vasodilation, intravascular pressure, or shear stress, could lead to changes in EC growth rate.[79] Actually, vasodilation is always seen in the early stages of angiogenesis.[90]

(2) Cell–to–Cell Interactions

Interactions between adjacent ECs have been postulated to regulate their growth. Cell membranes isolated from cultures of confluent EC can inhibit DNA synthesis in actively growing cultures of such cells.[93] It has also been postulated that pericytes can suppress the proliferation of ECs because neovascularization is often characterized by an absence of pericytes and because proliferation of ECs is inhibited by coculture with microvascular pericytes.[79]

(3) Extracellular Matrix

The role of the extracellular matrix in angiogenesis is not fully understood. Extracellular matrix has been shown to modulate the growth and differentiation of a wide variety of cells including the endothelial cells.[79] Components of the extracellular matrix alter the growth pattern of cultured ECs and affect the response of cultured ECs to angiogenic stimuli.[94,95] Growth factors may be sequestered in the extracellular matrix of tissue and may be released when the tissue is damaged.[89]

(4) Growth Factors

Growth factors are biologically active substances that have hormone–like functions in regulating cell proliferation and differentiation. The growth factors that are involved in angiogenesis have been discovered mainly under pathological conditions in adults, such as tumors, inflammation, wound healing, or regeneration.[96] A number of angiogenic

growth factors have been isolated and purified in recent years. They have been studied primarily *in vitro* on endothelial cells of different origins, smooth muscle cells, or fibroblasts as well as *in vivo* in rabbit cornea, chick chorioallantoic membrane, or various implanted chambers.[80,96] Currently, there has been no single well–accepted classification or nomenclature scheme or terminology for the angiogenic growth factors.

Heparin–binding endothelial cell growth factors:

Among the first few angiogenic growth factors to be described were basic fibroblast growth factor (basic FGF or bFGF) isolated from brain tissues and endothelial cell growth factor (ECGF) isolated from the hypothalamus.[30] Basic FGF was found to have an acidic homologue (acidic FGF, or aFGF) and ECGF is considered to be a precursor of aFGF. Many endothelial cell growth factors were polypeptides that were found to have a strong affinity for heparin and therefore, are so called "heparin–binding growth factors." The purification of these factors was greatly facilitated by heparin–affinity chromatography. Heparin–binding growth factor is classified as basic FGF and acidic FGF. Both forms of FGF are found to be structurally related, having a 35% absolute sequence homology and binding to the same receptor.[80,97] Both FGFs can not only stimulate endothelial cell proliferation *in vitro* but also promote angiogenesis *in vivo*.[80] It appears that members of this class of growth factors have been found in almost all normal tissues, but they are not secreted by cells under normal circumstances. However, they can be released after cell injury and may play a role in tissue repair.[98]

Transforming growth factors:

Transforming growth factors (TGF) are polypeptides that were first isolated on the basis of their ability to alter the phenotype of some normal cells to transformed cells. They are classified as two distinst molecules (TGF–α and TGF–β) with distinct structures and biologic properties. TGF–α is a growth promoting factor that shares a 35% homology to the epidermal growth factor (EFG). TGF–β is generally regarded as an inhibitor of cell growth *in vitro*. Of the 2 molecules, only TGF–α promotes endothelial cell growth, while TGF–β inhibits endothelial cell growth. The inability of TGF–β to directly stimulate endothelial cells *in vitro* opens the possibility that this factor may act as an indirect angiogenic agent *in vivo*.[80,99]

Tumor necrosis factors:

Tumor necrosis factors (TNF–α) are polypeptides that were originally isolated as agents that could cause necrosis and subsequent regression of certain solid tumors. TNF–α is considered likely to play a central role in the macrophage–induced angiogenesis that accompanies inflammation and wound healing.[99]

Angiogenin:

Angiogenin is a polypeptide isolated from a human adenocarcinoma cell line. It has angiogenic activity *in vivo* but does not stimulate endothelial cell proliferation. The target cells by which angiogenin promotes angiogenesis are unknown.[80,100]

Platelet derived growth factors:

Platelet–derived growth factor (PDGF) is a well known mitogen for endothelial cells and can be secreted from these cells. Platelet–derived endothelial cell growth factors (PD–ECGF) can stimulate growth and chemotaxis of endothelial cells *in vitro* and has angiogenic activity *in vivo*. In contrast to FGF, PD–ECGF does not stimulate the growth of fibroblasts *in vitro*.[98]

Vascular endothelial growth factor or Vascular permeability factor:

Both vascular endothelial growth factor (VEGF) and vascular permeability factor (VPF) have similar amino acid sequences. VEGF is a heparin–binding growth factor specific for vascular endothelial cells which is able to induce angiogenesis *in vivo*.[101] VPG has similar structure to PDGF and can increase a blood vessel's permeability, endothelial cell growth and angiogenesis.[102]

Prostaglandins:

Although most of the known angiogenic growth factors are polypeptides, certain arachidonic acid derivatives, such as PGE_1 and PGE_2 have also been documented to possess angiogenic activity. However, it is not clear how prostaglandins induce angiogenesis *in vivo*, because these lipids are chemotactic to endothelial cells but do not stimulate their proliferation *in vitro*.[80,99]

3.5 Mechanism of Angiogenic Stimulation

Despite the fact that growth factors are normally present in several tissues, vascular growth and development do not occur unless there is further stimulation. This implies that there is a control mechanism to regulate growth factor activation. It has been suggested that ischemia itself may induce changes that allow growth factors to come in contact with cellular receptors.[103] Various growth factors can stimulate angiogenesis *in vivo*. They, however, have quite different effects on *in vitro* capillary endothelial cell locomotion and proliferation that are the two key events necessary for the formation of a new capillary blood vessel *in vivo*. Some growth factors can stimulate endothelial cell locomotion or proliferation, or both. In contrast, others have no effect, or even inhibit endothelial cell proliferation.[80]

Angiogenic growth factors may operate either directly or indirectly on their putative targets *in vivo*. As Folkman and Klagsbrun have proposed, angiogenic factors which can stimulate locomotion or proliferation of vascular endothelial cell *in vitro* have the vascular endothelial cell as their target *in vivo*.[80] These factors may directly stimulate endothelial cells in pre–existing vessels to proliferate beyond the normal baseline, and new vessels are formed following a series of sequential steps during angiogenesis. Acidic FGF and basic FGF are examples of such direct angiogenic factors. On the other hand, some angiogenic factors that have no effect on endothelial cells *in vitro* can be categorized as acting by indirect pathways *in vivo*. The exact mechanisms of such

indirect pathways are not clear but there are at least 3 possible speculations. It is possible that certain indirect angiogenic factors could stimulate a nonspecific response *in vivo* by mobilizing macrophages and activating them to secret some direct angiogenic factors[104] or chemotactic factors[105] for endothelial cells or both. A second possibility is that indirect angiogenic factors could cause the release of some direct angiogenic factors (for example, bFGF) that are stored in the extracellular matrix.[106] A third possibility is that indirect angiogenic factors could release intracellular stores of some direct angiogenic factors.[106] Thus, different pathways for inducing angiogenesis should be considered since *in vivo* neovascular processes may utilize more than one pathway, for example, in tumor angiogenesis.[80]

4. PREVIOUS INVESTIGATIONS OF ANGIOGENIC STIMULATION

Angiogenic stimulation (or so called "therapeutic angiogenesis") is beginning to show the potential for its clinical applications. Although reports of the clinical as well as the experimental use of angiogenic factors are currently sparse, trials on a much larger scale are perhaps underway, and their arrival in the literature is imminent. Can purified angiogenic factors be administered *in vivo*, either locally or systemically, to accelerate wound healing and tissue repair, increase revascularization in ischemic tissues and organs, or enhance endothelialization of vascular grafts and denuded vessels? These questions can now be tested *in vivo* because of the availability of well-characterized angiogenic factors and the initial success of preliminary studies in using these factors to stimulate neovessel formation *in vivo*.[80,107,108] Progress in this area may cause a change in medical practice as significant as that caused by the introduction of antibiotics half a century ago.

4.1 Accelerating Wound Healing

Wound healing requires close control of degenerative and regenerative processes that involve numerous cell types and complex interactions between multiple biochemical cascades. There is considerable supporting data for the role of angiogenesis in wound healing and thus, angiogenic factors might be administered to accelerate wound healing by initiating the formation of new blood vessels in granulation tissue.[109] In experimental studies, McGee et al[110] demonstrated a modest but significant enhancement of normal skin incisional wound healing in a rat model, manifested by an increase in fresh wound tensile

strength, wound breaking energy, and evidence of accelerated histologic wound maturation following a single injection of bFGF into the incision area. Greenhalgh et al[111] examined the effects of recombinant growth factors in a mouse model of impaired wound healing. PDGF, bFGF, or combinations of both were applied directly to the wounds of the genetically diabetic mice for 5 to 14 days after wounding. Their results demonstrated the improved wound healing in PDGF or bFGF treated animals and combinations of both growth factors produced even better healing than either growth factor alone. A similar result was reported simultaneously by Tsuboi and Rifkin[112] using bFGF in the same animal model. To examine the potential effect of TNF on wound healing, Mooney et al[113] demonstrated that local administration of TNF in collagen to the wounds of normal and adriamycin–impaired mice resulted in an increased wound disruption strength and an improved histologic appearance, but the systemic administration of TNF had no effect on wound healing in this model over the dose range studied. Following successful purification and partial characterization of the placental growth factor, Burgos et al[114] in Britain reported that human uterine angiogenic factors could enhance angiogenesis of the wound bed in rats 3 days after meshed human dermis allograft implantation. This was accompanied by a greater amount of granulation tissue formation, enhanced granulation tissue penetration into the dermal grafts, and accelerated incorporation of the grafts. Basic FGF could improve the survival of ischemic skin flaps and prevent marginally perfused areas of the flap from undergoing necrosis by enhancing vascular connections between the bed and the flap, but it could not show beneficial effect in staged flap

transfers.[115] Local pretreatment with angiotropin, a polypeptide angiogenic factor isolated from activated peripheral monocytes, could also prevent skin flap necrosis and enhance dermal regeneration in a rabbit model.[116] Therefore, angiogenic stimulation may be a novel approach in the management of patients with deficient wound healing.

In preliminary clinical trials, Knighton et al[117] utilized autologous platelet-derived wound healing factors (PDWHF), a mixture of 5 platelet-produced growth factors that had been shown to stimulate angiogenesis in previous animal studies, to treat 49 patients with chronic nonhealing cutaneous ulcers of various etiologies. All patients healed their ulcers in an average of 10.6 weeks and the results first demonstrated that locally acting growth factors might promote healing of chronic cutaneous ulcers in the patients. This study was followed by a larger series of trials in 99 patients with chronic nonhealing ulcers from the same institution. Ninety percent achieved ulcer healing in an average of 7.4 weeks compared to an average of 96 weeks if treated by previous conventional treatments.[118] Based on their experimental studies in wound healing using epidermal growth factor (EGF),[119,120] Brown et al[121] conducted a prospective, randomized, double-blind trial to determine whether EGF could accelerate the rate of epidermal regeneration in humans. In their study, paired donor sites were created in 12 patients who required skin grafting. One donor site from each patient was treated topically with silver sulfadiazine cream containing EGF, and one was treated with silver sulfadiazine cream alone. The donor sites treated with the cream containing EGF had an accelerated rate of

epidermal regeneration in all 12 patients compared with that in the paired donor treated with the cream alone. Treatment with EGF significantly decreased the average length of time to regeneration and histologic evaluation 3 days after the onset of healing revealed almost completely regenerated epithelium. They concluded that topical application of a single biosynthetic growth factor, EGF, indeed accelerated the rate of healing of partial–thickness skin wounds in patients. A clinical study by Burgos et al[122] in Britain in 18 patients with chronic varicose ulcers also demonstrated that the addition of human placental angiogenic factors to dressings could cause a noticeable increase in both granulation tissue and epithelialization of ulcers in these patients. Recently, Robson et al[123] reported the first randomized, blinded, placebo–controlled human trial of bFGF for the treatment of chronic pressure sores in 50 patients. They also tested the safety and toxicity of bFGF in these patients. Their results not only demonstrated a greater healing effect for the bFGF–treated patients, with increased fibroblasts and capillaries in wound sections, but also showed no toxicity, no significant serum absorption or antibody formation. Therefore, topically applied recombinant bFGF is safe and may be effective in the treatment of chronic wounds. A similar result was also reported from the same center using PDGF for the treatment chronic pressure ulcers in 20 patients with the good results.[124]

4.2 Accelerating Healing in Poorly Vascularized Tissue

Angiogenesis is an essential part of normal growth and tissue repair. Factors that

mediate the angiogenic process may have important therapeutic implications in situations where an augmented vascular supply is needed, such as the healing of poorly vascularized or traumatized tissue. Although the direct cause–and–effect relationship of angiogenesis has yet to be fully demonstrated, the possibility of inducing accelerating angiogenesis poses an attractive investigational approach for augmenting tissue repair. In a bone graft repair model of the rabbit, Eppley et al[125] evaluated the effectiveness of bFGF in autogenous mandibular bone graft healing. Block cortical grafts harvested from the ilium were implanted into sites in the mandibular ramus or body. The bFGF was continuously infused over a period of 14 days through subcutaneous osmotic pumps. Increased vascularity, as assessed by vessel number and depth of penetration into the grafts, was noted at 10 days postoperatively in the bFGF treated side as compared to contralateral control side. In a bone healing model of the rat, Nottebaert et al[126] analyzed the efficacy of local administration of omental angiogenic lipid fraction on osseous neovascularization and bone repair. A segmental femoral defect was replaced by a demineralized allogenic bone graft exposed to continuous local delivery of omental lipid for 14 days via an implanted miniosmotic pump, while saline was delivered in the same way served for controls. Neovascularization and bone formation in the transplant were quantitatively evaluated. Compared with the control group, the omental lipid angiogenic fraction–treated specimens had significant increases in bone density as well as in regional blood perfusion, maximal at 2 weeks following surgery. At 6 weeks, the vascular response, determined by microangiography, was very intensive and diffuse in the treated group

involving the whole graft, while in the control group few vessels appeared, leaving the central portion of the graft relatively devoid of vasculature. At 12 weeks, biomechanical testing demonstrated significantly higher union rate and strength in the treated specimens as compared to the controls. Their data indicate that the omental lipid fraction has the ability, when administered locally, to enhance blood perfusion and vascular ingrowth as well as bone formation and healing in bone transplants. Therefore, angiogenic factors may have potential therapeutic value for the treatment of osseous problems such as a bone graft where angiogenesis is desired.

In other experimental studies, King and Vallee[127] implanted angiogenin, a potent angiogenic factor derived from human plasma, into experimentally injured menisci of 75 rabbits. During a 26 week study, localised neovascularization occurred in 52% of the angiogenin–treated animals compared to only 9% of the controls. Angiogenin induced neovascularization which could enhance the healing of injuries within the poorly vascularized meniscal fibrocartilage and improve the results of meniscal repair. Although the exact mechanism remains unknown, Cordeiro et al[128] demonstrated, in their recent study, that aFGF with heparin and type I collagen significantly enhanced peripheral nerve regeneration in an *in vivo* rat sciatic nerve model. The authors have postulated that aFGF might indirectly affect nerve regeneration by increasing the vascular supply to the nerve.

Donor airway ischemia and inadequate airway anastomotic healing have been the serious problems after lung transplantation. In order to solve these problems, Olech et al[129] examined the hypothesis that surface abrasion and topical application of bFGF would enhance omento–tracheal revascularization in a rabbit heterotopic autograft model. In their study, approaches to augment the rate and degree of angiogenesis in a devascularized tracheal segment were evaluated. However, neither surgical abrasion nor topical bFGF application were found to increase omento–treacheal revascularization of trachea in this model after 7 days. In the discussion regarding the deficiencies of their study, they postulated that Surgicel and Gelfoam used in their study might have been unable to retain bFGF at the surface of the trachea and omentum; heparin was not administered to the animals before extraction and subsequent implantation; the quantity of bFGF given in their animal model might not be sufficient to induce neovascularization; and bFGF might be capable of increasing neovascularization to tracheal segments, but their choice of time between operation and study did not permit this effect to be detected.

Folkman et al[130] described the development of another modality of therapeutic angiogenic stimulation: the accelerated healing of experimental duodenal ulcers by an orally administered angiogenic growth factor. In previous studies, they found that chronic duodenal ulcers share many similarities with chronic wounds. For example, both lack an epithelial covering and contain inflammatory cells, exposed collagen, necrotic debris, and granulation tissue.[131,132] Since granulation tissue is composed mainly of capillary blood

vessels, monocytes, and fibroblasts, and since bFGF can promote the formation of vascularized granulation tissue,[133,134] they hypothesized that the healing of chronic duodenal ulcers could be accelerated by stimulating angiogenesis in the ulcer bed. To test this hypothesis, an acid–stable form of bFGF was administered orally by intragastric gavage twice daily for 21 days and compared with similar administrations of recombinant bFGF, optimum dose of the H2–blocker cimetidine, or vehicle alone in a rat cysteamine–induced chronic duodenal ulcer model. The results showed that oral administration of acid–resistant bFGF stimulated a more than ninefold increase of angiogenesis in the ulcer bed and significantly accelerated ulcer healing compared with untreated control animals or with animals treated with cimetidine.[135,136] Therefore, angiogenic stimulation may provide a form of replacement therapy and a novel approach in the treatment of duodenal ulcers.[130]

4.3 Increasing Revascularization of Ischemic Organs

The use of angiogenic factors to enhance collateral vessel formation in the vascular territory of an occluded artery has obvious important clinical ramifications and will be a more foreseeable goal over the next decade. Many patients with coronary artery disease, cerebrovascular disease, or peripheral vascular disease who present with organ ischemia could potentially benefit from angiogenic therapy. For example, patients with diffuse and severe occlusive disease who are not suitable for surgical revascularization or balloon angioplasty might benefit from such therapy. However, the use of angiogenic

therapy to promote collateral formation or revascularization in ischemic organs could also have great applicability in patients with limited occlusive disease who have one or more unobstructed arteries feeding collaterals as the inflows to supply such organs. Could angiogenic therapy relieve organ ischemia by enhancing collateral formation or revascularization in the ischemic organ? This hypothesis has recently been tested in different organ ischemia models and the preliminary results, including those from our own studies, suggest that pharmacological potentiation of collateral formation in the ischemic organ may be possible and angiogenic therapy should be considered a novel approach for the treatment of organ ischemia.

In order to evaluate the efficacy of angiogenic factors as a potential treatment for patients suffering from cerebrovascular insufficiency, Lyons et al[137] designed an experiment to determine if intraventricular administration of bFGF could promote cerebral angiogenesis in a rat model of mild chronic forebrain ischemia. In this experiment, each Wistar rat underwent bilateral carotid artery ligation which induced the mild ischemia in forebrain which was confirmed by hippocampal neuronal counting. Animals received intraventricular injections of bFGF every 4 days for 28 days. Their results showed that bFGF caused a significant dose-dependent increase in capillary density compared to the non-ischemic control, the ischemic control, and the ischemic-vehicle control in all regions examined. These results support the hypothesis that an angiogenic factor, such as bFGF, when administered locally, could induce *in vivo* cerebral

angiogenesis and that this therapy may be useful for the patients who suffer from chronic cerebrovascular insufficiency.

With the ultimate goal to determine whether the angiogenic agents could promote myocardial revascularization in the ischemic heart, Unger et al[138] developed a canine ischemic myocardial model for such investigation. The surgical preparation of this model was based on the operation pioneered by Vineberg[139], in which the internal mammary artery (IMA) was tunnelled directly into the myocardium with the supposition that anastomoses were formed between it and the coronary circulation. In this canine model, they implanted the IMA as a potential source of collateral blood flow into the territory of the left anterior descending coronary artery (LAD) and gradually occluded the proximal LAD with an ameroid constrictor during a 2–3 week period. A tube situated in the distal IMA connected to an implanted pump provided for continuous intra–arterial infusion at the site of collateral formation. After 8 weeks, they demonstrated that collaterals were formed between the implanted IMA and the native coronary circulation in this model and these new formed collaterals could provide modest nutritive blood flow to a collateral–dependent region.[138] In a subsequent study using the same model, Unger et al[140] demonstrated that continuous infusion of heparin (15 or 150 U/h, for 8 weeks) significantly promoted collateral flow between the extracardiac artery and the myocardial circulation. In another subsequent study, Banai et al[141] placed a sponge saturated with aFGF between an extracardiac artery and ischemic myocardium to determine whether

direct application of aFGF to the heart could induce the formation of bridging collaterals between that extracardiac artery and the ischemic area of the heart in the same model. However, aFGF, delivered to the myocardium via an epicardial sponge 4 weeks after, did not cause an angiogenic response in viable myocardium but did cause vascular smooth muscle cell hyperplasia in areas subjected to ischemic injury. Thus, they suggest that careful studies are needed before it can be concluded that aFGF has either a salutary or a detrimental effect in this situation.

Yanagisawa–Miwa et al[142] have recently documented that intracoronary injection of bFGF could salvage infarcted myocardium in a canine experimental myocardial infarct model. In their study, an acute myocardial infarct was induced by the injection of an artificial thrombus into a segment of the LAD that had been made stenotic by laser ablation. At 30 minutes and 6 hours after LAD occlusion, bFGF (10 μg in 10 ml of saline) was infused into the left circumflex coronary artery (LCX). One week later when the study was terminated, they found that the animals treated with bFGF had significantly improved left ventricular ejection fraction, reduced myocardial infarct size, and had increased number of arterioles and capillaries in the infarcted myocardium compared to that of the control animals. Thus, they concluded that application of angiogenic agents, such as bFGF, might bring about a therapeutic modality for the treatment of infarcted myocardium.

The effects of angiogenic growth factor on angiogenesis and collateral vessel growth in ischemic limbs have recently been tested in our laboratory as well as others. In a recent report, Baffour et al[143] demonstrated enhanced angiogenesis and growth of collaterals by *in vivo* administration of bFGF in a rabbit model of acute lower limb ischemia. In their study, an animal ischemic hindlimb model was developed by a two–stage procedure in the pelvis and groin to produce severe ischemia of the left hindlimb. The ischemic hindlimb received intramuscular injections of saline, 1 μg bFGF, or 3 μg bFGF daily for 2 weeks. Angiography revealed extensive perfusion of the left hindlimb in all bFGF treated animals. Two bFGF–treated groups exhibited a much more rapid rise in transcutaneous oximetry than the control group. The capillaries per square millimeter and capillaries per muscle fiber ratios were significantly increased in all animals that received bFGF. Both bFGF–treated groups had a significant increase in thigh muscle viability compared with controls based on triphenyltetrazolium chloride reduction. There was a trend toward a dose–response effect for all measurements. Thus, their results demonstrate that *in vivo* administration of angiogenic growth factor might have the potential for being of therapeutic value in the acutely ischemic lower limb.

4.4 Enhancing Endothelialization of Vascular Graft or Denuded Vessel

The lack of endothelial regrowth and coverage in the surface of the grafts or denuded vessels is thought to contribute to the failure of vascular grafts due to thrombosis and restenosis. Recent reports from Clowes et al[144] and Greisler et al[145] have

demonstrated that alterations in graft design using more porous ePTFE material, for example, 60 μm internodal distance, could stimulate transmural capillary ingrowth, resulting in an enhanced reendothelialization of vascular grafts when implanted into animal models. Based on the understanding of the role of angiogenic mechanisms in graft endothelialization,[144] alternative approaches have been suggested whether vascular grafts impregnated with angiogenic growth factor might be invaded by capillaries and thus develop an endothelial cell lining more efficiently than conventional vascular prostheses. Recently, Greisler et al[146] reported their results for this approach. They affixed aFGF with fibrin glue to 60 μm internodal–distance ePTFE grafts and implanted these grafts in the aortoiliac position of dogs. When explanted at 28 days, these grafts had a grossly more substantial smooth and translucent neointima. Microscopy revealed a confluent endothelialized blood–contacting surface and extensive capillary ingrowth through the wall of the graft. No control grafts revealed such a surface during the same period. Scanning and transmission electron microscopy corroborated the above findings. Autoradiographic analysis of DNA synthesis in cells at the luminal surface revealed significantly increased labelled cells in the pretreated grafts. Thus, vascular grafts pretreated with one of the angiogenic growth factors may promote endothelialization of the graft surface through capillary ingrowth and increased endothelial cell proliferation.

Failure of the injured blood vessel to rapidly and completely reendothelialize following balloon angioplasty, atherectomy, and endarterectomy may also be a common

problem. Therefore, a means to stimulate reendothelialization might have potential to improve the clinical results of these procedures. Lindner et al[147] have recently tested this hypothesis in a rat model of the denuded artery. They found that the cessation of regrowth of endothelium in the devoid area of the left carotid artery following balloon catheter denudation could be overcome by systemic administration of bFGF through a catheter placed into the aortic arch via the right axillary artery. Administration of bFGF over an 8–hour period caused a highly significant increase in the replication rate of endothelial cells which resulted in a significant increase in the extent of endothelial outgrowth onto the denuded surface. Furthermore, total endothelial cell coverage on the denuded artery could be achieved within 10 weeks after balloon catheter denudation by continuous administration of bFGF twice a week for an 8–week period. In another injured rat carotid artery model, Bjornsson et al[148] demonstrated a dose–dependent inhibition of intimal thickening with a parallel increase of endothelial regeneration over the injured area 2 weeks after air–desiccation injury following i.v. administration of aFGF either as a constant–rate infusion for 2 weeks or as a single bolus injection immediately after completion of the injury. More recently, Stevens et al[149] showed that vascular permeability factor (VPF), an extremely potent polypeptide mitogen for endothelial cells but not for vascular smooth muscle cells or fibroblasts, could significantly accelerate endothelial repaving and inhibit intimal hyperplasia in rabbit carotid arteries following balloon catheter mechanical injury. Therefore, angiogenic growth factor may also be efficacious in the restoration of an intact, confluent endothelium and in the prevention of

restenosis following arterial injury.

5. METHODOLOGY FOR THE STUDY OF LIMB REVASCULARIZATION

5.1 Animal Model for the Study of Limb Ischemia

The search for a suitable animal model for the study of limb ischemia has been problematic. Most animal models reported from the literature have been more suitable for studying of acute rather than chronic limb ischemia. They have not resulted in stable hindlimb ischemia since normally many animals have a remarkable potential to form collateral pathways from their non–diseased proximal vessels and reconstitute in their normal distal arterial trees. Furthermore, it has been noted that ligation or division of even a major vessel often produces little observable effect on limb function and resting limb ischemia afterwards.

(1) Rat Ischemic Hindlimb Model

The muscles of small animals, such as the rat, are more sensitive to ischemia than the human muscles. For example, following identical periods of ischemia, rat muscle exhibits a more rapid and severe metabolic deterioration and a slower recovery from tourniquet ischemia than has been observed for human or canine muscle.[150] Thus, conclusions may be difficult to draw from studies when using rat ischemic hindlimb model.

Janda et al[151] reported a rat model of chronic hindlimb ischemia in which division of the common iliac artery produced a significant reduction of blood flow in the anterior

tibialis muscle during standard exercise until 7th weeks. The most severe histological and histochemical changes found in the muscles of the ischemic hindlimb, however, lasted for only 7 days.[152] Challiss et al[153] studied a rat hindlimb model of arterial insufficiency produced by division of the common femoral artery. They could not find any significant difference in the blood flow to the muscles of the ischemic limb compared with the normal contralateral limb at rest. Histological studies also revealed only minor morphological alterations in the calf muscles when examined 7 days after the division.[154] Although common iliac or femoral artery ligation alone in rats may be used as a model of moderate hindlimb ischemia, it is inadequate as a model of hindlimb ischemia at rest.

Mathien and Terjung[155,156] designed a model by inducing femoral artery stenosis to establish peripheral arterial insufficiency in the rat hindlimb. In this preparation, a ligature was placed tightly around the femoral artery and a 0.014" diameter stainless steel wire. The wire was then carefully removed to let the arterial driving pressure restore the patency of the vessel. The animals significantly reduced their exercise tolerance after this preparation while resting muscle blood flow of the hindlimb remained normal.

Seifert et al[157] described a two-stage operative procedure to induce resting arterial ischemia in the rat hindlimb. In this model, the first stage involved surgical interruption of collateral and re-entrant vessels, and the second stage involved femoral artery ligation 7 days later. The reduction of blood flow as well as the histologic appearance of

ischemia in the calf muscle were evident for at least 5 days. However, the complex and time consuming preparation of this small animal model has obvious disadvantages.

(2) Rabbit Ischemic Hindlimb Model

Chervu et al[158] developed a model of unilateral hindlimb ischemia in the rabbit for the study of limb ischemia and reperfusion. In this model, both circumflex iliac arteries and internal iliac arteries as well as the major iliolumbar arteries from the distal aorta were ligated in order to eliminate pelvic blood flow. To achieve ischemia in one hindlimb, the right common iliac and common femoral arteries were clamped. The adequate and consistent severe ischemia induced was confirmed by angiography as well as evidenced by the presence of marked acidosis and hyperkalemia in the venous effluent from the ischemic hindlimbs after 3 hours.

A rabbit model of partial hindlimb ischemia was studied by Hendricks et al[159] in which unilateral common iliac artery division was performed in 20 animals. Nine of 20 animals had objective evidence of functional limb impairment following surgery. The severity of ischemia were measured by quantitating muscle resting blood flow, femoral arteriovenous oxygen differences and calf arterial pressure in the ischemic hindlimbs compared with those in the contralateral limbs. The results demonstrated that common iliac artery division in this rabbit model could only produce persistent partial resting ischemia of the hindlimbs in about 3 weeks without muscle atrophy or tissue necrosis.

This model has been recently used by the same investigators to test the efficacy of a muscle pedicle flap to revascularize an ischemic limb 7 days after ischemic preparation.[68]

In a recent study, Baffour et al[143] designed a rabbit model of acute hindlimb ischemia to evaluate the effects of bFGF on revascularization in ischemic limbs. This model was developed by ligation and division of the caudal artery, both iliolumbar arteries, internal iliac arteries, and inferior epigastric arteries followed by excision of a 2 cm segment of the left femoral artery one week later. The rational for the two-stage procedure was thought to shorten an otherwise long operating time and allow some growth of native collaterals in the hindlimb before the induction of acute ischemia. Two weeks after this two-stage procedure, in total of 10 animals, 6 presented with limping, 4 had blackened toenails, and 6 developed muscle atrophy.

(3) Dog Ischemic Hindlimb Model

Although dogs have been widely used as models in experimental vascular surgery, the potential for rapid and complete collateral formation normally present in dogs has hampered their use as a good model in the studying of chronic limb ischemia. In order to induce a dog model of persistent hindlimb ischemia, as many as 14 separate ligations of branches of the iliac and common femoral arteries, and ligation of the profunda artery at its origin have been described. However, after a 6-week recovery period, the animals presented only with claudication but without severe ischemia. The distal-to-central

systolic blood pressure index was about 0.6 while resting blood flow was approximately normal.[150]

Johansen and Bernstein[47] developed a dog model of severe hindlimb ischemia in which an ischemic limb (right side) would undergo irreversible tissue death in the absence of a further therapeutic intervention. To achieve this, the surgical preparation involved ligatures of the right peripheral femoral artery and all femoral artery branches in the right thigh, as well as ligatures of the terminal aorta, both internal iliac arteries, the last right lumber artery, and the right deep circumflex iliac artery. All 7 animals presented with the cold, tender, and functionless hindlimbs within 6 days following the procedure. Histologic examination of skin and muscle from the ischemic hindlimbs revealed early mild to moderate tissue necrosis.

In our laboratory, Graham et al[48] developed a dog model of severe hindlimb ischemia. In order to ensure a severely ischemic preparation in the right hindlimb, the terminal aorta, right deep circumflex and 6th lumbar arteries, bilateral internal iliac, umbilical and 7th lumbar arteries, median sacral artery, right deep femoral artery, all branches of the right superficial femoral artery, and right distal femoral artery were ligated. All 5 animals had totally necrotic hindlimbs over a period of 24–48 hours and pathologic examination of these ischemic limbs revealed the gross and microscopic changes of acute ischemic necrosis. Baffour et al[49] modified the procedure to develop a

dog model of partial hindlimb ischemia by leaving only the deep femoral artery intact. These models have been used in our laboratory to study the role of arteriovenous reversal in revascularization of ischemic limbs.[48-50]

(4) Others

Aldman et al[160] reported a pig model with acutely induced subtotal ischemia of one hindlimb that might resemble the clinical situation in patients with acute thromboembolic occlusion. The ischemic preparation involved ligations of the medial sacral artery, the internal iliac artery, and the medial and lateral circumflex arteries and clamping of the femoral artery with a plastic cylinder so that hindlimb ischemia and reperfusion could be studied. The blood flow of the hindlimb was reduced to 5% of resting flow following the procedure in this model.

5.2 Evaluation of Arterial Perfusion to Ischemic Limbs

(1) Limb Blood Pressure Determination

Measurement of limb systolic blood pressure by Doppler flowmeter is one of the most useful technique to assess limb ischemia both clinically and experimentally. The method is noninvasive, relatively simple and can be employed in medium or large size animals. In an animal model of unilateral ischemia, the limb systolic blood pressure L/R (or R/L) ratio may reflect the ankle/brachial index (ABI) measured clinically to assess patients with peripheral vascular disease. This can be used as an index to indirectly

determine the degree of changes in arterial perfusion to the ischemic limb.

(2) Skin Perfusion Determination

Transcutaneous oximetry is a noninvasive and continuous method for monitoring skin oxygen tension in ischemic limbs and has currently been used to diagnose and manage the patients with peripheral vascular disease. Several studies demonstrated that transcutaneous oxygen pressure ($TcPO_2$) is a good indicator of tissue perfusion and correlates well with the degree of limb ischemia.[161-3] Previous studies from our laboratory[50] as well as from others[143] have shown the usefulness of $TcPO_2$ measurements in evaluating experimental limb ischemia. In addition, studies on limb ischemia and compartment syndrome from our laboratory have also demonstrated a close correlation between arterial blood gas and $TcPO_2$.[164,165] However, $TcPO_2$ measurements have some limitations since they can be influenced by any factor, such as marked edema, that can reduce the transmission of oxygen.[163]

Laser Doppler velocimetry (LDV) uses coherent helium–neon light which is directed via a fiberoptic conduit onto an area of skin or exposed tissue. The light reflected from circulating erythrocytes in capillaries undergoes a frequency shift proportional to average red cell velocity. This technique allows continuous noninvasive measurements of capillary blood flow in skin or exposed tissue and has been used clinically to predict healing of ischemic ulcer or to select the amputation level in an

ischemic limb.[166,167] Experimentally, it has also been used to detect changes of blood flow in flaps[168] or to assess changes of blood flow associated with angiogenesis in chronic inflammation.[169] However, LDV does not measure the actual blood flow per unit of tissue, rather it measures flow in relative terms. This technique can only measure the blood flow within a limit depth (1.5 mm) of tissue. There are also marked regional variations in laser Doppler determined blood flow within a single extremity, probably related to varying capillary densities in the skin.[150] It has been suggested that the diagnostic accuracy of LDV can be enhanced by its combination with the reactive hyperemia test and by measuring the cutaneous reactive hyperemia response in an ischemic limb.[170,171]

Other techniques have also been used to determine skin perfusion in an ischemic limb. These include skin fluorometry, a method which relies on an injection of fluorescein and is quantified visibly or by a fiberoptic fluorometer [172], and thermography[173] with variable success and major limitations.

(3) Muscle Metabolic Status Determination

Most routine noninvasive techniques do not directly assess the effect of tissue perfusion impairment on metabolic status of the limb that is responsible for the ischemic damage in the limb observed clinically. However, the metabolic status in an ischemic limb can be assessed by measuring the concentration of metabolic intermediates or

products in blood or tissue samples taken from the limb.

Venous blood samples from the ischemic limb can be used to determine the lactate, oxygen, and glucose levels. The impairment of tissue perfusion in the limb can therefore be assessed by the degree of increasing lactate release and glucose consumption or decreasing oxygen content in the venous effluent. [174] However, the impairment of tissue perfusion may not be shown in claudication patients[175] or in animals without resting hindlimb ischemia.[157,159]

Percutaneous or open muscle biopsies from an ischemic limb can be performed to analyze for ATP, ADP, phosphocreatine(CP), lactate, and pyruvate. In patients with peripheral vascular disease, chronic ischemic rest pain results in a depletion of resting levels of high-energy phosphates. This correlates with the severity of the disease as well as the efficacy of treatment.[176] Nuclear magnetic resonance (NMR) spectroscopy is a novel noninvasive technique for *in vivo* evaluation of tissue molecules containing phosphorous, hydrogen, and carbon, such as ATP, CP, and inorganic phosphate. The technique is expensive but it can provide total limb (skin and muscle) data on tissue perfusion and can be repeatedly used in an intact limb.[177]

Muscle surface pH measurement, a percutaneous technique that has been used clinically to directly measure extracellular fluid pH at the muscle surface, is an alternative

approach to tissue biopsy for lactate measurement and it can quantitatively determine tissue acidosis in an ischemic limb.[174] Transmembrance potential measurement is an invasive technique with minimal injury to the muscle. It can provide a more sensitive index of skeletal muscle cell injury or repair than the simple measurement of tissue metabolites.[178]

(4) Limb Perfusion or Blood Flow Determination

Standard techniques successfully used to measure extremity blood flow include plethysmography, electromagnetic flowmetry, tracer washout techniques, and injection of radioactive microspheres or tracers.[150] An objective assessment of the extent of perfusion in an ischemic limb can therefore be reliably obtained.

Plethysmographic techniques are well described and are now part of the standard clinical armamentarium in the noninvasive vascular laboratory. Total blood flow to an limb can be conveniently recorded with a mercury–in–Silastic strain gauge. This is based on the detection of minute changes in the electric resistance of the mercury column, which depends on its length. These techniques, can not, however, differentiate the relative distribution of perfusion to skin, subcutaneous tissue, and muscle.[179]

Electromagnetic probes can also reliably measure blood flow when they are placed directly around vessels as small as 0.5 mm in diameter. Blood flow is measured using

the principle of electromagnetic induction, where the flow of blood through a magnetic field induces a measurable voltage that is directly proportional to flow.[180]

The washout of inert radioactive tracer substances gently injected into tissues has been used extensively to estimate tissue or organ perfusion.[151,157] The most widely used tracer method for the assessment of limb perfusion is Xenon 133, although similar data can be obtained using other indicators. The techniques are highly reproducible and have the distinct advantage that absolute flow values from a specific tissue can be obtained.[181]

Measurement of regional blood flow by the injection of radioactive tracers is a highly reliable, well–described technique that has been used in experimental studies as well as in the clinical evaluation of peripheral vascular disease.[48,68,150,153,159,182] The basic principle of this technique for determining the regional distribution of blood flow is that radioactive particles, larger in diameter than a capillary, are trapped within the first capillary bed they encounter and thus are distributed in proportion to the perfusion of that capillary bed.[182] Following injection of the radioactive tracers, such as ^{99m}Tc–labelled albumin microspheres or ^{99m}Tc–labelled macro aggregates, in a high flow location upstream of the body (ideally into the left atrium), the whole or part of body are scanned and counted on a Gamma Camera. The relative quantification of arterial perfusion to an limb can then be assessed.

(5) Histologic or Histochemical Determination

Histologic and histochemical evidence of muscle cell abnormalities due to the impaired arterial perfusion can usually be demonstrated in the acutely rather than chronically ischemic limbs.[183] In acute ischemia, ground or scattered necrotic muscle fibers undergoing phagocytosis as well as regenerating fibers can be demonstrated by light microscopy. Trilaminar plates in the intracristal space of mitochondria and interruption of the plasma membrane with dissolution of the Z-discs can be evidenced on electro microscopy.[184] In chronic ischemia, inflammatory cell infiltration and active myophagia can be seen and they are the most specific indications in severe chronic ischemia. [83,157,184] Special histochemical staining can identify damaged cells despite a grossly normal appearance. The triphenyltetrazolium chloride (TTC) reduction method, in which colorless TTC can stain normal skeletal muscle cells but not stain irreversible injured or necrotic muscle cells, is one such example.[186]

5.3 Evaluation of New Vessel Formation in Ischemic Limbs

Determination of new vessel formation in an animal ischemic hindlimb model is difficult. The use of the available techniques to differentiate the newly formed vessels from the just enlarged pre-existing channels secondary to ischemia in an ischemic limb still presents a challenge. Gross or histologic technique can be used to assess new vessel formation in an ischemic limb and each approach represents an investigation at different levels.

(1) Gross Examination

Conventional angiography or combined with digital subtraction remains one of the best approaches to determine the extent and degree of vascularization in an ischemic limb of large animals. In a limb or an organ such as heart, the number of blood vessels visualized on the angiograms can also be counted in order to permit scores to compare with different degrees of revascularization.[142,143] However, this imaging technique can only visualize the blood vessels on angiograms and can not directly differentiate the newly formed vessels from the pre–existing channels in an ischemic limb.

Other techniques such as microangiography,[126] direct counting of vessels after particle perfusion,[125] and preparation of vascular casts[187] can also be used in an ischemic limb of small animals. The last two techniques can be studied on the gross level or the subgross level with the aid of a dissecting microscope.

(2) Histologic Examination

Histologic quantification of new vessel formation in the skeletal muscle can be studied using the microscopic angiogenesis grading system developed by Brem et al,[188] who have used this method to quantify angiogenesis in a variety of tumors. With hematoxylin and eosin staining, the microvessels are counted at a magnification of X200. The field examined at this magnification encompasses an area of 2.54 mm^2. The histologic slides are first scanned at low magnification and only the area of maximum

vascular density is selected for grading. Vascular density is defined as the highest number of vascular lumens per field (X200) encountered in the sample. Ten different fields in the most vascular area of the tissue are counted, and only the one having the highest vascular density is used for analysis. A similar method, but using elastin van Gieson staining and X400 magnification, has been used to evaluate new vessel formation in a canine model of myocardial infarction after intracoronary administrations of bFGF.[142]

The discovery of alkaline phosphatase activity in the walls of blood vessels has led to the histochemical techniques which label alkaline phosphatase in the capillary endothelium so that the number of capillaries in the skeletal muscle can be counted. The technique of capillary density (capillaries/mm^2) determination has recently been used in experimental animals which have been subjected to various stimuli to evaluate new vessel formation in the skeletal muscle and myocardium.[50,143,189] After alkaline phosphatase staining, the histologic slides are microscopically examined at X200 magnification. Capillaries are counted manually within 10 randomly chosen fields (0.0625 mm^2 each) for each muscle section and then averaged. Capillary density is finally expressed as capillaries/mm^2.[189] With the aid of a computerized image analysis system, this technique becomes more accurate and less time–consuming.

Tritiated–thymidine uptake is an established and reliable technique for measuring cell proliferation. Normally, adult vascular endothelium has an extremely slow turnover

rate, rarely dividing, so that mitoses are seldom encountered by this method. However, new vessel formation is associated with proliferation of vascular endothelial cells and there is a positive correlation between the proliferation and the uptake of tritiated thymidine.[88,190] The presence of tritiated–thymidine labelled endothelial nuclei can be confirmed by autoradiography under the microscope and the labelling index (defined as the ratio of labelled to nonlabelled endothelial nuclei) can be used as an indicator to assess the degree of vascular proliferation *in vivo*. [81,191,192] The technique has previously been used in our laboratory to confirm vascular proliferation after AVR in a canine ischemic hindlimb model. However, the demonstration of significant endothelial cell proliferation still does not distinguish between new vessel development and an increase of in size of the pre–existing vessels in an ischemic limb.[50] More recently, a double–staining technique with a monoclonal antibody to Bromodeoxyuridine, a purine analog, has been introduced and used by some investigators. This method can provide a rapid, reproducible, and nontoxic means to measure cellular kinetics. The labelled endothelial cells may give us more conclusive evidence of new vessel formation than classic autoradiography with tritiated thymidine.[193]

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CHAPTER II

HYPOTHESES AND PURPOSES OF THE PRESENT STUDY

Limb salvage in patients with extensive, diffuse peripheral vascular disease who are not suitable candidates for direct surgical revascularization or endovascular procedures, or effective nonoperative therapy for patients with limited peripheral vascular disease who have ischemic symptoms remains a challenge in modern vascular surgery. Efforts to identify the factors involved in initiating and controlling the growth of blood vessels and the successful isolation and purification of some angiogenic stimulants have now made it possible to use such agents as a pharmacological means to relieve tissue or organ ischemia by enhancing collateral vascular growth or angiogenesis. In our laboratory, we have been interested in the concept that angiogenic factors might potentially promote collateral vessel growth in ischemic organs. Based on our understanding of angiogenesis and its stimulants, as well as on a few preliminary reports that *in vivo* administration of one of the angiogenic agents indeed directly stimulated the new vessel growth in rat sponge models, we hypothesized that revascularization of ischemic limbs would be enhanced by *in vivo* administration of one of the angiogenic factors recently developed.

The experimental studies performed during my entire Ph.D. training were designed to test the original hypotheses in a rabbit ischemic hindlimb model. They were carried out by four step–by–step complete experiments and the purpose of each study is as follows:

(1) To develop a rabbit ischemic hindlimb model in which persistent ischemia of the limb could be maintained for a few months, and in which the severity of the ischemia and the response to treatment could be measured. Such a model should be ideally suited for the study of angiogenic stimulation in ischemic limbs by measuring arterial perfusion and collateral vessel formation.

(2) To test our hypothesis that administration of one of the angiogenic factors, endothelial cell growth factor (ECGF), would significantly improve arterial perfusion and collateral vessel growth in the rabbit ischemic hindlimb model.

(3) To further test our hypothesis in the rabbit model of bilateral hindlimb ischemia and to determine whether direct administration of ECGF to a single hindlimb of animal would significantly improve revascularization of that limb compared with its contralateral limb.

(4) To further our understanding of this potential alternative therapy for the treatment of limb ischemia, that is to determine whether a dose–response relationship would be demonstrated for ECGF, whether it would be effective when administered systemically, and finally whether it would affect vascularization to a non–ischemic limb.

In summary, the purposes of the present study were to test our original hypotheses, to establish the concept of using angiogenic stimulation to relieve organ ischemia, and to further understand this potential therapy for the treatment of limb ischemia. The ultimate goal of the present study would be to find an alternative approach to treat the moderately ischemic limb nonoperatively and to salvage the severely ischemic limb which is not currently treatable by direct surgical techniques.

CHAPTER III

ANIMAL MODEL OF THE PRESENT STUDY

A PERSISTENT HINDLIMB ISCHEMIA MODEL IN THE RABBIT

Presented in part at the Eighth Annual Meeting of the Academy of Surgical Research, St. Charles, IL, September 19–22, 1992

ABSTRACT

The study of new approaches for the treatment of limb–threatening ischemia has been hampered by lack of a suitable animal model of persistent limb ischemia. We describe the development and evaluation of an animal model of persistent hindlimb ischemia. Ischemia was induced in the left hindlimb of 28 NZW rabbits by ligation of the distal external iliac artery and excision of the common and superficial femoral arteries. The severity of the ischemia and its relief in each animal was evaluated every 10 days postoperatively until day 40 (all animals) or day 90 day (5 animals). Nine animals developed superficial tissue necrosis in the distal calf or foot, but no deaths were attributable to the ischemia–inducing procedure. Angiography demonstrated minimal collateralization and sluggish filling of distal vessels up to postoperative day 90, which was accompanied by a decrease at rest in calf blood flow ratio ($p< 0.005$ vs. day 0), an increase in lactic acid in the femoral venous blood (left vs. right side, $p< 0.002$) up to postoperative day 40, and a decrease in calf blood pressure ratio ($p< 0.0001$ vs.day 0) up to day 90. Histologic study of the distal anterior tibial muscles demonstrated evidence of atrophy and fibrosis in the left hindlimb. This model can be used to evaluate direct and indirect approaches to the treatment of chronic limb ischemia.

INTRODUCTION

With increasing life expectancy in North America, a growing population of patients with atherosclerotic occlusive disease need treatment for limb salvage.[1] Despite the considerable advances in direct arterial revascularization over the past decade, there still remains a group of patients, who cannot be helped by these procedures because of extensive obliteration of the distal arterial tree. Therefore, research has been stimulated on other possible treatment modalities to avoid amputation.[2-6] The study of alternative therapies for chronic limb threatening ischemia and the achievement of a better understanding of the process by which collateral formation occurs have been hampered by the lack of a suitable animal model. Most models of limb ischemia either are more suitable for studying the effects of acute rather than chronic occlusive disease, or they do not result in a stable ischemic preparation because of the extensive collaterals normally present in animals such as dogs.

This report describes the development of a reproducible animal model in which persistent ischemia of the limb was maintained up to 3 months, and in which the severity of the ischemia and the response to treatment could be measured.

MATERIALS AND METHODS

SURGICAL TECHNIQUE

Twenty–eight New Zealand white rabbits (male, mean 4 kg) were anesthetized with intramuscular Ketamine (50 mg/kg) and Xylazine (5 mg/kg). A longitudinal incision was performed in the left thigh from the inguinal ligament toward the knee. With the aid of surgical loops, the femoral artery was totally dissected and its branches including the profunda, lateral circumflex, superficial epigastric arteries were dissected as far as possible. The popliteal and saphenous arteries were further dissected distally. Ischemia was then induced by ligation of the distal external iliac artery(just above the level of the inguinal ligament), the inferior epigastric artery, and the branches of the femoral artery. The proximal portion of the popliteal and saphenous arteries were then ligated followed by excision of the entire common and superficial femoral arteries from just above the inguinal ligament to the level of the proximal popliteal and saphenous arteries.(Figure 1). All animals received 0.9% sodium chloride 50 ml intravenously during the surgery, and Cefazolin (15 mg/kg/per day) intramuscularly for 4 days started the day of surgery. The pain reliever (Bupreorphine 0.04 mg/kg) was also administered daily during the first 10 days after surgery in each animal. Animals in which superficial skin necrosis was evident were given pain relievers throughout the study. The approval for the use of the rabbits in this study was granted by our institutional animal care committee. The care of the animals complied with the guidelines of the Canadian Council of Animal Care, and the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory

Animals (NIH publication No. 80–23, revised 1985).

Each animal was evaluated preoperatively (day 0), and postoperative 1, 10, 20, 30, and 40 days following the surgery by clinical assessment, angiography, measurement of calf blood pressure and femoral venous lactate levels, and calf radioisotopic perfusion. In 5 animals, the study was prolonged to 90 days, with calf blood pressure measured at each 10–day interval after day 40, and femoral venous lactate assessed and angiography performed on postoperative day 90. The animals were sacrificed on postoperative day 40 or day 90, and muscle samples from both calves were prepared for histologic studies.

ASSESSMENTS

Angiography

Conventional angiograms was performed on postoperative day 1, 40, and 90 (3 randomly at each time intervals). With the animal under the same general anesthesia described earlier, the left common carotid artery was exposed through a ventral incision in the neck. A 4–French catheter was introduced into the exposed artery with use of the Seldinger technique and advanced to a position about 3 cm proximal to the aortic bifurcation. A total of 10 ml of contrast agent (60% iothalamate meglumine) was injected at the rate of 3 ml/second, with serial filming of both hindlimbs.

Calf blood pressure

The calf blood pressure was measured by Doppler Flowmeter (Model 1059, Parks Medical Electronics, Aloha, Oregon) on preoperative (day 0), and postoperatively at each 10–day intervals until completion of the experiment on day 40 or day 90. Both hindlimbs were shaved and cleaned. With the rabbit under light general anesthesia, the pulse of the posterior tibial artery in the lower calf was detected by the doppler probe with contact gel, and the systolic blood pressure of the calf was measured according to the standard technique. The calf blood pressure ratio was defined as the ratio of the pressure in the left hindlimb to that in the right hindlimb (L/R ratio).

Femoral venous lactate

Preoperatively (day 0), and on postoperative days 10, 40, and 90, a 0.5 ml blood sample from both femoral veins was withdrawn into a 3–ml heparinized syringe after exposure of the vessels through a cutdown performed with the animal under general anesthesia. The lactic acid content was determined with a lactate analyzer (Model 23L, YSI Incorporated, Yellow Springs, Ohio), used according to the specifications of the manufacturer.

Calf radioisotopic perfusion scanning

Relative quantification of arterial perfusion in the calf was studied by radioisotopes (15–30 μ in diameter) using ^{99m}Technetium Macroaggregates (^{99m}TcMAA, E.I. du Pont

de Nemours & Co., Boston, MA). Preoperatively (day 0) and on postoperative days 20, 30, and 40, with the rabbit under anesthesia, the left ventricle was punctured through the 4th intercostal space and 1 mCi of ^{99m}TcMAA (in 2 ml saline) was injected. Scanning was then done and radioactive counting with a gamma camera (Omega 500, Technicare Co., Cleveland, Ohio) interfaced with a computer system (MCS 560). The counts were stored on a computer disk for later data retrieval and analysis. At analysis, the radioactive counts from each calf were generated from approximately placed regions of interest. The ratio of the counts between the calves (L/R) was calculated to represent an index of relative calf perfusion.

Histologic studies

The distal tibialis cranialis muscles were dissected and removed from both legs before the animal was sacrificed. The muscle specimen was fixed in 10% buffered formalin, embedded in paraffin, and cut in 10 microns cross sections. Each section was stained with hematoxylin and eosin, and evaluated by one of the authors (S.C.) according to a single–blind protocol.

Statistical analysis

All data are expressed as the mean ± one standard error of the mean (SEM). Statistical comparisons between groups was done with the unpaired Student's t–test. Values obtained from the ischemic and the normal limb in the same animal were

compared with the paired Student's t–test. A p value <0.05 was considered statistically significant.

RESULTS

Clinical assessment

All 28 animals had limping in their left hindlimbs at day 1 postoperatively. After day 10, 9 animals developed varying degrees of skin necrosis in the distal portion of their left limb (ankle and foot). Three had nonfunctional hindlimbs. The remaining animals had either with mild hindlimb limping or a functionally normal leg but noticeable muscle atrophy after day 10. Six animals died before the termination of the study because of the anesthesia, radioisotopic perfusion scanning or angiography. No deaths were attributable to the ischemia–inducing procedure in this study.

Angiography

Angiography performed on postoperative day 1 demonstrated markedly retarded filling of the arterial tree beyond the excised femoral artery as compared with the control limb.(Figure 2) Angiograms obtained on postoperative day 40 also showed substantially slower filling and reconstitution of the left popliteal artery with a few collaterals seen in the thigh.(Figure 3) Angiograms from postoperative day 90 continued to show slower filling and reconstitution of distal arterial tree with minimal collaterals in the thigh.(Figure 4)

Calf blood pressure

The measurement of calf blood pressure ratio (L/R) at all time intervals

postoperatively showed a significant decrease as compared with the values obtained on day 0.(Figure 5) Nevertheless, there was a progressive increase in the ratio from day 10 to day 20 (0.19 ± 0.03 vs 0.36 ± 0.03, $p< 0.0001$), followed by a slower increase from day 20 to day 30 (0.36 ± 0.03 vs 0.45 ± 0.02, $p< 0.02$). The values then became relatively stable, with only minimal changes from day 30 until day 90 (0.45 ± 0.02 to o.63 ± 0.04).(Figure 5)

Femoral venous lactate

Measurement of blood lactate levels in femoral venous effluents from both sides showed no significant difference between the left and right hindlimbs (1.75 ± 0.14 vs 1.78 ± 0.14 mmol/L, NS) before the surgery (day 0). Ten days postoperatively, however, Lactate production in the left hindlimb was very significantly higher than that in the right (2.30 ± 0.21 vs 1.75 ± 0.13 mmol/L, $p< 0.0002$). By day 40, although the lactate production in the left hindlimb had diminished somewhat, it was still significantly higher than that in the right (2.16 ± 0.14 vs 1.88 ± 0.12 mmol/L, $p< 0.002$). By day 90, however, there was no longer a significant difference between lactate producation in the two limbs (1.74 ± 0.25 vs 1.70 ± 0.23 mmol/L, NS).(Figure 6)

Calf radioisotopic perfusion scanning

The results of the radioisotopic scanning were evaluated as an index of total arterial perfusion to each limb at each time interval. Preoperatively, the ratio of arterial

perfusion between the left and right calves was 1.01 ± 0.02. The ratios remained significantly lower ($p < 0.005$) for at least 40 days.(Table 1) The mean calf perfusion ratio was 0.72 on day 20, 0.76 on day 30, and 0.83 on day 40.

Histologic studies

When sections of the tibial cranialis muscle were examined microscopically, those from the right calf had normal skeletal muscle architecture.(Figure 7A) Muscle sections from the left calf, however, showed different histologic changes, such as atrophy, regeneration, patchy fibrosis and necrosis of muscle tissue, and even normal appearing muscle tissue. The most striking findings, as shown in Figure 7B, were tissue atrophy, fibrosis, or incomplete regeneration which were indicative of persistent ischemia up to 90 days postoperatively.

DISCUSSION

In preparing an ideal animal model of persistent limb ischemia that is comparable to a patient with chronic critical ischemia, the following 4 criteria must be respected: a significant degree of ischemia is induced, which is reproducible and can be tolerated by the animal; the ischemia becomes stable after the ischemic procedure and is maintained for an appreciable period; the ischemia in the limb can be detected and measured quantitatively by most conventional tests; and direct comparison is possible between the ischemic and contralateral normal limb. In contrast to models of acute limb ischemia,[7] representations of persistent limb ischemia are difficult to create because even a proximal arterial ligation or division usually results in little measurable change in the resting limb, over time, in most laboratory animals. This is because of the animal's remarkable ability to form collateral pathways from their non–diseased proximal vessels or to reconstitute the normal arterial trees and thereby relieve the ischemia within a short period.

A canine model of "chronic" ischemia has been described which required as many as 14 separate ligations of the pelvic arteries, the external iliac and common femoral arteries and their branches. After a 6–week recovery period, the dogs had approximally normal resting blood flow, and the mean distal to central systolic blood pressure index was 0.60.[7] A common iliac artery division in a rabbit model produced resting limb ischemia for only 17 days.[8] Combined common iliac and femoral artery ligation alone could not produce an adequate ischemia at rest in rat hindlimbs.[9-11] Ligation of common

iliac artery resulted in only inconspicuous histologic changes in the hindlimb muscle 7 days after the procedure.[12] Our preliminary studies also failed to produce angiographically severe ischemia in rabbit hindlimbs 30 days after ligation of the common iliac or femoral artery alone.(unpublished observations)

We have now developed an animal model of persistent hindlimb ischemia by ligating the distal external iliac artery and excising the common and superficial femoral arteries in the left hindlimb of rabbits.(Figure 1) The surgical preparation produced substantial persistent ischemia demonstrable at rest up to 90 days, at which point the study was terminated. Changes in arterial perfusion in the two hindlimbs were compared with use of assessment of calf blood pressure and femoral venous lactate levels, as well as calf radioisotopic perfusion scanning. These evaluations not only confirmed the production of ischemia in this model, but also demonstrated its persistence at rest during the 40–day study period.(Table 1; Figures 5,6) A prolonged study measuring calf blood pressure (L/R ratio) also demonstrated relatively stable ischemia from postoperative days 30 to 90.(Figure 5) The results were supported by angiographic and histologic findings.(Figures 3,4,7B)

We chose rabbits for this study because they are easily available and manageable, and they are sufficiently large to allow performance of the necessary surgical procedure (although magnifying loops are helpful). In addition, conventional angiography, and

noninvasive hindlimb blood pressure measurement are possible. Inter–individual differences are minimal because animals from the same strain can be used. The rabbit model is superior to the dog model because collateral formation is much less complete. Furthermore, rabbits have an acceptable purchase and maintenance cost, thereby allowing the use of a large numbers and long–term follow–up.

The surgical technique involved in preparing this model may be responsible for the different results obtained and some of the special features in comparison with those achieved in other available models.[7-9] Our model produces severe ischemia through ligation of the distal external iliac artery and excision of the common and superficial femoral arteries––procedures which also delay reconstitution by collaterals in distal arterial tree. The model avoids proximal arterial ligation (for example, of the common iliac artery), which requires a laparotomy and is less effective in producing ischemia. It preserves the inflow to the thigh, especially from the internal iliac and circumflex arteries, an important source for collateral development after the surgery to maintain limb survival.(Figures 3,4)

The model also makes it possible to quantitate the collateral vessels in the thigh with use of angiography and pathological or histologic techniques, such as counting latex–perfused vessels grossly[13] or measuring capillary density microscopically.[3] This should allow the model to be utilized to test such approaches as angiogenic stimulation in the

ischemic limb, because the response of neovascularization can be measured directly in the thigh. In our investigation, serial measurements of calf blood pressure showed an initially rapid increase in blood pressure in the limbs up to 20 days postoperatively that probably represented recruitment and dilation of existing collaterals in response to the produced ischemia. After day 20 (Figure 5; Table 1), only slow, minimal increases in blood pressure and perfusion were observed—similar to findings in patients with chronic superficial femoral artery occlusion.

The tests chosen to evaluate the ischemia in rabbit hindlimbs in this study can be compared with certain evaluations done in patients. Calf blood pressure measured noninvasively and evaluated by expression of a L/R ratio reflects the ankle/brachial index determined in patients with peripheral arterial occlusive disease. In addition, increases in lactate release produced by hypoxic tissue cells through anaerobic glycolysis have been documented in resting limbs of patient with rest pain, ischemic gangrene, or claudication after exercise.[14-16] The lactate accumulation confirmed by the measurement of femoral venous effluent in our rabbit model has a close relationship with the metabolic delay and the severity of ischemia in patients and is a good biochemical marker of tissue ischemia.[14-16] Thus, in this study, we found that femoral venous lactate was a useful biochemical marker of severe tissue ischemia.

For our sequential and survival studies, we chose the radioisotopic particle–distribution method for determining regional distribution of blood flow in rabbit calves.[7,17] The ^{99m}TcMAAs are larger in diameter than a capillary and thus are trapped in the first capillary bed they encounter and distributed in proportion to the perfusion in that bed. This method has been used clinically for determining regional pulmonary perfusion, as well as perfusion in the heart and extremities.[17] We evaluate the regional blood flow in the calf, rather than that in the entire hindlimb, in order to exclude the effects of local trauma and wound healing in the thigh.

The chief difference between our animal model and chronic limb ischemia in patients is that, in the model, ischemia is produced after an acute ischemic insult, whereas, in patients, it follows a relatively slow progressive vessel occlusion. Thus, the severe tissue changes observed in the rabbit—particular the degree of skeletal muscle necrosis and fibrosis—is not typically seen in patients with chronic critical ischemia. The histologic evidence of muscle necrosis and the biochemical finding of increased femoral venous lactate are probably more in keeping with acute ischemia occurring early in the evolution of the ischemic process (first 10 days).[18] Thus, our model becomes a true representation of persistent ischemia only beyond that time.[19]

CONCLUSION

The surgical preparation we used produced persistent hindlimb ischemia in rabbits for at least 90 days. We believe that this model approximates the clinical state of rest pain in the first few weeks after its preparation and of claudication due to occlusive arterial disease of the superficial femoral artery afterward. The model is easily reproducible, its characteristics are readily measurable, it is relatively inexpensive, it allows comparison between limbs in the same animal (so each rabbit serves as its own control), and it can be used for the study of various approaches to the treatment of chronic limb ischemia that focus on improvement of arterial perfusion and collateral formation.[20] The model is ideally suitable for studying angiogenic stimulation in an ischemic limb[21], and appears better for this purpose than other models.[7-11] In addition, it can provide valuable information on the metabolic and physiological consequences of chronic limb ischemia.

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TABLE

TABLE 1

Calf Radioisotopic (^{99m}TcMAA) Perfusion Scan Ratio (L/R)

DAY 0	20	30	40
1.01 ± 0.02	0.72 ± 0.05*	0.76±0.05*	0.83±0.04*

Note: mean ± SEM, *p< 0.005 vs. day 0

FIGURES

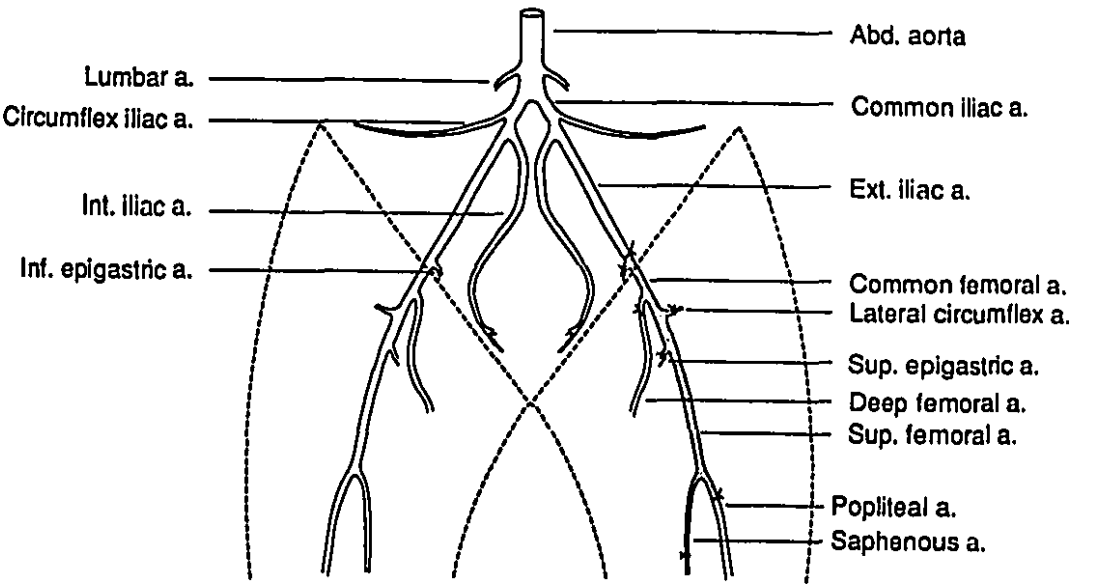

Figure 1. Rabbit left hindlimb ischemia preparation. All shaded vessels are ligated and excised.

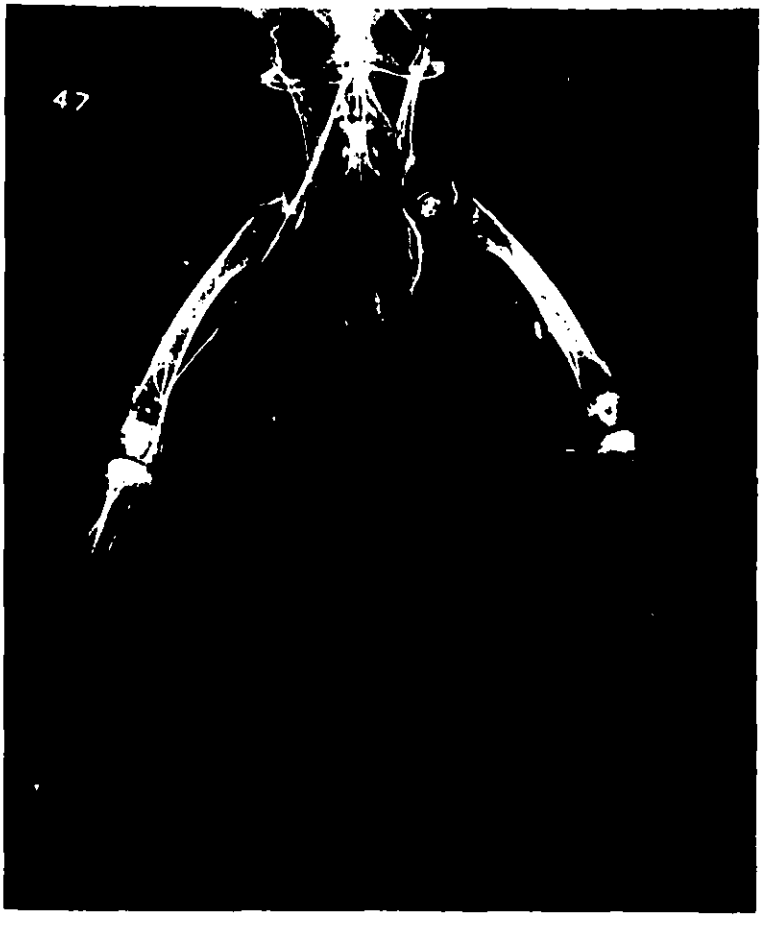

Figure 2. Angiogram (day 1) shows an almost complete lack of filling of the distal arteries in the left hindlimb.

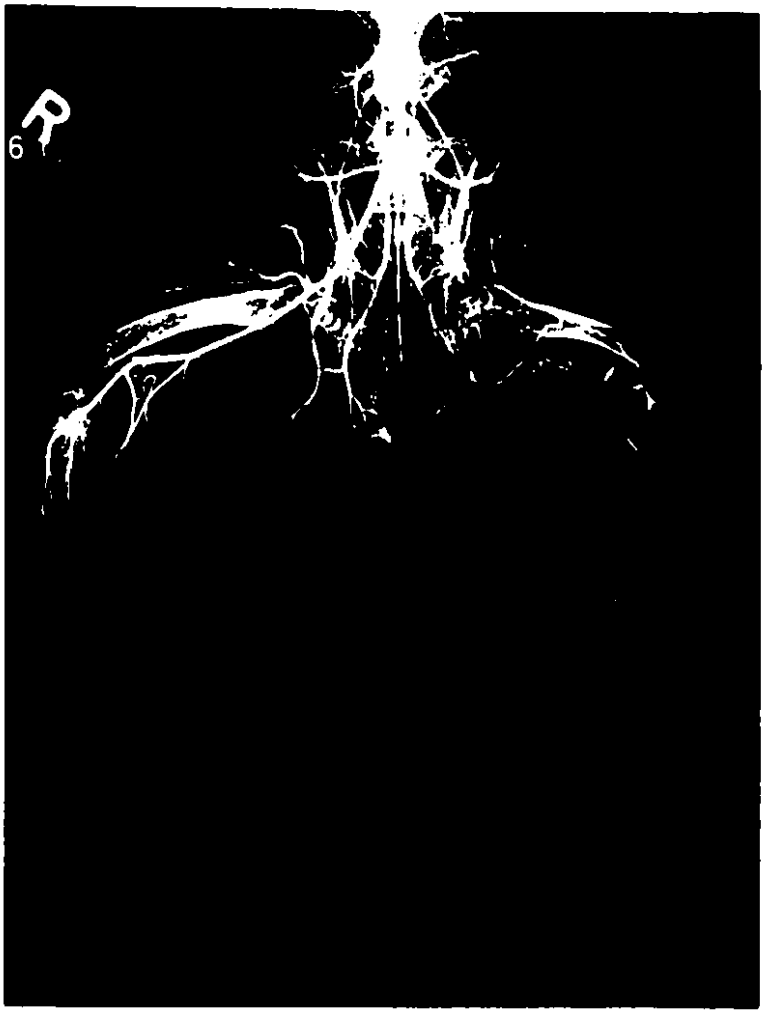

Figure 3. Angiogram (day 40) shows slow reconstitution of the distal arteries with a fewer collaterals in the thigh of the left hindlimb.

Figure 4. Angiogram (day 90) demonstrates persistently slower reconstitution of the distal arteries with minimal collateral formation in the thigh of the left hindlimb.

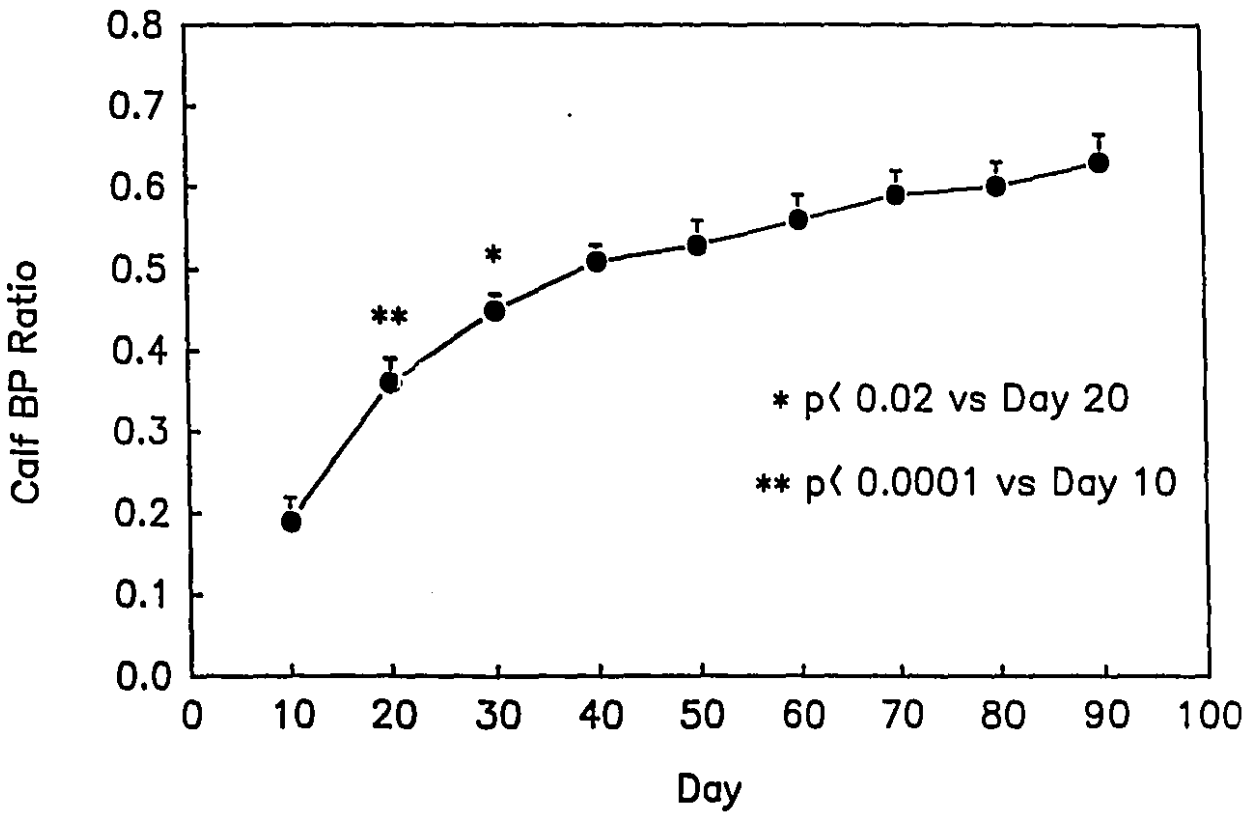

Figure 5. Postoperative measurement of calf blood pressure (L/R ratio) reveals a marked increase of the ratio from day 10 to day 20, a more gradual increase from day 20 to day 30, and then stabilization, with only a minimal increase, from day 30 to day 90.

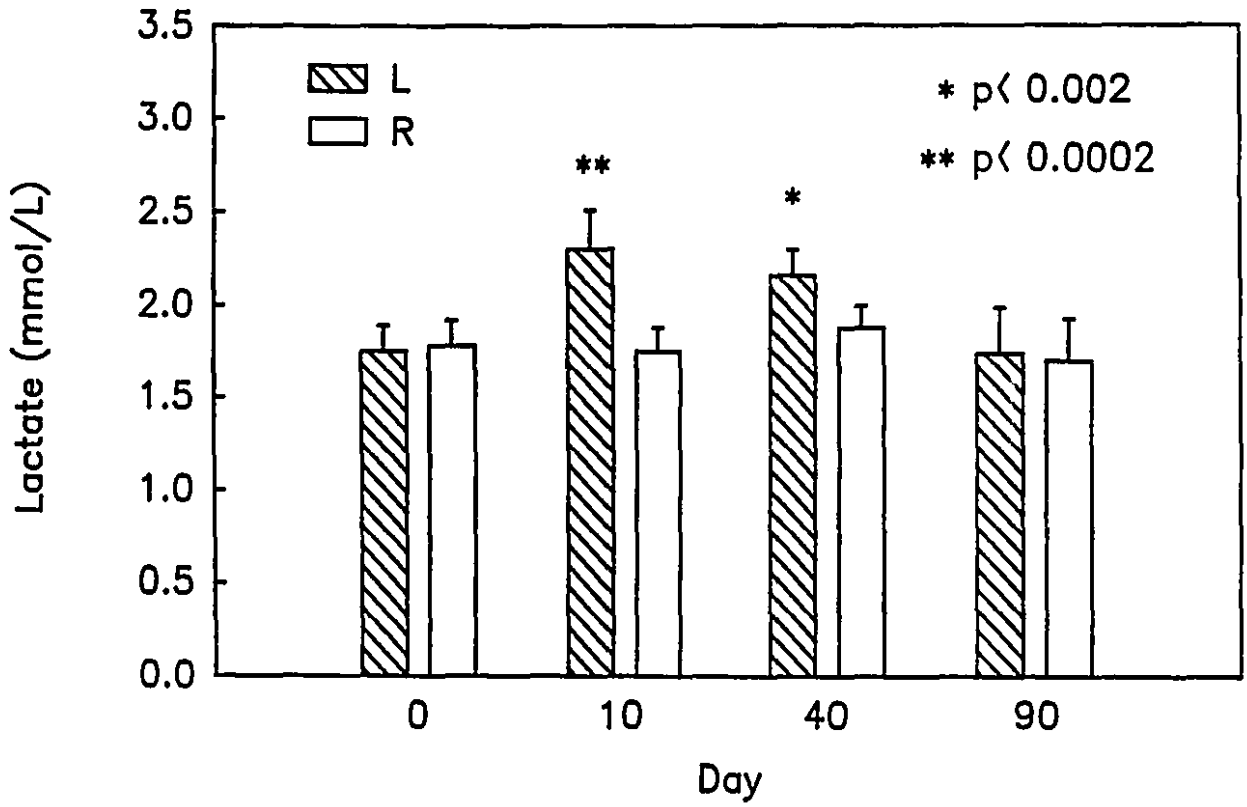

Figure 6. Femoral venous lactate concentration as an indicator of ongoing severe ischemia in the hindlimb (L vs R) to day 40 following ischemia–inducing surgery.

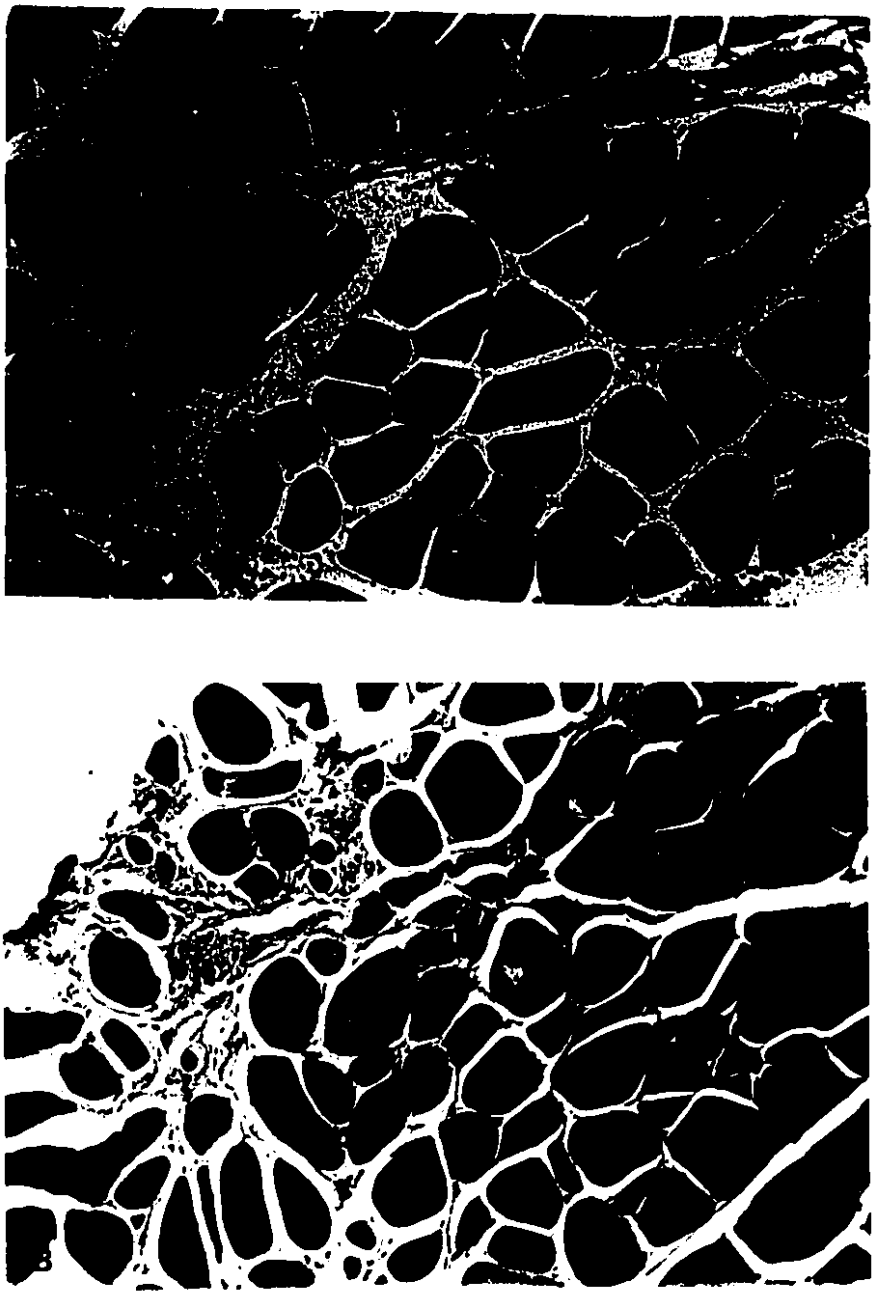

Figure 7. Cross section of distal tibialis cranialis muscle from the same animal. A, the normal side (*right*), shows normal appearing skeletal muscle. B, the ischemic side (*left*), shows atrophic fibers, interstitial fibrosis, and incomplete regeneration of muscle tissue.(Hemotoxylin and eosin, X 550)

CHAPTER IV

ANGIOGENIC STIMULATION: A NEW APPROACH FOR SEVERE CHRONIC LIMB ISCHEMIA

ENHANCED REVASCULARIZATION OF THE ISCHEMIC LIMB BY MEANS OF ANGIOGENIC THERAPY

Presented in part at the 42th Surgical Forum during the 77th Clinical Congress of the American College of Surgeons, Chicago, IL, October 20–25, 1991.

ABSTRACT

This study tests the efficacy of an angiogenic growth factor, endothelial cell growth factor (ECGF), in a rabbit model of persistent hindlimb ischemia. Ischemia was induced in the left hindlimb of 22 New Zealand white rabbits by ligation of the distal external iliac artery and complete excision of the common and superficial femoral arteries. Two groups of animals were studied: Group I consisted of 11 animals who for 10 days received daily intramuscular injections of 4 mg of ECGF beginning on post–operative day 11; Group II consisted of 11 animals who also underwent the same surgical ischemic procedure, but received only injections of saline daily for the same post–operative period. Perfusion of the ischemic left limb was compared with the normal right limb in each animal on post–operative days 10, 20, 30 and 40, utilizing the calf blood pressure ratio, ^{99m}TcMAA radioisotopic perfusion scans and serial angiography. Neovascularization in the left thigh at day 40 was quantified from the angiograms. Each technique documented that animals in group I had significantly better perfusion than animals in Group II. That is, the calf pressure ratio was higher in Group I than Group II (0.56 vs 0.32 at day 20, 0.64 vs 0.44 at day 30, and 0.70 vs 0.50 at day 40, $p<0.0001$); and the calf radioisotopic perfusion ratio was also higher in Group I than Group II (0.88 vs 0.74 at day 20, $p<0.02$; 0.93 vs 0.76 at day 30, and 0.96 vs 0.79 at day 40, $p<0.008$). Angiographic studies correlated well with these results demonstrating much earlier distal arterial reconstitution and enhanced neovascularization (23.8 vs 9.0, No. of vessels, $p<0.007$). The data clearly indicate that an angiogenic growth factor, ECGF, promotes revascularization in this experimental ischemic hindlimb model raising the possibility that in the future such agents

might be of value in humans.

INTRODUCTION

The number of people who suffer from lower extremity ischemia is increasing with a corresponding increase in the need for limb salvage vascular surgery.[1] Currently, severe chronic limb ischemia is treated by a variety of arterial revascularization techniques. These include bypass procedures, using autogenous and synthetic conduits, endarterectomy and percutaneous techniques such as balloon angioplasty and atherectomy. Unfortunately, about 20% with limb threatening ischemia have disease which is so extensive that direct revascularization procedures cannot be undertaken successfully.[2] Too often, the only alternative for these patients is limb amputation with its attendant morbidity and mortality.

A number of innovative approaches have been reported clinically and experimentally. These include pedal vessel bypass,[3] conventional bypass with adjunctive distal arteriovenous fistula,[4,5] staged arteriovenous reversal,[6–9] omental or muscle flap transfer,[10–12] and others.[13] However, the effectiveness and practicality of all such procedures has yet to be clearly established. Powerful testament to the size of the problem that remains comes from the Maryland study by Tunis et al,[14] which showed no decrease in the amputation rate despite the increase in bypass surgery and angioplasty.

Angiogenesis is the term which describes the formation of new blood vessels. Folkman and others[15–17] have not only elucidated some of the fundamental biologic

mechanisms involved in angiogenesis, but have pioneered the isolation and description of a number of angiogenic factors.[18,19] Recently, the studies by Thompson et al,[20,21] Goldsmith et al,[22,23] and others[24–27] in different animal models demonstrated dramatic angiogenesis in response to the administration of one or other of the angiogenic factors. These results, stimulated our interest in the possibility of employing such an approach in the treatment of severe chronic limb ischemia. The purpose of the present study was to test the hypothesis that administration of one of these factors, endothelial cell growth factor (ECGF), would significantly improve perfusion in a rabbit ischemic hindlimb model.

MATERIALS AND METHODS

Animal hindlimb ischemic model

Twenty–two adult New Zealand male white rabbits (mean weight 4 kg) were studied. All animals were anesthetized with intramuscular Ketamine (50 mg/kg) and Xylazine (5 mg/kg) and then a longitudinal incision was made in the left groin from the inguinal ligament to above the knee. With the aid of surgical loops, the femoral artery was dissected and its branches, the profunda, the lateral circumflex and the superficial epigastric were exposed as completely as possible. The proximal popliteal and saphenous arteries were also dissected free. Ischemia was then induced in the left hindlimb by ligation of the distal external iliac artery just above the inguinal ligament, the inferior epigastric artery, all the branches of the femoral artery, as well as the proximal popliteal and saphenous arteries. This was followed by excision of the common and superficial femoral arteries (Figure 1). All animals received 50 ml of 0.9% sodium chloride during surgery and Cefazolin (15 mg/kg/day) intramuscularly for 4 days starting on the day of the surgery. An analgesic, Bupreorphine 0.04 mg/kg, was administered daily for the first 10 days after surgery. Approval for animal use in this study was granted by the Institutional Animal Care Committee, and the care of these animals complied with the guidelines of the Canadian Council of Animal Care, and the Principles of Laboratory Animal Care, and the Guide for the Care and Use of Laboratory Animals (NIH publication No. 80–23, revised 1985).

Endothelial cell growth factor

Endothelial cell growth factor (ECGF) is purified from bovine retina and hypothalamus and is one of the heparin binding growth factors.[18,19] ECGF is an acidic fibroblast growth factor (aFGF) which has been shown to promote endothelial cell proliferation *in vitro* (at a concentration of 1 ng/ml) and to stimulate angiogenesis *in vivo* (at levels of 10–100 ng in the chick chorioallanoic membrane and corneal bioassays).[18,28]

Experimental protocol

Two groups of 11 rabbits each were studied. Group I received 4 mg ECGF (Endo Gro™, VECTEC Inc., Albany, NY, 100 mg + 10 units Heparin/per vial) (in 3 ml saline) intramuscularly in the left thigh daily for 10 days commencing on post–op day 11. Each injection was given intramuscularly in 3 different sites in the left thigh (2 ml in the medial thigh, 0.5 ml in the lateral thigh, and 0.5 ml in the distal thigh near the kness). Group II animals received intramuscularly a sham injection of 3 ml saline according to the same manner. The animal was evaluated preoperatively (day 0) and on postoperative days 10, 20, 30 and 40, by clinical assessment, calf blood pressure, calf radioisotopic perfusion and angiography. The study was terminated on postoperative day 40.

Calf blood pressure measurement

The calf blood pressure was measured in both limbs by Doppler Flowmeter (Model 1059, Parks Medical Electronics, Aloha, Oregon). The hindlimbs were shaved and

cleaned, and under anesthesia as described above, the pulse of the posterial tibial artery in the lower calf was detected by the doppler probe and the systolic blood pressure then determined. The calf blood pressure is defined as the ratio of the left calf to right calf systolic pressure (L/R ratio).

Calf radioisotopic perfusion scanning

Arterial perfusion was determined radioisotopically using ^{99m}Technetium macroaggregates which measured 15–30 μ in diameter (EI du Pont de Nemours & Co, Boston, MA). The aggregates are designed to be so large that they will be trapped in the capillary bed. Under anesthesia, the left ventricle was punctured through the 4th intercostal space and 1 mCi of ^{99m}TcMAA in 2 ml saline was injected. Following the injection, the limbs of the animal were scanned and counted on a Gamma Camera (Omega 500, Technicare Co., Cleveland, Ohio) which was interfaced to a MCS 560 computer system. The accumulated counts were stored on a disk for later retrieval and analysis. At that time, the radioactive counts from each calf were generated from corresponding regions of interest and the ratio of the counts between the calves (L/R) was calculated as an index of relative calf perfusion.

Angiographic evaulation

Angiograms were performed using standard techniques. Two animals were studied for each group at post–operative day 20 and 4 from each group at day 40. Under

anesthesia, the left common carotid artery was exposed through a ventral incision in the neck. Using the Seldinger technique a 4–French catheter was introduced into the exposed artery and advanced to a position 3 cm proximal to the aortic bifurcation. Ten ml of contrast agent (MD–76, diatrizoate meglumine) was injected at a rate of 3 ml/sec and serial filming of both hindlimbs performed.

Vascularization in the left thigh was determined from the angiograms at day 40 and defined as the number of collateral vessels extending along a vertically drawn line which passed through the centre of the femur. The 4 second post injection film was analyzed on two separate occasions by the same observer and the results averaged. If the two differed by more than 10%, a third count was made and the three averaged.

Statistical analysis

All data are expressed as means ± one standard error of the mean (SEM). Comparisons between Group I and II were performed using a computer statistical package using unpaired Student's t–test. A difference was considered statistically significant if the p value was less than 0.05.

RESULTS

All animals demonstrated weakness of the left limb on the first post–operative day. After day 10, 4 of the animals in Group II developed varying degrees of superficial tissue necrosis in their distal calves or toes. No similar abnormalities appeared in any animals in Group I.

Comparison of calf blood pressure ratios (L/R) determined by doppler demonstrated no difference between the two groups on either day 0 post–op or day 10. In both, severe ischemia was evident on post–op day 10 in the left hindlimb compared to day 0 pre–op.($p<0.0001$) However, as shown in Table 1, on all subsequent examinations, although the ratio rose progressively in both groups, the value in Group I was always substantially higher than in Group II with the differences easily statistically significant.($p<0.0001$)

The results of the radionuclide perfusion study are given in Table 2. As anticipated, there was no pre–operative difference in perfusion between the limbs or between the groups. At the time of the first post–operative examination (day 20), evidence of significantly better perfusion in Group I than Group II was present (0.88 vs 0.74, $p<0.02$). This difference was even greater on the succeeding examinations such that by day 40, the ratio of flow between the operated and non–operated limbs in the treatment group approached unity (0.96 vs 0.79, $p<0.008$).

Satisfactory angiographic examinations were obtained in all animals. The examples that follow are representative of the differences that occurred between Group I and Group II animals. Figures 2 and 3 were taken on post-operative day 20. The first is from an animal in Group I, the second from an animal in Group II. Distal arterial reconstitution in the left limb is obvious in the Group I animal, but barely evident in the corresponding angiogram in the Group II animal. These differences are, if anything, even more obvious in the angiograms focusing on the left thigh. Revascularization is easily evident in Group I animals (Figure 2), but virtually absent in those in Group II (Figure 3).

The angiograms performed at the end of the study (day 40), are also of considerable interest, again demonstrating substantially more distal arterial reconstitution of the left hindlimb in Group I animals (Figure 4) than in Group II animals (Figure 5). As well more collateral vessels were present in Group I than in Group II animals (Figures 4 and 5 respectively). Of considerable interest as shown in Figure 6, a few of the most rapidly "growing" collateral vessels were shown to extend all the way from their original source to near the ankle where they reconstituted the distal arterial tree in that animal.

Quantitative analysis of new vessel formation in the left thigh at day 40, demonstrated more than twice number of nutrient vessels in Group I compared to Group II animals (23.8 ± 3.4 vs 9.0 ± 1.5 vessels, $p<0.007$).

DISCUSSION

Biotechnology now makes it possible to isolate and purify factors which can stimulate new blood vessel growth or angiogenesis.[18,19] In this study, several techniques were used to examine and quantify limb perfusion. The results were consistent throughout with in every instance, evidence of substantially improved perfusion and revascularization in the group which received the angiogenic factor during the post–operative period.

The agent used in the present study is one of the heparin–binding growth factors. This group has been divided into a basic fibroblast growth factor (bFGF) and an acidic fibroblast growth factor (aFGF). The genes and protein structure of the two forms are similar and both bind to the same receptor.[29,30] In most systems examined bFGF is anywhere from 10–100 fold more potent than aFGF.[31] Although heparin has been shown to promote angiogenesis in a large animal model following continuous intravascular infusion,[32] the apparent dose required far exceeded ($2x10^4$ units per animal) that used in this study (4 units per animal). It is highly unlikely, therefore, that the angiogenic effect we observed was related to the small amount of heparin that was bound to the ECGF. On the other hand, in the presence of heparin, the ability of aFGF to stimulate endothelial cell proliferation increases 100 fold[31] and under these circumstances the ED_{50} of aFGF is just about the same as bFGF.[33] In this study, we used ECGF which is an aFGF. We did so because it is cheaper than bFGF and since it is commercially available bound to

heparin, we believed it was likely to be efficacious. Larger doses as well could be administered. The doses of the ECGF used in this study were based on previous experience obtained during *in vivo* studied by Thompson et al,[20] and Andrade et al.[24] The format and schedule of administration of the agent was based on Goldsmith's lipid angiogenic factor study,[23] as well as, preliminary experience from our laboratory.[34] Administration of the agent was delayed to the 11 post–operative day to minimize confounding effects of the host response to acute ischemic injury and the surgical procedure.

Angiogenesis is a complex process involving capillaries and venules, and the exact mechanism by which it was achieved in this study remains unknown. At least four steps have been demonstrated to be involved in the development of a new capillary. These include: enzymatic degradation of the basement membrane of the parent vessel to allow formation of a capillary sprout; migration of endothelial cells toward the angiogenic stimulus; proliferation of endothelial cells just behind the leading front of migrating cells; and finally maturation and organization of endothelial cells into capillary tubes.[35] ECGF might have acted through either one or several pathways to promote this process. For example, as suggested by Folkman and Klagsbrun,[18] it might stimulate and mobilize macrophages which then secrete angiogenic growth factors or chemotactic agents for vascular endothelial cells. Another possibility is that ECGF causes the release of such factors from intracellular sites in cells within the ischemic tissue itself. Alternatively, of

course, it might act directly on the capillaries and arterioles of the ischemic vessels to promote the growth and development of new vascular channels.

In this study, we obtained quantitative angiographic evidence of revascularization in the ischemic hindlimb of group I compared to group II on angiography. The accurate quantitation of revascularization in an ischemic hindlimb model is difficult, but we believe that the method we used in this study is simpler and more reliable than other techniques which are used to quantitatively investigate *in vivo* angiogenesis in an ischemic limb model of large animals.[25,34] However, the use of the available techniques to determine whether the increased collateral vessels seen on angiograms are newly formed or just enlarged pre–existing vessels still presents a challenge.

The cellular events in the new vessel formation process are currently thought to be under the control of locally acting growth factors. The study by Mooney et al[36] suggested that local administration of an angiogenic factor (tumor necrosis factor) demonstrated beneficial effects superior to its systemic application in a rat wound healing model. The topical application of angiogenic factors has been used by several investigators to enhance wound healing,[36,37] bone graft healing,[25,26] vascular graft endothelialization,[37,38] bronchial anastomotic healing after lung transplantation,[39] duodenal ulcer healing,[40] and peripheral never regeneration[41] by hoping to increase angiogenesis in these tissues. However, whether the angiogenic factors have a systemic effect or not is

still uncertain.

Whatever the mechanism, the results of the present study strongly suggest that agents such as ECGF have the potential to markedly enhance angiogenesis in the presence of limb ischemia. Each technique used to assess this phenomenon confirmed the efficacy of this agent in this experimental model of persistent limb ischemia. The angiographic studies in particular, provide graphic evidence of enhanced neovessel formation in the group receiving ECGF. Curiously, until quite recently, this class of agents had not been tested experimentally for its capacity to relieve organ ischemia. Some evidence is now available, however, with regard to both the heart and hind limbs. Banai et al,[42], for example were unable to demonstrate benefit from delivery of an acidic fibroblast growth factor to ischemic myocardium from an epicardial sponge when regional ischemia was gradually produced by application of an anaeroid constrictor to the left anterior descending coronary artery. By contrast, Yanigisawa–Muwa et al[43] demonstrated significantly enhanced collateralization with intracoronary injection of a basic fibroblast growth factor. The first report, however, of successful revascularization of the inschemic hindlimb due to administraction of an angiogenic factor came from our laboratory[44] and dealt with the ischemic hindlimb, an observation that has since been confirmed by Baffour and his colleagues using, however, a basic fibroblast growth factor.[45]

CONCLUSION

Clearly much remains to be done to further clarify and confirm the efficacy of this approach to limb, as well as, other tissue revascularization. In addition the safety and potential adverse effects of administration of such agents must be assessed since the animals in the present study were not systematically assessed for evidence of hematological, renal, or hepatic toxicity after administration of ECGF. Nevertheless, given this present evidence of *in vivo* efficacy and the obvious need to improve our therapy of severe arterial insufficiency in patients, clinical application in the future of angiogenic agents similar to this should now be considered a real possibility.

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TABLES

TABLE 1

CALF BLOOD PRESSURE (L/R) RATIO

DAY	0	10	20	30	40
Grp I	1.02±0.02	0.19±0.03*	0.56±0.02+	0.64±0.02+	0.73±0.02+
Grp II	1.01±0.02	0.17±0.02*	0.32±0.02	0.44±0.02	0.50±0.02

Note: mean ± SEM, *p<0.0001 Day 10 vs Day 0, +p<0.0001 Grp I vs Grp II

TABLE 2

CALF BLOOD FLOW (L/R) RATIO

Radioisotopic (^{99m}TcMAA) Perfusion Scan

DAY	0	20	30	40
Grp I	0.99±0.02	0.88±0.03*	0.93±0.04+	096±0.02+
Grp II	1.01±0.02	0.74±0.05	0.76±0.05	0.79±0.05

Note: mean ± SEM, *p<0.02, +p<0.008, Grp I vs Grp II

FIGURES

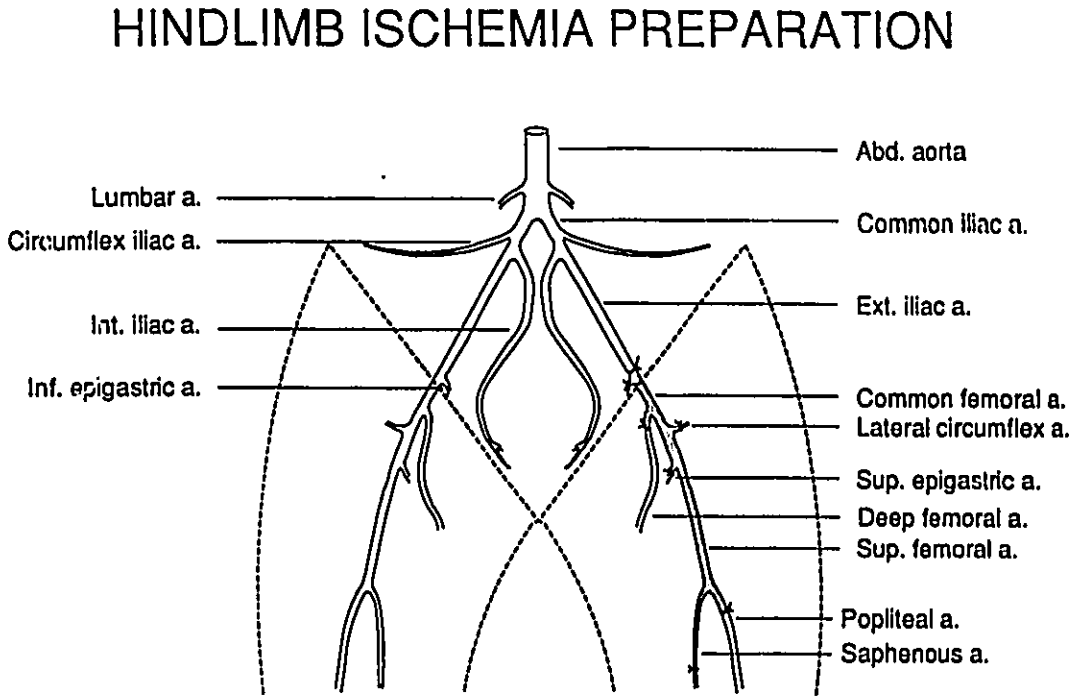

Figure 1. A diagram of the rabbit hindlimb ischemia preparation. The ligated vessels are indicated and all shaded vessels are excised.

Figure 2. Angiogram obtained on postoperative day 20 from group I (just after ECGF administrations, the 4th second film) shows early distal arterial reconstitution in the left hindlimb and more prominent neovascularization in the left thigh compared with those in group II.

Figure 3. Angiogram obtained on postoperative day 20 from group II (the 4th second film) shows the absence of the distal arterial reconstitution in the left hindlimb and only a few collateral vessels in the left thigh.

Figure 4. Angiogram obtained on postoperative day 40 from group I (20 days after ECGF administrations, the 4th second film) shows early distal arterial reconstitution in the left hindlimb and more visualized neovascularization in the left thigh compared with those in group II.

Figure 5. Angiogram obtained on postoperative day 40 from group II (the 4th second film) shows the poorly developed collateral formation (*arrows* indicate the sources of these collaterals) and distal arterial reconstitution in the left hindlimb.

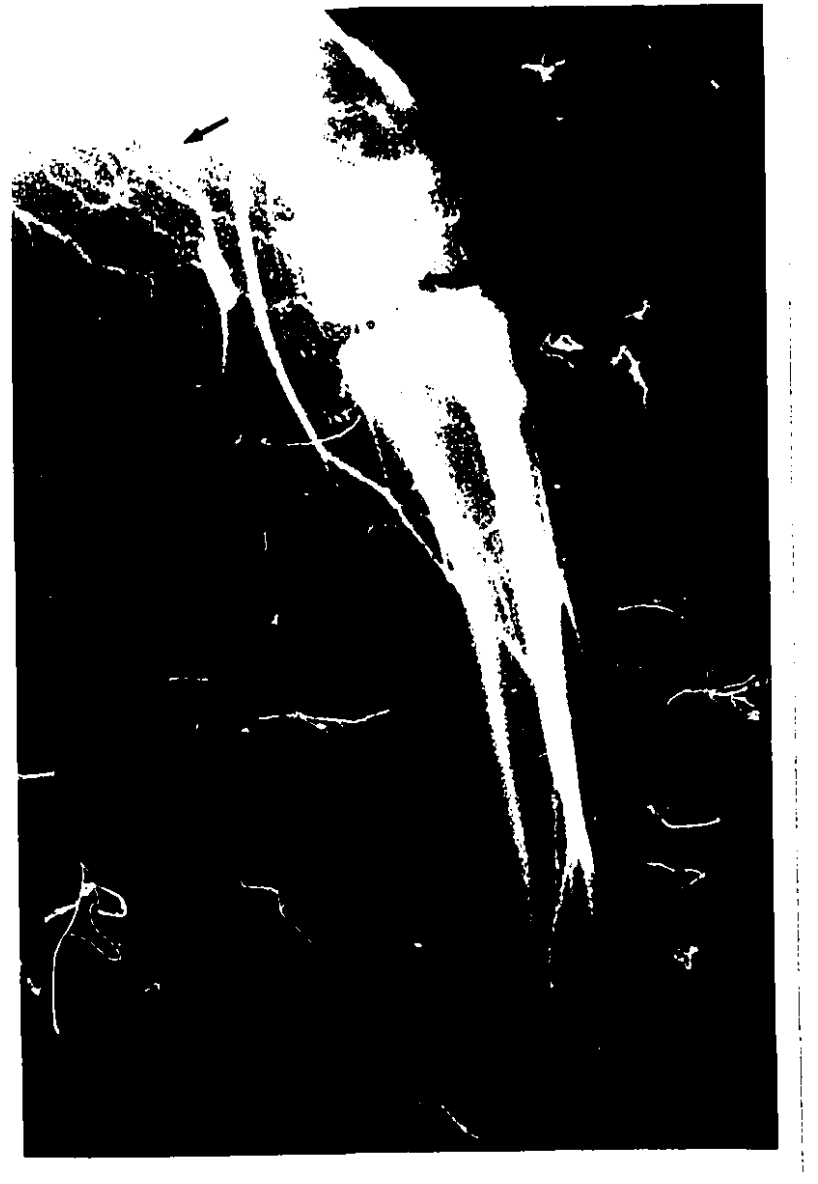

Figure 6. An angiogram obtained on postoperative day 40 from a group I animal (the 5th second film) shows the rapid "growing" collateral vessels *(arrows)* from the thigh towards the ankle to reconstitute the distal native arterial trees.

CHAPTER V

ANGIOGENIC STIMULATION: A ROLE IN REVASCULARIZATION OF THE ISCHEMIC LIMB

ANGIOGENIC STIMULATION PRODUCES ENHANCED REVASCULARIZATION IN A RABBIT MODEL OF BILATERAL LIMB ISCHEMIA

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ABSTRACT

We previously showed that administration of endothelial cell growth factor (ECGF) accelerates revascularization in a rabbit model of unilateral severe limb ischemia. The present study was undertaken to further test this hypothesis in a model of bilateral severe limb ischemia. Ischemia was induced in 11 NZW rabbits by surgical interruption of the arterial inflow to both hindlimbs. Five intramuscular injections of 8 mg of ECGF in 3 ml saline were given in the left hindlimb every other day beginning on postoperative day 11, while the right (control) hindlimb was injected with 3 ml of saline. Calf systolic blood pressures in both limbs were measured on postoperative days 10, 30 and 50. On day 50, angiography was performed and vascularization assessed by counting the number of vessels crossing a line drawn across the mid–thigh on the films. The muscle samples were then obtained for quantitation of capillary density and for histologic studies. The mean calf systolic blood pressure in the left limb injected with ECGF was significantly higher than in the right (68.9±3.1 vs 45.0± 2.9 mmHg on day 30 and 83.0±3.0 vs 57.0±1.7 on day 50; $p<0.0001$ for both comparisons). Vascularization was significantly improved in the left limb as indicated by angiography (17.2±1.6 vs 11.0±0.8 vessels, $p<0.0006$) and capillary density (225.9±11.4 vs 159.6±12.9 capillaries/mm^2, $p<0.002$) compared to the right. Thus, our results demonstrate that ECGF can markedly enhance collateral development leading to a significant improvement in perfusion in severely ischemic limbs.

INTRODUCTION

Despite enormous technical advances in arterial revascularization techniques, limb salvage and effective relief of disabling ischemic pain remains a difficult problem in many patients with diffuse peripheral vascular disease, and amputation is still the only treatment option for such patients. In addition, a far larger population of patients with stable but disabling claudication have no choice but to "live with" their symptomes because the durability of revascularization after the procedures currently employed is inadequate to warrant their use in those not faced with limb loss and because no medical alternative has been proved effective.

Several new approaches to this problem have been reported, including omental transplantation,[1] arteriovenous reversal,[2] and muscle pedicle flap transfer,[3] but their efficacy and practicality have not been established. Recently, however, experimental work from several laboratories has suggested that enhanced vascularization resulting from administration of an angiogenic factor might be of value in improving perfusion in a variety of ischemic tissues.[4-7] For example, we demonstrated that arterial perfusion to a chronically ischemic rabbit hindlimb was significantly enhanced after administration of endothelial cell growth factor (ECGF).[4] In that study, only one hindlimb was rendered surgically ischemic and no histologic assessment of the tissue was done. The current study was undertaken to evaluate this angiogenic approach further in a rabbit model of bilateral severe hindlimb ischemia and to determine whether direct administration of

ECGF to a single limb of the animal would significantly improved revascularization to that limb compared with its contralateral limb.

MATERIALS AND METHODS

Approval for the experimental protocol was obtained from our Institutional Animal Care Committee. The care of rabbits complied with all guidelines of the Canadian Council for Animal Care and the "Principles of Laboratory Animal Care" and the "Guide for the Care and Use of Laboratory Animals"(NIH publication No. 80–23, revised 1985).

Bilateral hindlimb ischemia preparation

Severe ischemia was produced in both hindlimbs of 11 male New Zealand white rabbits of average weight 4 kg with use of a surgical procedure that produces a markedly ischemic limb which remains viable for up to 90 days.[8] Each rabbit was anesthetized with intramuscular Ketamine (50 mg/kg) and Xylazine (5 mg/kg). Longitudinal incisions were made in both groins from the inguinal ligament on each side towards the knee. With the aid of surgical loops, each femoral artery was completely dissected and its branches, including the profunda, lateral circumflex, and superficial epigastric arteries, were dissected as far as possible. The proximal popliteal and saphenous arteries were exposed. Ischemia was induced in both hindlimbs by ligation just above the inguinal ligament of the distal external iliac arteries, the inferior epigastric arteries, all identified branches of the femoral arteries, and the proximal popliteal and saphenous arteries. The common and superficial femoral arteries were then completely excised.(Figure 1)

All animals received 50 ml of 0.9% sodium chloride during surgery. Cefazolin (15 mg/kg) was administered intramuscularly for four days beginning the day of surgery. Bupreorphine at a dosage of 0.04 mg/kg/day was given for the first 10 days after surgery and longer if necrosis developed in a hindlimb. Each animal was evaluated as detailed below and the experiment was terminated on postoperative day 50.

Administration of endothelial cell growth factor

Endothelial cell growth factor (ECGF), which is purified from bovine retina, is one of the heparin–binding growth factors.[9] It is also considered as an acidic fibroblast growth factor (aFGF) and has been shown to promote endothelial cell proliferation *in vitro* (at levels of 1 ng/ml) and to stimulate angiogenesis *in vivo* (at levels of 10–100 ng in the chick chorioallantoic membrane and cornea bioassays).[9,10] The ECGF used in this study (Endo Gro™, 100 mg plus 10 units of heparin/per vial) was commercially purchased(VEC TEC Inc., Albany, NY).

Eight mg of ECGF diluted in 3 ml saline was injected intramuscularly into the left thigh every other day starting on postoperative day 11 until five doses had been administered. Each injection was given in 3 different sites in the left thigh (2 ml in the medial thigh, 0.5 ml in the lateral thigh, and 0.5 ml in the distal thigh near the knee). A sham injection of 3 ml saline was given in the right thigh in the same manner.

Calf blood pressure

Calf systolic blood pressure, used as an index of arterial perfusion to each hindlimb, was measured in both limbs by a Doppler Flowmeter (Model 1059, Parks Medical Electronics, Aloha, Oregon) on postoperative days 10, 30, and 50. Both hindlimbs were shaved and cleaned. With the animal under anesthesia, the pulse of the posterior tibial artery in the lower calf was detected with a Doppler probe and blood pressure was measured in the standard fashion. The measurements were done on two separate occasions by the same observer and the results averaged. If they differed by more than 10%, a third assessment was done and the three values averaged.

Angiographic evaluation

Bilateral hindlimb arterial angiogram was performed on postoperative day 50 in each animal with use of standard techniques to determine the rate of reconstitution of distal arterial tree and the extent of vascularization of both ischemic hindlimbs. With the animal under anesthesia, the left common carotid artery was exposed through a ventral incision in the neck. The Seldinger technique was employed to introduce a 4–French catheter into the exposed artery and advance it to about 3 cm proximal to the aortic bifurcation. A total of 10 ml of contrast agent (MD–76, diatrizoate meglumine and sodium) was injected at the rate of 3 ml/second, with serial filming of both hindlimbs.

Vascularization was quantitated by determining the number of vessels visualized in each thigh that crossed a vertical line drawn across both mid–femurs on angiograms obtained 4 seconds after injection of contrast into the distal aorta. Vessel counts were done on two separate occasions by the same observer and the results averaged. If the two counts differed by more than 10%, a third count was made and the three values were averaged.

Capillary density measurements

A histochemical technique that labels alkaline phosphatase in capillary endothelium was used to count the number of capillaries in hindlimb specimens. Muscle samples (about 1 cm^3 each) were removed from both adductors in the mid–thigh and frozen in methylbutane (precooled by dry ice) on postoperative day 50, just before the animals were killed. Cryostat sections (10 μ thick) from each muscle sample were cut transversely to the directions of the fibers and fixed in acetone at 4°C for 5 minutes and then air dried before staining. For each muscle section, staining for alkaline phosphatase with an indoxyl–tetrazolium method[11] was done for 1 hour at 37°C, using a incubation medium and adjustment to pH 9.2 to 9.4 with boric acid. After rinsing and a brief post–fixation in neutral buffered 10% formalin, the slides were counterstained with 0.5% eosin and then subjected to alcohol dehydration, xylene clearing, and mounting. This technique stains capillaries black while leaving the rest of the muscle a light pink.

Capillary density was measured by one of the authors (G.R.G.) according to a single–blind protocol using a computerized image analysis system (Optimas, Bioscan, Inc., Edmonds, WA). At magnification X 100, capillaries in 10 randomly selected fields (0.1744 mm^2/field) were counted for each muscle section and the results averaged. The capillary density was then calculated and expressed as capillaries/mm^2.

Histologic studies

Muscle samples were removed from the adductors in the mid–thighs for routine histologic studies when the rabbits were killed. The specimens were fixed in 10% buffered formalin, embedded in paraffin, and cut in 10 μ cross–sections. Each section was then stained with hematoxylin and eosin for the studies.

Statistical analysis

All values are reported as means ± one standard error of mean (SEM). Results in the two hindlimbs were compared with the paired Student's t–test. A p value of less than 0.05 was considered statistically significant.

RESULTS

In all 11 animals, both hindlimbs were weak when assessed clinically on postoperative day 10, with gradual recovery of function thereafter. Two rabbits developed superficial tissue necrosis in the distal toe and obvious limb muscle atrophy in the right hindlimb as compared with the left. No gross evidence of tissue inflammation was present in any animal at the time it was killed.

Calf blood pressure

There was no significant difference in calf systolic blood pressure between the two ischemic hindlimbs on postoperative day 10 before the administration of ECGF. However, as shown in Figure 2, after the ECGF was given, the calf blood pressure was found to be significantly higher in the left hindlimb. On postoperative day 30, the mean pressure (mmHg) was 68.9±3.1 in the left hindlimb and 45.0±2.9 in the right ($p<0.0001$). On day 50, the values were 83.0±3.0 vs. 57.0±1.7 ($p<0.0001$).

Revascularization on angiography

Satisfactory angiograms were obtained in all animals, and there was an obvious difference between the two hindlimbs in each animal in that reconstitution of the distal vessels in the left hindlimb was substantially more pronounced than that in the right.(Figure 3A) In Figure 3B, the area of the thigh has been selected and neovascularization is considerably more advanced in the left limb compared to the right.

The treated limb contains arterial vessels with a corkscrew–like path in the region in which the femoral vessels had been excised. These may be newly formed vessels, and it is interesting that they are anastomosed to the distal native arterial channels that were left in place.

The angiographic features shown in Figure 3 were found consistently in the treated limbs, whereas vascularization in the control limbs was variable. In all rabbits, there appeared to be fewer vessels in the right hindlimb and, in fact, quantitation of the vessels supplying the limbs revealed significantly more vessels in the left limb compared to the right (17.2±1.6 vs 11.0±0.8 vessels, $p<0.006$).

Capillary density

Representative sections from both adductors in the mid–thigh that were assessed for capillary density are shown in Figure 4. More capillaries are evident in the specimen from the left thigh than in that from the right. Quantitative analyses of capillary density showed 225.9±11.4 capillaries/mm^2 in muscles from the left thigh and 159.6±12.9 capillaries/mm^2 in those from the right($p<0.002$).

Histologic studies

Histologic assessment of adductors in the mid–thigh revealed primarily normal skeletal muscle in both hindlimbs, with no evidence of inflammatory cell infiltration 30

days after administration ECGF or saline. However, microscopical examination of the muscle samples showed more intramuscular arterioles in the specimens from the left thigh than those from the right.(Figure 5)

DISCUSSION

The experimental technique used in this study produced severe stable ischemia in both hindlimbs of New Zealand white rabbits. Ten days were allowed to elapse after the arterial inflow to the hindlimbs was markedly reduced to permit acute responses to the operative procedure to dissipate. Intramuscular ECGF was then injected into only one limb of each animal. Subsequently, the rabbits were monitored clinically and by means of invasive and non–invasive measures for 50 days to determine whether arterial flow to the treated limb was enhanced. Every evaluation revealed an improvement in perfusion in the limb in which ECGF had been injected.

These findings confirm the results of our previous investigation of the use of ECGF and provide additional support for its efficacy. In our earlier study, only one hindlimb was rendered ischemic and two separate groups of rabbits were compared– one that received local injections of ECGF and one that did not. We found higher calf systolic blood pressure (expressed as a Left–to–right ratio) and more angiographically visible vessels in the hindlimb of the animals given ECGF.[4] Our use of a bilateral ischemic hindlimb model in the current study removed any unrecognized bias that may have arisen from comparing two separate groups of rabbits. In addition, we here quantitated the extent of neovascularization both angiographically and histologically.

A variety of angiogenic factors have been isolated and studied *in vitro*. Of these, both basic and acidic heparin–binding or fibroblast growth factors have been identified, and the two forms have been found to share similar gene and protein structures, in addition to binding to the same receptor.[12,13] In most systems, basic FGF is between 10– and 100–fold more potent than acidic FGF, although this difference disappears in the presence of heparin .[13,14] The ECGF (an acidic FGF) was bound to heparin to enhance its effectiveness. In addition, its relatively low price as compared with that of basic FGF allows large amounts to be used.

Although heparin itself has been shown to produce angiogenesis,[15] the doses required far exceed those used in this study (0.4 units/injection). It is highly unlikely, therefore, that the angiogenic effect we observed was related to the small amount of heparin bound to the ECGF. Indeed, studies of heparin–induced angiogenesis suggest that its effect is most likely related to its interaction with acidic and/or basic FGF.[16-18]

This study was not designed to identify the mechanism or mechanisms by which the ECGF induced angiogenesis. It may have acted directly on endothelial cells of preexisting vessels in the ischemic limbs to stimulate cell locomotion and mitosis and thereby initiate the formation of new vessels. However, the angiograms we obtained demonstrate that the vascular response to ischemia is complex and several components are apparently involved. Successful revascularization involves enlargement of native

vessels from which collateral flow can occur, development of adequate collateral vessels from these native vessels (a phenomenon that may represent enlargement of preexisting channels), neovascularization through the area from which the native vessels were removed, and finally, anastomosis of these channels to the native distal arterial tree. Therefore, ECGF may act on by opening and enlarging preexisting vessels, as well as by stimulating growth of new vessels in the ischemic limb.[19]

Although the exact mechanisms of the activity of angiogenic factors remain to be determined, these agents have been used experimentally with variable success to enhance the healing of wounds,[20] bone grafts,[21] the bronchial anastomosis after lung transplantation,[22] and duodenal ulcers,[23] as well as to endothelialize vascular grafts[24] and to enhance skin–graft survival.[25] Until recently, the ability of angiogenic agents to relieve organ or limb ischemia had not been tested.[4-7,26] Now, however, reports of its use in both cardiac and hindlimb ischemia are available. Banai et al[26] were unable to demonstrate any benefit from delivery of an acidic FGF to ischemic myocardium from an epicardial sponge when regional ischemia was produced gradually by application of an aneroid constrictor to the left anterior descending coronary artery. In contrast, Yanagisawa–Miwa and coworkers[7] demonstrated significantly enhanced collaterization with intracoronary administration of basic FGF. The factor was injected twice into the circumflex coronary artery 30 minutes and 6 hours after the left anterior descending was occluded with thrombus.

In studies in lower limbs, Baffour et al[6] showed that daily injections of a recombinant basic FGF significantly improved vascular flow in a rabbit model of acute lower limb ischemia. Their model, protocol, and experimental design, however, were different from those used in the current study. Our animal model may be more analogous to patients with chronic lower extremity ischemia (such as that resulting from occlusion of a superficial femoral artery [SFA]) than other rabbit models because the pelvic blood supply is left intact as a source of collaterals to the distal limb, while the femorals (which have more branches in animals than does the SFA in humans) are removed.[3,6] The results of angiogenic stimulation in this study may also be more pertinent than those of investigations of acute ischemia in determining the potential benefit of this therapy for chronic tissue or organ ischemia[5–7] This is because in our model, acute ischemia is created but the animal's condition is allowed to stabilize for 10 days before the study is begun. As a result, the normal response to ischemia and the inflammatory response to the surgical procedure have already occured before instituting therapy. Use of the bilateral ischemia model further controls for these effects.

Our findings provide further support for the use angiogenic stimulation to revascularize ischemic tissue in order to, for example, salvage ischemic limbs affected by diffuse small–vessel disease not treatable by direct surgical technique, enhance distal run–off in conjunction with a direct bypass procedure, or improve collateral development in patients not considered to be operative candidates.

CONCLUSION

We believe that the results of the study, as well as those of our previous work, demonstrate unequivocally that chronic severe ischemia of a hindlimb can respond dramatically to an appropriate dosage of ECGF. Clearly, much work remains to be done to identify the mechanisms by which such improved vascularization occurs, to determine appropriate dosage and optimal route of administration, and to evaluate the toxicity of the agents used. Nevertheless, the potential of such an approach in the treatment of tissue or organ ischemia in humans must now be acknowledged.

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FIGURES

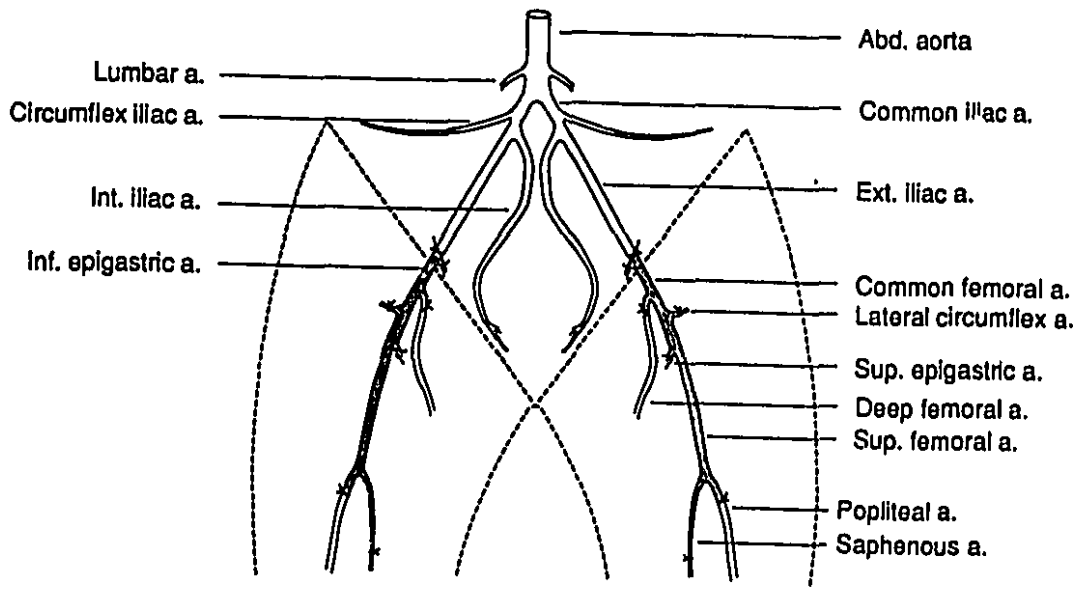

Figure 1. Technique used to produce bilateral hindlimb ischemia in rabbits. Ligations sites are shown; shading indicates vessels that are excised.

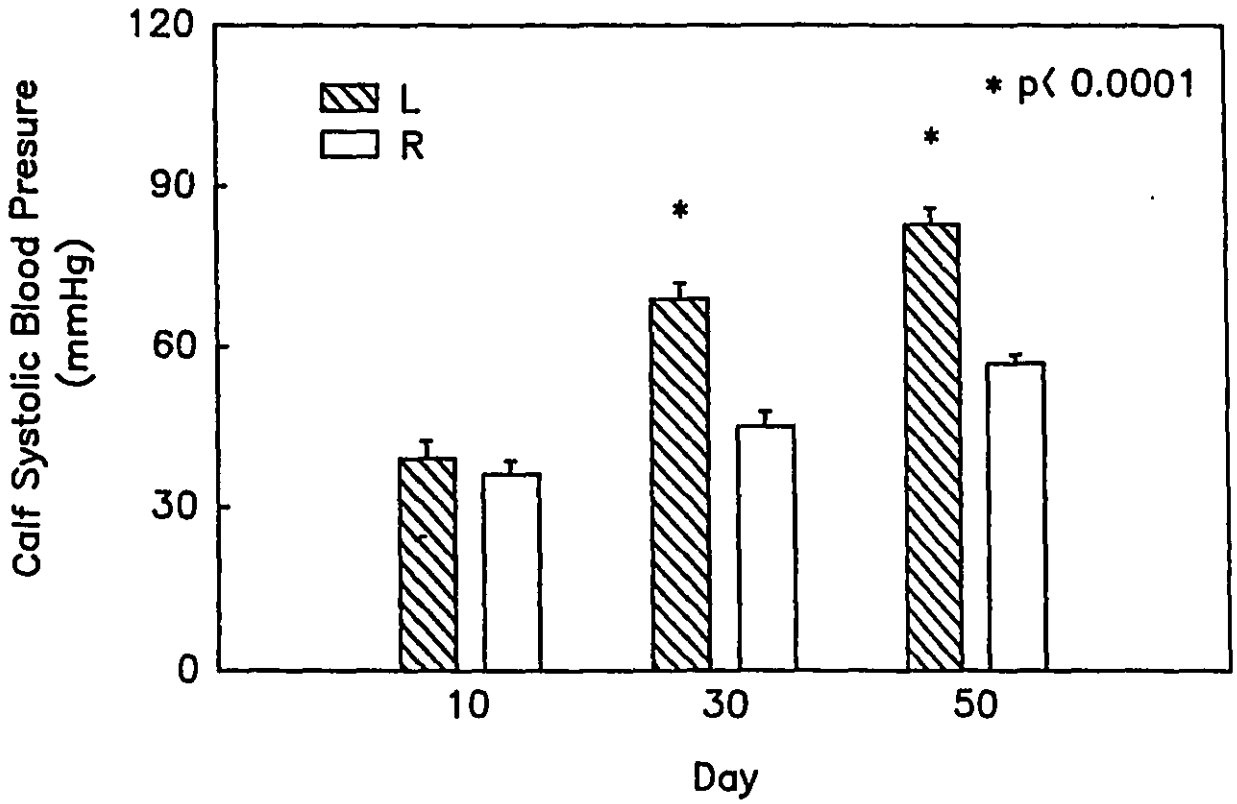

Figure 2. Measurement of calf systolic blood pressure (Left vs Right) demonstrates a significant increase of the blood pressure in the left hindlimb compared to the right on postoperative days 30 and 50.

Figure 3A. Angiogram obtained on postoperative day 50 (the 4th second film) shows earlier and more advanced distal arterial reconstitution in the left hindlimb compared to the right as indicated by the presence of more visualized vessels in the left thigh than in the right.

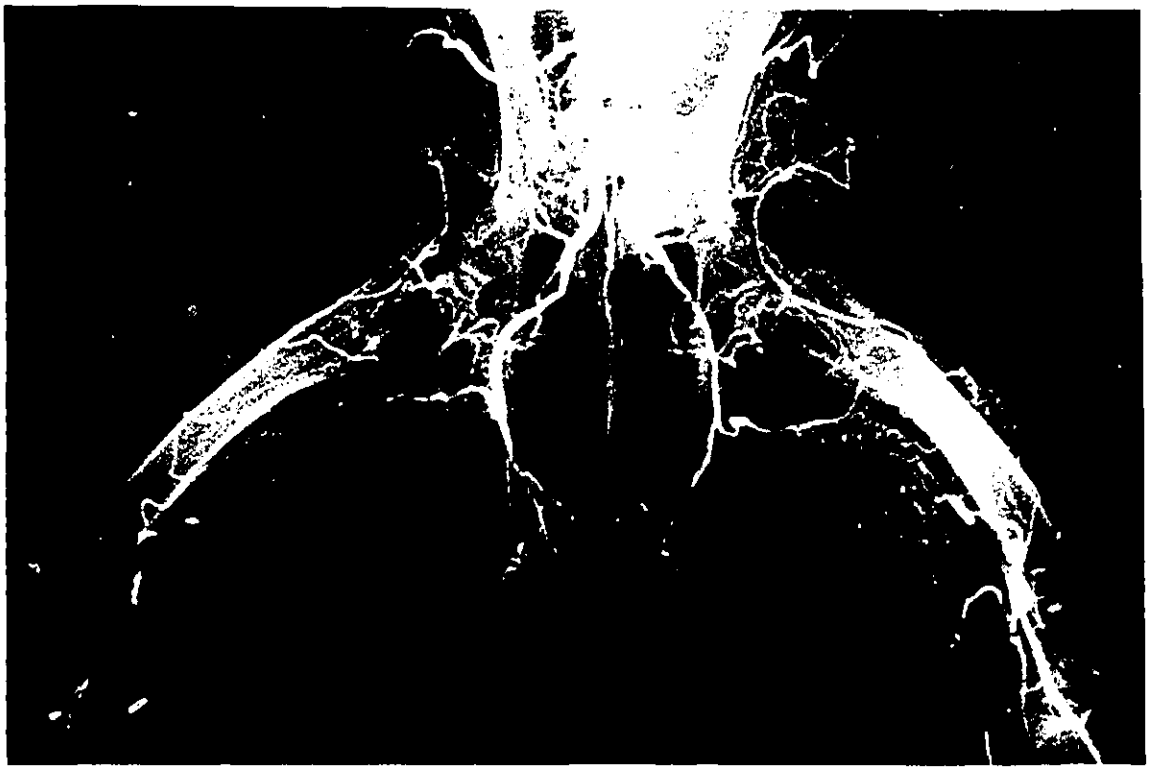

Figure 3B. A magnified version of Figure 3A.

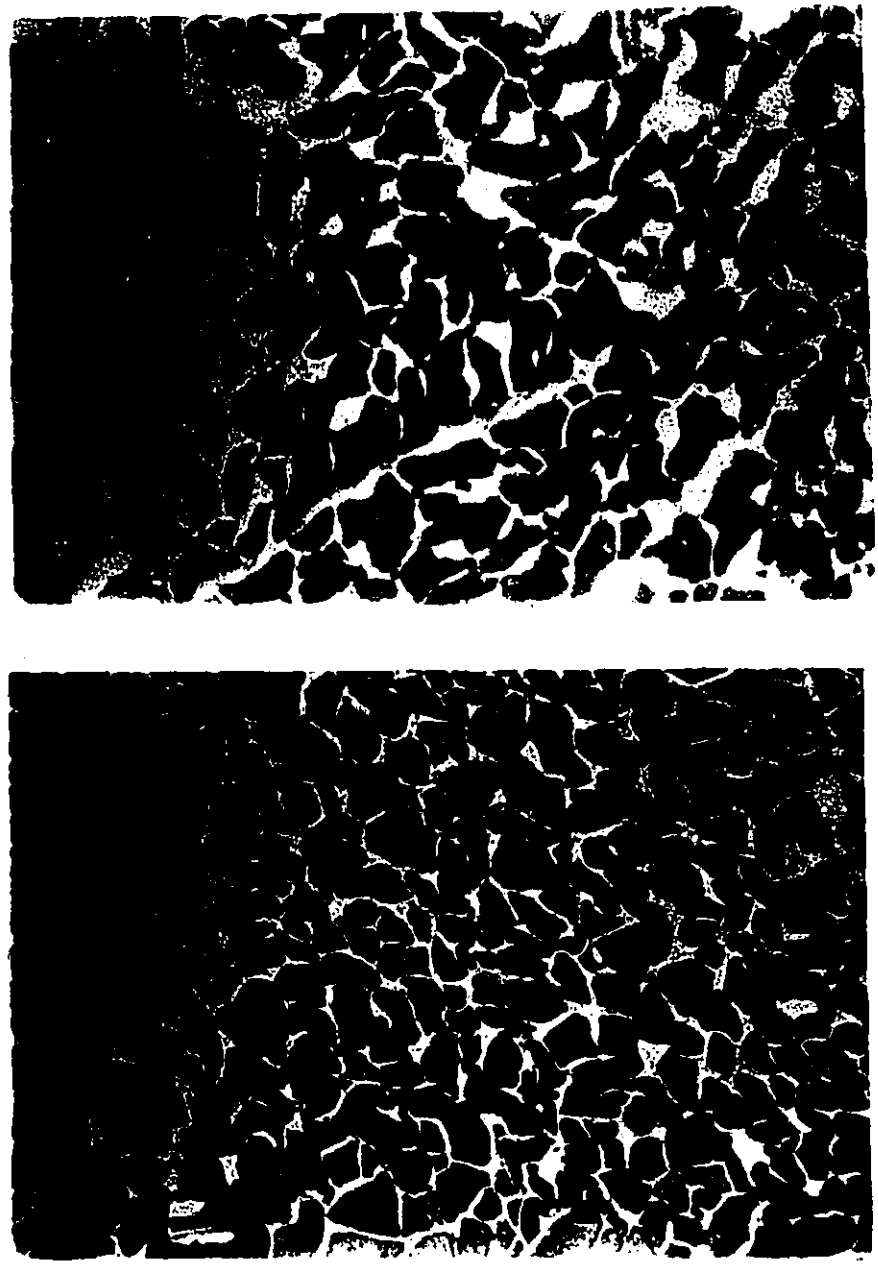

Figure 4. Microscopical cross–section of left (A) and right (B) thigh muscles from the same animal. More capillaries (dark sports) are evident in the left thigh. (Alkaline phosphatase stain; original magnification X 100)

Figure 5. Microscopical cross–section of left (A) and right (B) thigh muscles from the same animal. Histological studies show normal tissue, with no evidence of inflammatory cells infiltration, in both thighs. However, more intramuscular arterioles (*arrows*) are evident in the left thigh. (Hemotoxylin and eosin stain; original magnification X 100)

CHAPTER VI

A BETTER UNDERSTANDING OF ANGIOGENIC STIMULATION FOR THE TREATMENT OF ISCHEMIC LIMB

ANGIOGENIC GROWTH FACTOR AND REVASCULARIZATION OF THE ISCHEMIC LIMB: EVALUATION IN A RABBIT MODEL

Presented in part at the 26th Annual Meeting of the Association for Academic Surgery, Montreal, PQ, November 18–21, 1992.

ABSTRACT

We have previously shown that administration of endothelial cell growth factor (ECGF) significantly accelerates revascularization in the ischemic rabbit hindlimb model. Nevertheless much remains to be learned as to the effectiveness and limitations of this approach. The present study was designed, therefore, to determine whether a dose–response relationship could be demonstrated for this agent, whether it was effective if systemically administered, and finally, whether it affected vascularization in a non–ischemic limb. Our established unilateral ischemic rabbit hindlimb model and ECGF administration protocol were used to examine these questions. Three groups of animals were studied to determine a dose–response relationship of ECGF in the ischemic limb. Revascularization was assessed by measurement of calf blood pressure L/R ratio before (day 0) and after ECGF injections (days 1, 10, and 20) and quantitative vascularization was assessed by angiography at day 20 when the study was terminated. The results of the dose–response study were:

	Calf Blood Pressure (L/R) Ratio (mean ± SEM)				No. of Vessels
Day	0	1	10	20	Day 20
Grp 1(ECGF,4mg)	.26±.01	.59±.03*	.68±.04*	.76±.04*	26.6±2.4*
Grp 2(ECGF,1mg)	.23±.02	.50±.02+	.58±.03+	.64±.02+	18.6±2.2+
Grp 3(Control)	.26±.03	.42±.02	.51±.03	.55±.02	11.0±2.1

*p<.005 vs Grp 3, p<.05 vs Grp 2; +p<.05 vs Grp 3

Two other groups of animals were also studied. In Grp 4, intramuscular ECGF was injected in left front limb remote from the ischemic hindlimb and in Grp 5 it was

injected into left hindlimb of a normal animal. In neither group was any significant effect on vascularization evident. Thus, our data suggest that the relationship of ECGF and revascularization of the ischemic limb is dose dependent and is demonstrable only when it is administered directly into the limb in the presence of ischemia.

INTRODUCTION

Direct revascularization techniques for patients with severe chronic limb–threatening ischemia may be unsuccessful due to unavailability of an adequate conduit for arterial reconstruction, diffuse and progressive arterial disease, diabetic small vessel disease, or high surgical risk in the elderly patient. These patients may eventually require limb amputation with significant morbidity and mortality.[1,2] The need for limb salvage for this group of patients has stimulated research into alternative treatment modalities to avoid limb amputation.[3–5]

Angiogenic stimulation, which has been described by Folkman[6] and others,[7–9] may have the potential for neovascularization of ischemic tissue. The initial applications using one of the angiogenic growth factors (basic fibroblast growth factor or bFGF) have been reported to accelerate the healing of experimental bone grafts[10], chronic wounds[11] and duodenal ulcers.[12] More recently, however, a few groups, including our own have shown the angiogenic growth factor to be effective in experimental models of myocardial and hindlimb ischemia.[13–16] In preliminary studies, we have demonstrated enhanced arterial perfusion and vessel growth in a rabbit model of persistent hindlimb ischemia with the administration of endothelial cell growth factor (ECGF).[15,16] The objective of the present studies was to further our understanding of this potential alternative therapy for the treatment of tissue ischemia. We used our established rabbit ischemic hindlimb model and ECGF administration protocol to determine whether a dose–response relationship for

ECGF could be demonstrated, whether the agent was effective when injected at a site remote from the ischemic region, and finally whether it affected the vascular supply to a non–ischemic hindlimb.

MATERIALS AND METHODS

Experimental design. Twenty–five adult New Zealand white rabbits (male, mean 4kg) were used in this study and divided into 3 different experiments (Figure 1). Experiment A was designed to determine if a dose–response relationship could be demonstrated for ECGF. To do so, three groups (N=5 in each) were used. In all, the left hindlimb was made ischemic (for surgical procedure see below). Group 1 and Group 2 received injections of 4 and 1 mg ECGF respectively into the ischemic hindlimb whereas Group 3 received injections of saline only. Experiment B (Group 4, N=5) was designed to determine whether ECGF administered at a site remote from the ischemic zone favourably affected revascularization while Experiment C (Group 5, N=5) was to determine if ECGF affected vascularization of a normal limb. All animals were evaluated clinically before (day 0) and after ECGF injections (days 1, 10 and 20). Calf blood pressure was also measured at all timepoints noted above and on day 20 angiography was undertaken. The experiments were terminated on day 20 after ECGF injections and muscle samples from appropriate sites were examined histologically.

Animal hindlimb ischemia model preparation. A persistent hindlimb ischemia model in the rabbit was established in our laboratory and used in the present study. The surgical preparation in this model produced significant ischemia which persists up to at least 90 days.[17] Briefly, under intramuscular anesthesia (Ketamine, 50 mg/kg and Xylazine, 5 mg/kg), a longitudinal incision was made in the left groin of the rabbit from

the inguinal ligament toward the knee. With the aid of surgical loops, the femoral artery was totally dissected and its branches including the profunda, lateral circumflex, and superficial epigastric arteries were dissected as far as possible. The proximal popliteal and saphenous arteries were also dissected. Severe ischemia was induced in the left hindlimb of the animal by ligations of the distal external iliac artery (just above the level of the inguinal ligament), the inferior epigastric artery, the branches of the femoral arteries, the proximal popliteal and saphenous arteries followed by excision of the common and superficial femoral arteries. Each animal received 0.9% sodium chloride 50 ml intravenously during the surgery, and Cefazolin (15 mg/kg/per day) intramuscularly for 4 days started the day of surgery. The pain reliever (Bupreorphine 0.04 mg/kg) was also administered daily during the 10 days after surgery for each animal. The approval for animal use in this study was granted by our Institutional Animal Care Committee. The care of animals complied with the guideline of the Canadian Council of Animal Care, and the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals (NIH publication No. 80–23, revised 1985).

Endothelial cell growth factor administrations. ECGF was obtained from a commercial source (Endo Gro™, VEC TEC Inc., Albany NY, 100 mg plus 10 units heparin per vial). With regard to Experiment A (3 different groups, N=5 in each), the appropriate amount of ECGF (4 mg in group 1, 1 mg in Group 2) was diluted in normal saline (3 ml solution per injection). Each injection was given intramuscularly in 3

different sites in the left thigh (2 ml in the medial thigh, 0.5 ml in the lateral thigh, and another additional 0.5 ml in the distal thigh near the knee). A sham injection of saline was given in the same fashion in group 3 animals. The injections were made for 10 consecutive days beginning at postoperative day 11. In Experiment B (Group 4), 4 mg ECGF was administered intramuscularly into the left front limb (a remote non–ischemic site) daily also for 10 consecutive days beginning on post–operative day 11 in 5 animals with an ischemic left hindlimb. In Experiment C (Group 5), 5 animals who did not undergo any surgery received intramuscular injections of 4 mg ECGF into their left thigh for 10 consecutive days but received sham saline injections in their contralateral right thigh.

Calf blood pressure. Calf blood pressure, as an indicator of arterial perfusion to each ischemic hindlimb, was measured before and after ECGF administrations. The calf systolic blood pressure was determined noninvasively in both hindlimbs by Doppler Flowmeter (Model 1059, Parks Medical Electronics, Aloha, Oregon). To do so, both hindlimbs were shaved and cleaned. Under anesthesia, the posterior tibial artery pulse in the lower calf was detected by the Doppler probe with contact gel and the calf systolic blood pressure measured according to the standard technique. The calf blood pressure ratio was defined as a ratio of left to right calf systolic blood pressure (L/R ratio) and was used to determine the changes of arterial perfusion to the left hindlimb. The lower the ratio, the more impaired the arterial perfusion of the left hindlimb.

Angiography. A series of conventional angiograms was performed by standard techniques to visualize simultaneously the vasculature in both hindlimbs of each animal on day 20 after ECGF or saline injections when the study was terminated. Under anesthesia, the left common carotid artery was exposed through a ventral incision in the neck. Using the Seldinger technique a 4–French catheter was introduced into the exposed artery and advanced to about 3 cm proximal to the aortic bifurcation. A total of 10 ml of contrast agent (MD–76, diatrizoate meglumine and sodium) was injected at the rate of 3 ml/second with serial filming of both hindlimbs.

Vascularization of the left thigh was quantitated by direct counting the number of vessels (No. of vessels) crossing a line drawn vertically across the mid–thigh (passing through the center of the femur) on the film taken 4 seconds after injection of contrast in each series of angiograms. Vessel counts were made on two separate occasions by the same observer and averaged. If the variation between the two values was greater than a 10%, a third count was taken and the three counts averaged.

Histologic examinations. Muscle samples from the left adductors in the mid–thigh, where ECGF or saline were administered, were taken for routine histologic studies when animals were sacrificed. The muscle specimen was fixed in 10% buffered formalin, embedded in paraffin, and cut in 10 μ cross–sectionally. Each section was stained with hematoxylin and eosin, and was examined microscopically.

Statistical analysis. All data are expressed as the mean ± one standard error of mean (SEM). Statistical comparison among the three groups or between the two groups was performed on a computer (with statistical package) using the one–way analysis of variance and the unpaired Student's t–test, but between the two hindlimbs of each animal was performed using the paired Student's t–test. A result was considered significant if p value was less than 0.05.

RESULTS

1. Evidence for a dose-dependent effect of ECGF on revascularization (Exp. A)

1) Clinical examination: Three of the 5 animals which received only saline (group 3) developed obvious muscle atrophy in their left hindlimbs or superficial tissue necrosis in their distal toes. One animal in group 2 (ie the low ECGF dose) also developed muscle atrophy in the left hindlimb. By contrast, none of the animals in group 1 (ie the high ECGF dose) manifested any clinical problems in the left hindlimb.

2) Calf blood pressure ratio: The results for calf blood pressure ratio are given in Figure 2. The lower the value obtained, the greater the disparity in arterial perfusion of the two limbs. Note that it is low and indicative of severe ischemia but virtually identical in all groups on day 0 before ECGF injections. In the control group (group 3), it is slightly higher on days 1, 10 and 20. In the low dose group of ECGF (group 2), it is significantly higher than control at all three timepoints after day 0. Of considerable interest, it is significantly higher yet again in the group with the higher dose of ECGF (group 1) establishing for this parameter a dose–response relationship between this agent and arterial pressure in the left hindlimb.

3) Angiographic results: Satisfactory angiograms were obtained in all animals and the findings were consistent. Representative 4 second angiograms from one animal in each group are reproduced in Figure 3. The result obtained in an animal who received

only saline into the left hindlimb is shown in Figure 3A. Note that only a few vessels are seen traversing the left thigh. A typical result from an animal with low dose ECGF is shown in Figure 3B. Now a greater number of vessels are visualized coming out of the pelvis and running through the left thigh. One of these anastomoses to the distal native supply of the hindlimb. In Figure 3C, a result from the high dose ECGF is shown. Even more vessels are seen in the left thigh and once again reanastomosis to the distal vessels is seen. The quantitative results are given in Figure 4. The average number of vessels crossing the midthigh was 11.0 ± 2.1 in the control group and 18.6 ± 2.2 in the low dose ECGF group ($p<0.05$). A further increase was observed in the high dose ECGF group compared to the low dose group (26.6 ± 2.4, $p<0.05$).

Histologic examination

Microscopic examination of the left adductors in the mid–thigh revealed normal appearing skeletal muscle without evidence of inflammatory cells infiltration in all groups 20 days after ECGF or saline direct administrations.

2. Remote administration of ECGF in animals with an ischemic hindlimb (Exp. B)

In these animals (Group 4) in whom the arterial supply to the left hindlimb was interrupted as described above, 4 mg ECGF was injected in the left front limb daily for 10 days beginning on postoperative day 11. Two animals (2/5) developed obvious muscle atrophy in their left hindlimbs. The measurement of calf blood pressure ratio (L/R ratio)

demonstrated no significant difference compared to the control group (group 3) in Experiment A (Table 1). That is to say, in contrast to what was observed when ECGF was injected into the local ischemic region, no significant improvement in arterial perfusion was documented. This result was confirmed by the angiographic studies. A typical result is seen in Figure 5. This does not differ from that observed in the control group (Figure 3A). The quantitative analyses fully support this impression in that the number of vessels did not differ significantly from that present in the control group (10.5 ± 1.4 vs 11.0 ± 2.1, NS). This result is also significantly less than that observed in the low dose ECGF local administration group even though the amount of ECGF in the remote protocol was four times greater (10.5 ± 1.4 vs 18.6 ± 2.2, $p<0.05$). Histologic examination also revealed normal appearing skeletal muscle in the left adductors.

3. Effect on a normal limb (Exp. C)

Five animals (Group 5) were kept for 10 days and then injected with 4 mg ECGF daily into a normal left thigh and 3 ml saline into a contralateral right thigh. The measurement of calf blood pressure ratio (L/R ratio) demonstrated no significant changes before and 1, 10, and 20 days after ECGF injections (Table 2). Angiography performed at the end of this period did not reveal remarkable differences between both normal hindlimbs (Figure 6). The quantitative analyses also support this impression in that the number of vessels did not differ significantly in the left treated limb compared to the untreated right limb (17.0 ± 1.2 vs 15.5 ± 1.1, NS). Histologic examination again

revealed normal appearing skeletal muscle in the left adductors.

DISCUSSION

The results of this study significantly extend the evidence that ECGF substantially increases revascularization to an ischemic hindlimb. In addition to clinical inspection, the adequacy of arterial inflow was determined by two objective parameters: calf blood pressure ratio and quantitative angiography. Evidence of enhanced vascularization compared to control was found with the low dose ECGF local injection. For the first time, however, we have found evidence of a significant dose–response relationship in our rabbit model of persistent hindlimb ischemia. Revascularization was shown by both objective parameters used in this study to be enhanced at the high dose ECGF compared to the low dose ECGF. On the other hand, ECGF was effective only if given directly to the ischemic area and it had no significant detectable effect when injected into a normal limb.

In the present study, we have used our previously developed rabbit ischemic hindlimb model to learn more about the effects of angiogenic growth factor for limb revascularization and to better understand the potential of this alternative therapy for the treatment of limb ischemia. ECGF was used in this study and its administration was based on our previously successful protocol, in which the administration of ECGF was started at postoperative day 11 in the hope of minimizing the effects arising from the host response to acute ischemia and the surgery itself. Four mg of ECGF was administered daily for 10 consecutive days and the study was terminated 20 days later(on postoperative

day 40).[15] To assess the dose–dependent response of ECGF in the ischemic hindlimb, one fourth of our previously used dosage was chosen as a lower dosage (1 mg daily) so that two different doses (4 mg or 1 mg) were administered daily to each group (Group 1 or 2) of animals compared to saline control administration (Group 3) according to the same manner and schedule. For determining the effect of ECGF on the remote ischemic hindlimb (Group 4), the distant site (left front limb) administration was performed as Goldsmith et al[4] did in a study of the lipid angiogenic factor in a cat model.

Growth of blood vessels or angiogenesis is necessary for normal growth and tissue development. It also plays a central role in tissue repair and wound healing. Our understanding of the factors which regulate blood vessel growth has advanced enormously but is still incomplete. Evidence has accumulated pointing variously to mechanical factors such as shear stress, interactions between adjacent endothelial cell, changes in the vessel extracellular matrix, and growth factors– all of which, individually or together– may play critical roles in inducing and supporting vessel development.[7] Growth factors have been found in tumor or normal tissue and have been shown to stimulate angiogenesis both directly and indirectly *in vivo*.[6–9] Basic FGF, acidic FGF, and ECGF are examples of such factors which may directly act on receptors on the surface of endothelial cells to stimulate their migration, proliferation and production of enzymes capable of modifying the extracellular matrix followed by invasion of capillary sprout to form new vessels.[6,23] These factors have been successfully isolated and purified and therefore, it would be

possible to administer one of the angiogenic growth factors directly into ischemic tissues to promote collateralization or vessel growth.[6,24]

The potential of angiogenic stimulation has been demonstrated recently in several experimental studies for a variety of clinical problems by stimulating angiogenesis and subsequently increasing blood supply to ischemic tissues. For example, in a bone autograft healing model, Eppley et al[10] demonstrated increased vascularity of the graft at postoperative day 14 following continuous local infiltration of bFGF. By infiltrating omental lipid angiogenic fraction, Nottebaert et al[18] demonstrated microangiographically enhanced neovascularization as well as enhanced bone blood perfusion and regeneration of the graft. In an experimentally injured menisci model, King et al[19] demonstrated neovascularization within the poorly vascularized meniscal fibrocartilage accompanied by improved results of meniscal repair using a locally inserted disc containing angiogenin. The administration of one of the angiogenic growth factors has also been shown to accelerate wound healing in several animal models.[11,20,21] In a cycteamine–HCL induced duodenal ulcer model, Folkman et al[12] demonstrated enhanced angiogenesis in the ulcer bed and accelerated ulcer healing following orally administered bFGF compared to cimetidine.

Considerably less work has been done using angiogenic growth factor to improve vascular supply for the treatment of ischemic organs such as the heart or an extremity.

Recently, Yanagisawa–Miwa et al[13] demonstrated significantly enhanced collaterization with two intracoronary injections of bFGF in an acute canine myocardial ischemia model. Baffour et al[14] have shown that daily injection of bFGF significantly improved collateral vessel growth in a rabbit acute hindlimb ischemia model. In our laboratory, Pu et al[15,16] also demonstrated enhanced arterial perfusion and revascularization in a persistent rabbit hindlimb ischemia model following direct administration of ECGF for a period of 10 days. However, delivering aFGF via an epicardial sponge in a chronic canine myocardial ischemia model failed to show improved vascularization in the myocardium after 4 weeks.[22] Since angiogenic growth factor is considered a pharmacologic agent, the negative results from this study might relate to factors such as the short half–life and comparably small dosage of the agent. That is, multiple or continuous direct administrations of angiogenic growth factor using higher dosage may be necessary to produce an angiogenic effect in the *in vivo* setting.

The angiogenic effect of ECGF in this study is dose dependent and is demonstrable only when administered directly into the ischemic limb. Its administration is not accompanied by evidence of an inflammatory reaction. This finding may suggest that ECGF acts directly on ischemic limbs to stimulate new vessel formation and probably does not have a systemic effect on remote tissues of the body. The beneficial effect of growth factor administered locally versus systemically was also confirmed by the study of Mooney et al[21] in a skin wound healing model using tumor necrosis factor (TNF). The

minimal systemic effect of these growth factors *in vivo* when they are absorbed into the circulation may result from their short half–life and accumulation in some "filtering" organs in the body such as liver and/or kidney.[25] Since angiogenesis has been shown to play a role in atherosclerotic lesion formation and contribute to intraplaque hemorrhages in these lesions, especially in the carotid artery,[26,27] and also to play a role in a group of diseases so called "angiogenic disease", such as diabetic retinopathy, rheumatoid arthritis, hemangioma, etc.,[6] the avoidance of the unwanted systemic effects of angiogenic growth factors when administered *in vivo* in atherosclerotic patients for treatment of ischemic problems may be crucial. Further, as a pharmacological stimulator, angiogenic growth factor is probably most effective if delivered locally into ischemic tissues[28] and therefore, its local rather than systemic administration may be necessary.

Whether angiogenic growth factor does have an effect on non–ischemic normal tissues remains uncertain. It can be postulated based on our recently acquired knowledge of molecular biology that effective gene expression of locally acting growth factor may be suppressed in normal tissue which lacks a strong stimulus such as ischemia. An ischemic episode alone may cause gene expression and transcription of the growth factor and this cascade may lead to mitosis in endothelial cells and subsequent formation of collaterals or new vessels in ischemic tissue.[24] A study by Goldsmith et al[4] in a cat normal hindlimb where omental lipid angiogenic fraction was administered revealed that the effectiveness of the fraction determined by measuring vascular perfusion in the thigh

was only demonstrable in the presence of ischemia. However, a study by Kim et al[29] indicated that bFGF alone would have an angiogenic effect on normally perfused tissue demonstrated microscopically in the mouse ear model, but bFGF together with ischemia might act synergistically to produce a much greater angiogenic response. Our data did not reveal any significant angiogenic effect of ECGF on a normal limb where vascularization was determined by calf blood pressure ratio and quantitative angiography.

CONCLUSION

The results from the present study demonstrate that ECGF accelerates revascularization of the ischemic rabbit hindlimb only when administered locally in the presence of severe ischemia. Further, a clear relationship exists between the dose of ECGF and the degree of vascularization achieved, and the agent appears to have no significant effect on a normal limb. Our findings suggest that angiogenic growth factor, when administered locally, may have potential for the treatment of ischemic limbs by improving collateral vessel growth bridging obstructive lesions as well as perhaps improving the outflow for direct arterial reconstruction.

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TABLES

TABLE 1

CALF BLOOD PRESSURE (L/R) RATIO

DAY	0	1	10	20
Group 4	0.30±0.02	0.40±0.04	0.50±0.02	0.56±0.02
Group 3	0.26±0.03	0.42±0.02	0.51±0.03	0.55±0.02

Note. Group 4, remote administrations of ECGF; Group 3, the control group in Experiment A. Group 4 vs Group 3, NS. Data was expressed as mean ± SEM.

TABLE 2

CALF BLOOD PRESSURE (L/R) RATIO

DAY	0	1	10	20
Group 5	1.02±0.02	1.08±0.02	1.09±0.03	1.10±0.05

Note. Group 5, direct administrations of ECGF into a normal limb. Days 1, 10, and 20 (after ECGF injections) vs Day 0 (before), NS. Data was expressed as mean ± SEM.

FIGURES

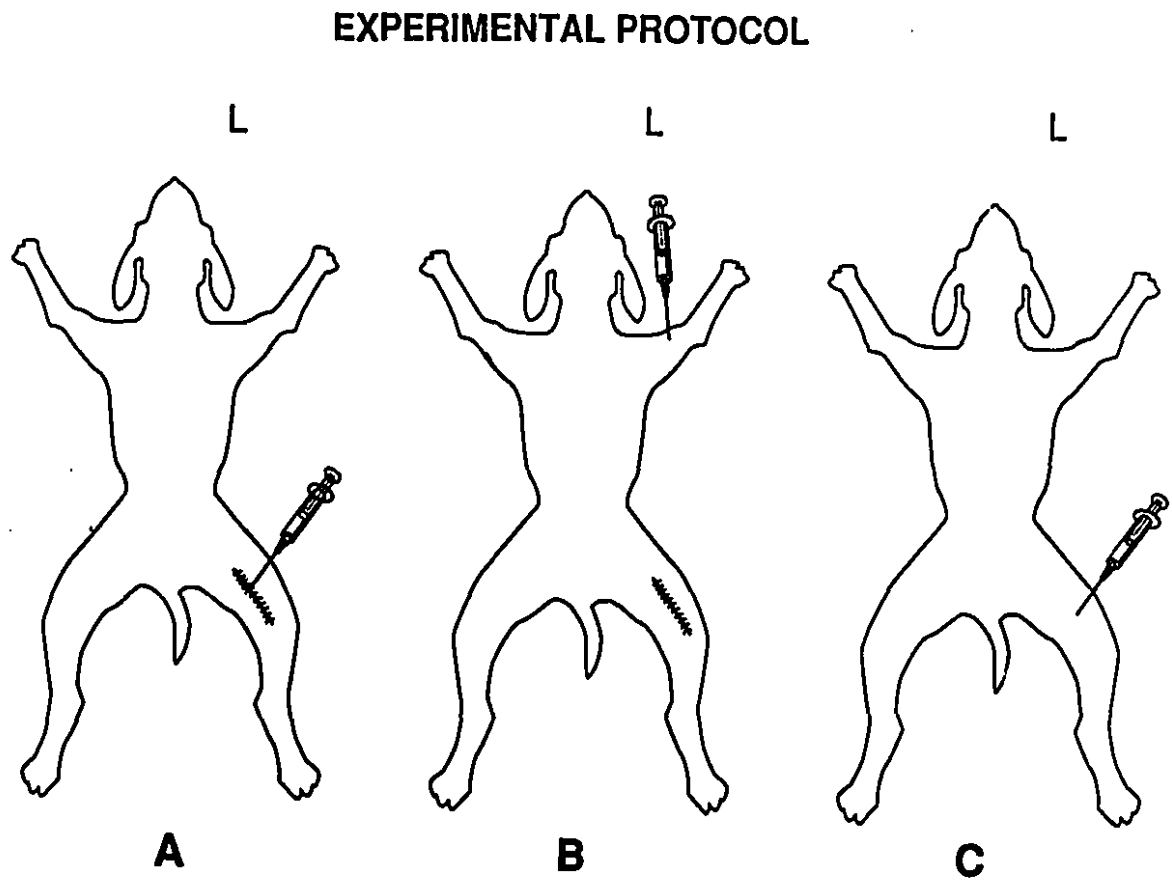

Figure 1. Composite drawing of experimental design. A, surgical creation of the left hindlimb ischemia model and direct administrations of ECGF into the ischemic limb. B, surgical creation of the left hindlimb ischemia model and distant site (left front limb) administration of ECGF. C, direct administration of ECGF into the normal left hindlimb.

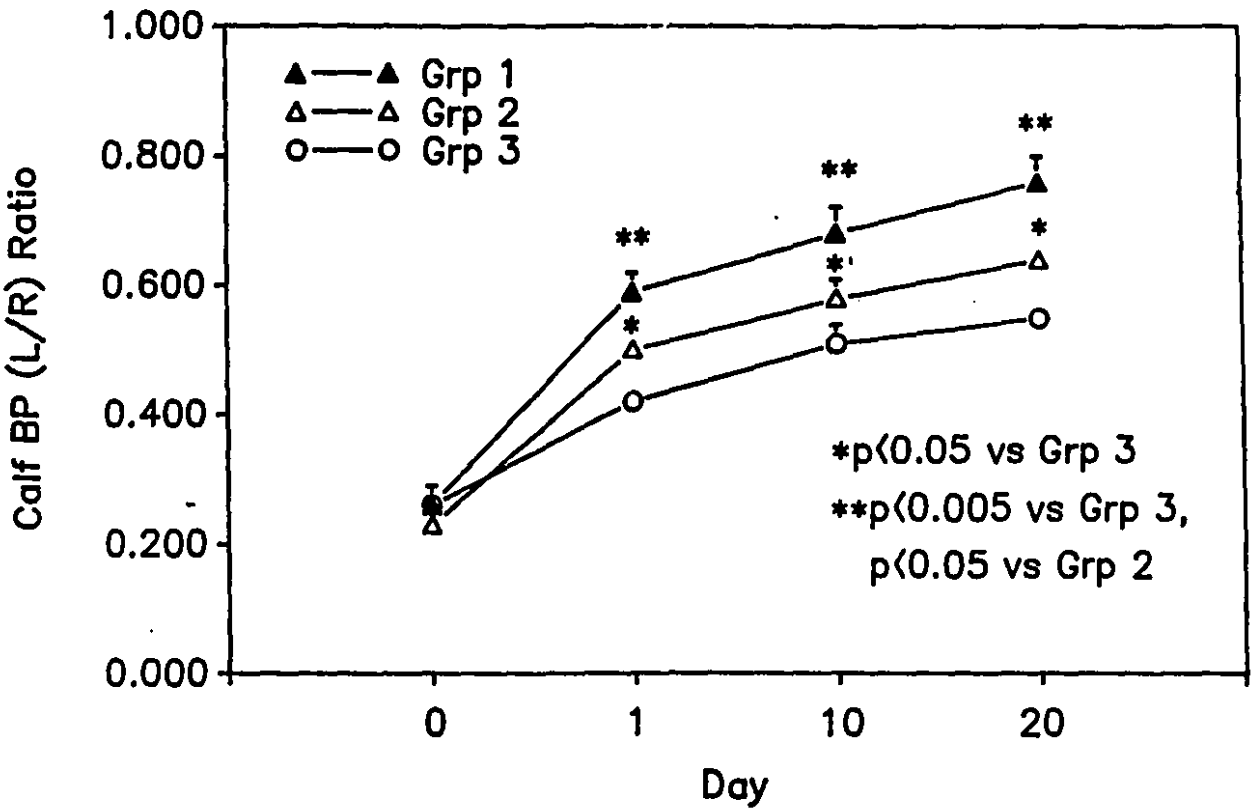

Figure 2. Measurements of calf blood pressure ratio (expressed as L/R ratio) demonstrate a dose-dependent increase of the ratios by ECGF when administered directly into the ischemic limb (Grp 1, 4 mg; Grp 2, 1 mg; and Grp 3, saline control) from Experiment A.

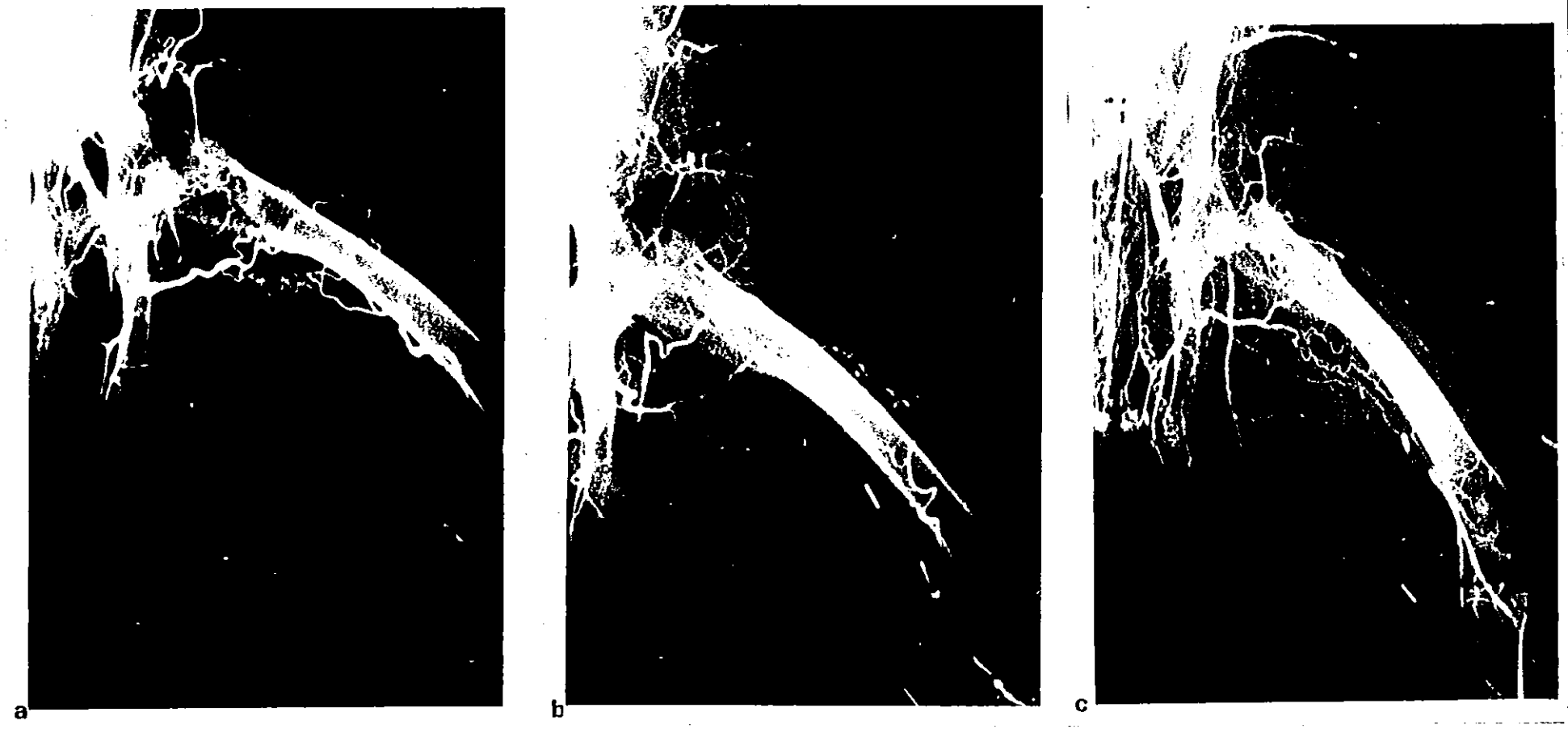

Figure 3. Angiograms (all 4th second films) at day 20 after ECGF administration comparing the vascularity of the ischemic limb in Experiment A. (a) Grp 3, saline control; (b) Grp 2, 1 mg of ECGF; and (c) Grp 1, 4 mg of ECGF.

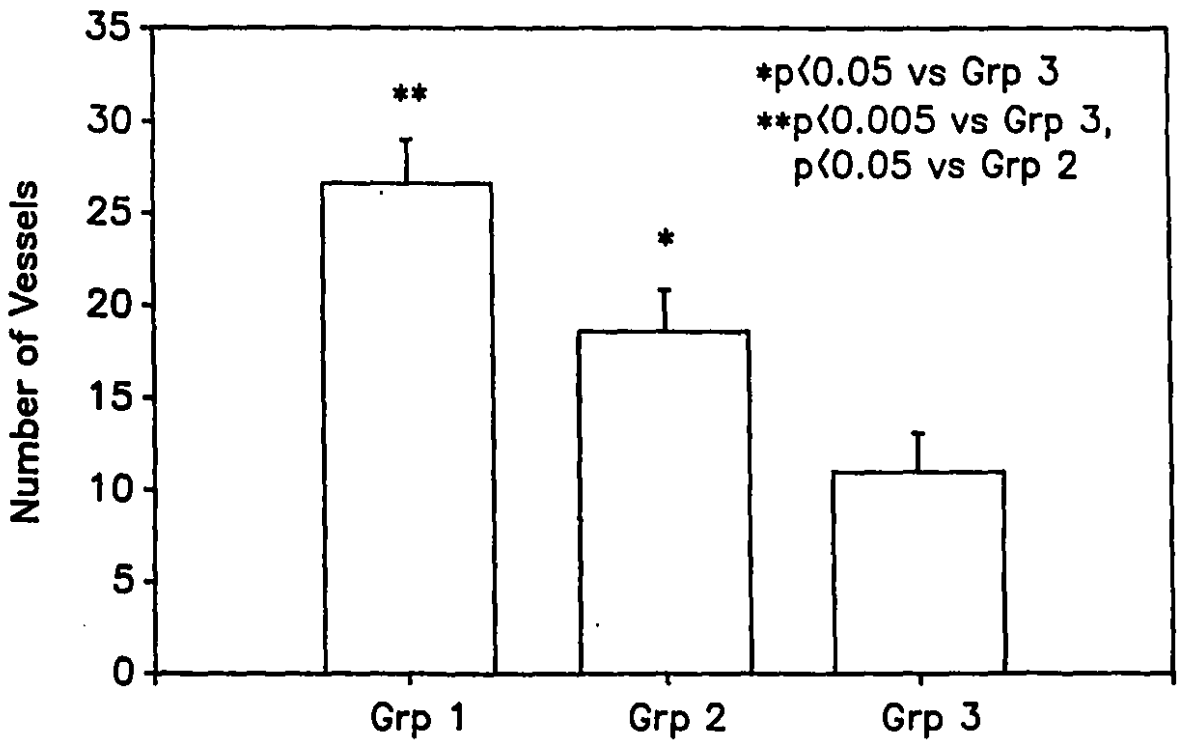

Figure 4. Quantitative measurements of vascularization (No. of vessels) on the angiograms of the left thigh at day 20 after ECGF administration demonstrate a dose–dependent increase in arterial vessels among the 3 groups (Grp 1, 4 mg; Grp 2, 1 mg; and Grp 3, saline control) from Experiment A.

Figure 5. Angiogram (the 4th second film) on day 20 after remote administration of ECGF from an Experiment B animal (Grp 4) reveals only a few developed collateral vessels secondary to ischemia.

Figure 6. Angiogram (the 4th second film) on day 20 after ECGF administration from an Experiment C animal (Grp 5) reveals no significant increase of vascularity in the left limb (ECGF–treated) compared to its contralateral right limb (Saline control).

CHAPTER VII

CONCLUSIONS AND CONTRIBUTIONS OF THE PRESENT STUDY

1. OVERALL CONCLUSIONS

In the present study, the first experiment was designed to develop a suitable animal ischemic hindlimb model for the study of angiogenic stimulation in ischemic limbs. This work was then followed by several subsequent investigations to test the original hypothesis we have raised that *in vivo* adminstration of one of the angiogenic factors would enhance revascularization of ischemic limbs. In both the second and third experiments, the hypothesis was extensively examined by a number of techniques using our rabbit models of unilateral hindlimb ischemia as well as bilateral hindlimb ischemia. Finally, a fourth experiment was designed to further our understanding of angiogenic stimulation for the treatment of ischemic limbs. The major conclusions from these investigations are as follows:

(1) The surgical preparation we have designed produces the persistent hindlimb ischemia in the rabbit for periods up to at least 90 days. This rabbit model approximates the clinical state of ischemic rest pain in the first few weeks and ischemic claudication thereafter. The model can be used for the study of various approaches for the treatment of chronic limb ischemia by measuring the improvement of arterial perfusion and collateral vessel formation in the ischemic limb.

(2) In the first experimental trial using the unilateral ischemic hindlimb model, the results clearly demonstrate that an angiogenic factor, ECGF, when administered intramuscularly into the ischemic limb, enhances revascularization in the ischemic limb, evidenced by significantly improved arterial perfusion and collateral vessel growth. This finding raises the possibility that angiogenic therapy may be a novel approach for patients with chronic limb ischemia.

(3) In the second experimental trial using the bilateral ischemic hindlimb model, the results unequivocally demonstrate that an angiogenic factor, ECGF, when administered intramuscularly into the ischemic limb compared with the contralateral ischemic limb, enhances the development of both collateral and capillary vessels which contribute to the marked improvement of arterial perfusion to that limb. This finding confirms the role of angiogenic stimulation in revascularization of ischemic limbs and again suggests that such an approach may have the potential for the treatment of patients with chronic limb ischemia.

(4) In the third experimental trial using the unilateral ischemic hindlimb model, the results demonstrate that the relationship between administration of an angiogenic factor (ECGF) and revascularization of the ischemic limb, evidenced by improvement of arterial perfusion and collateral vessel growth, is dose–dependent and that the angiogenic effect of ECGF is demonstrable only when it is

administered directly into the limb in the presence of severe ischemia.

In summary, the present study shows that the direct *in vivo* administration of one of the angiogenic factors, ECGF, into ischemic muscles indeed enhances revascularization in our experimental ischemic hindlimb models. This agent has a direct effect on the ischemic limb and appears to have no significant effect on the normal limb. Angiogenic stimulation may be a novel approach for the treatment of ischemic limbs by increasing collateral vessel growth to bypass the obstructive lesions as well as to improve the outflow for direct arterial reconstruction. This therapy may have the potential for the treatment of patients with ischemic limb claudication or for the salvage of patients with severe chronic limb ischemia in place of, or as an adjuvant to, direct revascularization procedures. Further work remains to determine the mechanisms of angiogenic stimulation in revascularization of ischemic limbs and to confirm the efficacy of this approach to an ischemic limb as well as to other ischemic tissues or organs. In addition, the safety and potential toxicity of this therapy as well as the appropriate dosage and optimal route to administer these angiogenic agents should also be defined before starting a clinical trial.

2. CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

The following statements are viewed by the author as being contributions to original scientific knowledge.

(1) The surgical preparation designed by the author in a rabbit produces the persistent hindlimb ischemia for periods up to at least 90 days. This animal ischemic hindlimb model is reproducible, measurable, relatively inexpensive and still allows one to perform most conventional measurements.

(2) The rabbit ischemic hindlimb model has been extensively investigated by the author using a number of techniques. This model is ideally suited for the study of the effect of *in vivo* administration of an angiogenic factor in the ischemic limb by measuring arterial perfusion and collateral vessel formation in that limb.

(3) The author, for the first time, confirms the original hypothesis that revascularization of ischemic limbs can be enhanced by the *in vivo* administration of one of the angiogenic factors and objectively demonstrates the relief of limb ischemia by means of angiogenic therapy.

(4) The author demonstrates both improved arterial perfusion and increased collateral vessel formation in the ischemic limb by a number of techniques following intramuscular injections of ECGF in the thigh.

(5) The author, for the first time, demonstrates that the *in vivo* intramuscular administration of one of the angiogenic factors in a single ischemic limb can significantly improve revascularization to that limb compared with its contralateral ischemic limb.

(6) The author demonstrates that both collaterals and capillaries increase in the ischemic limb that received intramuscular administrations of ECGF in the thigh compared with its contralateral ischemic limb by both quantitative angiography and histochemical technique.

(7) The author demonstrates that the dosage of one of the angiogenic factors, ECGF, and the degree of revascularization achieved in the ischemic limb have a clear dose–dependent relationship.

(8) The author demonstrates that one of the angiogenic factors, ECGF, can significantly enhance revascularization of an ischemic limb only when the agent is administered locally into that limb.

(9) The author demonstrates that one of the angiogenic factors, ECGF, appears to have no significant angiogenic effect on a non–ischemic normal limb when the agent is administered directly into that limb.

3. PUBLICATIONS

Portions of these investigations described in this thesis have appeared in the following original abstracts or minipapers:

1. Pu LQ, Lachapelle KJ, Graham AM, Lisbona R, Brassard R, Symes JF. Angiogenic stimulation: A new approach for severe chronic limb ischemia. Surg Forum 1991;42:365–7.

2. Pu LQ, Arekat Z, Brassard R, Carpenter S, Symes JF. Evaluation of a chronic hindlimb ischemia model in the rabbit. J Invest Surg 1992;5:278

3. Pu LQ, Arekat Z, Brassard R, Symes JF. A demonstration of significantly enhanced neovascularization by angiogenic stimulation in the ischemic limb. Surg Forum 1992;43:368–70.

4. Pu LQ, Arekat Z, Graham AM, Brassard R, Symes JF. Angiogenic growth factor and neovascularization of the ischemic limb: Evaluation in a rabbit model. Presented at the 26th Annual Meeting of the Association for Academic Surgery, Montreal, Canada, November 18–21, 1992, p115.

Manuscripts presented in chapters 3, 4, 5, and 6 will be published or have been submitted for publication in the following periodicals:

1. Pu LQ, Jackson S, Lachapelle KJ, Arekat Z, Graham AM, Lisbona R, Brassard R, Carpenter S, Symes JF. A persistent hindlimb ischemia model in the rabbit. J Invest Surg 1993 in press

2. Pu LQ, Sniderman AD, Brassard R, Lachapelle KJ, Graham AM, Lisbona R, Symes JF. Enhanced revascularization of the ischemic limb by means of angiogenic therapy. Circulation 1993 in press

3. Pu LQ, Sniderman AD,Arekat Z, Graham AM, Ricci MA, Gadowski GR, Brassard R, Symes JF. Angiogenic stimulation produces enhanced revascularization in a rabbit model of bilateral limb ischemia. Submitted

4. Pu LQ, Sniderman AD, Arekat Z, Graham AM, Brassard R, Symes JF. Angiogenic growth factor and revascularization of the ischemic limb: Evaluation in a rabbit model. Submitted