The Moran Foundation
DEPARTMENT OF PATHOLOGY
BAYLOR COLLEGE OF MEDICINE
TEXAS MEDICAL CENTER
HOUSTON, TEXAS 77030

June 23, 1992

Mark W. Majesky, Ph.D.
Department of Pathology
Baylor College of Medicine

Dear Dr. Majesky:

Please update me on the status of your Moran Foundation project (2-90-0047) entitled "a-Thrombin: Structure-Function Relationship in the Control of PDGF B-Chain Gene Expression".

Since approval and funding is generally for a one-year period, all projects approved in or prior to June 1991 should now be "complete", or nearly so.

I need a progress and/or final report regarding your project, including dates and times of any presentations, and information regarding any publications.

Please submit this to me within the next 30 days.

Sincerely yours,

Philip J. Migliore, M.D.
Research Director

PJM/ms

c: Dr. Michael Lieberman
Mr. John Moran

Final Report: Moran Foundation Project # 2-90-0047

Investigator: Mark W. Majesky, Ph.D.
Department of Pathology
Baylor College of Medicine

I originally proposed to examine the structure-function relationship for the regulation of PDGF-B gene expression by the multifunctional enzyme α-thrombin. In the project application, I stated that the proposed experiments had two objectives: **(1)** To examine the structural features of the α-thrombin molecule that are important for regulation of PDGF-B gene expression in arterial smooth muscle cells (SMC) in culture, and **(2)** to probe the molecular mechanisms that maintain stable differences in PDGF-B gene transcription in two types of arterial SMC that we had previously isolated from diseased arteries.

Objective 1:

Studies related to Objective #1 were completed by the end of December, 1991. We found that α-thrombin stimulated PDGF-B gene expression in smooth muscle cells (SMC) cultured from rat aorta in a dose and time-dependent manner. The ability of α-thrombin to increase PDGF-B mRNA levels required an intact enzyme active site of the protease. α-Thrombin inactivated by prior treatment with Phe-Pro-Arg-chloromethylketone (PPACK-thrombin), or by hirudin (a leech anticoagulant with an extremely high affinity for α-thrombin), failed to increase PDGF-B transcript levels in cultured SMC. Consistent with this interpretation, we found that γ-thrombin was much less potent (~100X) than α-thrombin at producing increases in PDGF-B mRNA in rat aortic SMC. These data clearly showed that the enzyme active site was required, while the anion binding exosite was dispensable, for the ability of α-thrombin to increase PDGF-B gene expression in aortic SMC. However, the anion binding exosite appears to contribute to the overall affinity of α-thrombin for a receptor on SMC that mediates PDGF-B gene expression. Moreover, using a synthetic peptide identical to the first 10 amino acids of the tethered ligand sequence of the thrombin receptor, we obtained full agonist stimulation of PDGF-B gene expression in SMC. The same 10 amino acids in a scrambled sequence had no effect. The EC_{50} for the agonist peptide (~30μM) compared to α-thrombin (~0.3nM) is consistent with the idea that α-thrombin (an enzyme) can catalyze the activation of many receptor molecules per α-thrombin molecule.

Publications Resulting From Work on Objective 1:

These results are represented in an Abstract for the 1990 Cell Biology Society Meetings (see attached abstract) and in a manuscript entitled "Regulation of Platelet-Derived Growth Factor Ligand and Receptor Gene Expression by α-Thrombin in Vascular Smooth Muscle Cells" by Okazaki, Majesky, Harker and Schwartz. This manuscript has been accepted for publication in *Circulation Research*.

Objective 2:

In pilot experiments supported by funds from this Project an entirely new direction was explored to address Objective 2. The new direction was prompted by finding a lack of PDGF-B gene expression by SMC in normal or diseased vessel wall *in vivo*. This new direction has proven to be highly successful. It has led to the publication of one Abstract, to the submission of one Manuscript and the preparation of a Grant Application to the National Institutes of Health targeted for submission in October, 1992.

After finding that PDGF-B was not expressed by SMC *in vivo*, we decided to study a modular, extracellular glycoprotein called tenascin because of its proposed role in promoting cell migration. Repair of vascular injury requires that normally stationary, quiescent SMC acquire a motile phenotype and proliferate. We hypothesized that a specialized set of matrix proteins, their cell surface receptors and selected matrix proteases would be required to accomplish this transition from a stationary to a motile phenotype *in vivo* and thought that tenascin would be an important member of this set of proteins. We first found that α-thrombin stimulates the synthesis and secretion of tenascin in cultured SMC. Since α-thrombin is known to be present at sites of acute vascular injury *in vivo*, it may be an important stimulus for activation of tenascin synthesis by SMC in injured arteries. We then found that SMC isolated from the neointima of diseased arteries synthesized tenascin at higher rates than SMC from normal artery both *in vivo* and *in vitro*. Finally, we found that cultured neointimal SMC produced about 5-fold higher levels of tenascin mRNA than did normal medial cells when both cell populations were maximally stimulated by α-thrombin. Thus, using α-thrombin as a probe, we have identified a gene (tenascin) whose expression in neointimal versus normal medial SMC seems to reflect some intrinsic difference between these two types of SMC. Since most occlusive vascular disease is a disorder of the intima with little or no involvement of the medial compartment, this difference in tenascin gene expression in the two SMC types could be very important. On the one hand, it provides a useful experimental tool. We can now use basic molecular biology techniques to determine why the tenascin gene is being differentially expressed in neointimal versus medial SMC. This may lead to the identification of regulatory molecules that maintain the neointimal versus medial identity in SMC. These regulatory molecules might be expected to control the expression of a set of genes (in addition to tenascin) that specify the neointimal SMC type. On the other hand, since SMC migration *in vivo* is an important feature of neointima formation after vascular injury, we can learn how this important component of the extracellular matrix is regulated in SMC both *in vivo* and *in vitro*.

Future Directions: Human Vascular Disease

Vascular injury is produced by nearly all forms of surgical reconstructions including balloon angioplasty, endarterectomy, atherectormy and vein graft bypass replacements. Restenosis is a major post-surgical complication that limits the long-term success of these procedures. A failure rate of 20 to 40% within 5 years due to vascular restenosis is commonly reported. Synthesis of tenascin by neointimal SMC may be an important event in SMC migration and matrix production that leads to restenosis. I have initiated a study with Dr. Luon Troung (Pathology), Dr. Don Weilbaecher (Pathology) and Dr. Gerald Lawrie (Vascular Surgery) to obtain human restenotic tissue at time of surgery and begin to test how these possibilities may apply to human vascular disease. We

obtained our first surgical specimen two weeks ago and are setting up to do immunohistochemistry, Northern blot analysis and *in situ* hybridization assays for tenascin in these tissues.

Publications Resulting From Work on Objective 2:

The recent work on tenascin synthesis by SMC has been published in Abstract Form (see attached copy). A manuscript entitled "α-Thrombin Stimulates the Synthesis and Secretion of Tenascin, an Anti-Adhesive Glycoprotein, by Vascular Smooth Muscle Cells" by Majesky, Dong and Pindur is being prepared for submission to the Journal of Biological Chemistry. Another manuscript on the expression of tenascin in vascular tissues and mesangial cells of diseased human kidneys is being prepared in collaboration with Dr. Truong. Finally, a grant application to the National Institutes of Health, Heart, Lung and Blood Institute is being written for submission in October 1992.

Presentations:

Funds from this project supported work that was presented in the following seminars or Meetings:

(1). "The Role of Smooth Muscle Cell Diversity in Arterial Wound Repair", a talk presented at the 1992 FASEB Annual Meeting, April 5-9, 1992, Anaheim, CA.

(2). "Paracrine Mechanisms of Intimal Smooth Muscle Cell Growth Control", Department of Medical Physiology, Texas A&M University, College Station, TX, May 6, 1992.

(3). "Smooth Muscle Diversity in Artery Wall Formation and Repair", University of Alabama, 4th Annual Vascular Biology and Hypertension Research Retreat, Sandestin, Florida, October 9-11, 1992.

1990 ASCB ABSTRACT FORM

Start here: TITLE and TEXT MUST fit within the blue lines J. Cell Biol. 111: 445a (1990).

OFFICE USE ONLY

α-Thrombin regulates PDGF and PDGF receptor gene expression in cultured vascular smooth muscle cells. [1]H. Okazaki, [2]M.W. Majesky and [1]S.M. Schwartz. [1]Department of Pathology, University of Washington, Seattle, WA 98195. [2]Department of Pathology, Baylor College of Medicine, Houston, TX 77030.

Cultured adult rat arterial smooth muscle cells (SMC) express PDGF A-chain, PDGF α-receptor and PDGF ß-receptor transcripts. We have examined whether expression of these genes are regulated in SMC by vasoactive factors to which vascular smooth muscle cells may be exposed at site of injury. As vasoactive factors, we have tested α- and ß-adrenergic agonist, Serotonin, Histamine, Angiotensin II, Endothelin, PDGF-AA, PDGF-BB, bFGF, IGF-I, EGF, and α-thrombin. Among these compounds, α-thrombin strikingly regulates both PDGF ligand and PDGF receptor mRNA expression in cultured SMC. α-thrombin increased the expression of PDGF A-chain mRNA, which peaked 4 hr after exposure, remained elevated 12 hr after treatment. PDGF α-receptor mRNA levels were maximally reduced at 6 hr and gradually returned to the basal level by 48 hr. PDGF ß-receptor mRNA levels were also reduced at 6 hr and remained below basal levels 48 hr after α-thrombin exposure. PDGF B-chain mRNA levels were undetectable in these cultured SMC. The effects of α-thrombin were observed between 0.1 nM and 100 nM. Previously, we have observed a similar increase in PDGF A-chain mRNA levels and reduction in PDGF ß-receptor mRNA levels in response to balloon catheter injury in rat carotid arteries. These data suggest a role for α-thrombin as a regulator of PDGF A-chain and PDGF ß-receptor gene expression at sites of injury in vivo. (Supported by NIH grants HL-03174 and HL-26405, and Kirin Brewery Co., LTD.)

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REGULATION OF PLATELET-DERIVED GROWTH FACTOR LIGAND AND RECEPTOR GENE EXPRESSION BY α-THROMBIN IN VASCULAR SMOOTH MUSCLE CELLS

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Running head: PDGF Regulated by α-Thrombin

Recent Abstract: Presented at the Vascular Biology Gordon Conference, June 29-July 3, 1992.

ABSTRACT

α-Thrombin Stimulates the Synthesis and Secretion of Tenascin by Vascular Smooth Muscle Cells. Mark W. Majesky, Xiu-Rong Dong and Jana Pindur, Departments of Pathology and Cell Biology, Baylor College of Medicine, Houston, TX 77030.

Repair of vascular injury requires that normally stationary, quiescent smooth muscle cells (SMC) acquire a motile phenotype. A family of specialized matrix proteins, their cell surface receptors and certain matrix proteases function to allow transient formation and breakdown of cell-extracellular matrix contacts necessary for cell migration. Tenascin (also called cytotactin) is a modular, secreted glycoprotein with "anti-adhesive" properties that is transiently expressed at sites of active cell migration in the embryo and during repair of connective tissue injury in the adult. We found that tenascin mRNA levels (undetectable in normal rat carotid artery) were greatly increased in the first 6 hours after balloon angioplasty injury *in vivo*. To explore possible mechanisms for activation of tenascin gene expression in injured arteries, we exposed arterial SMC *in vitro* to α-thrombin, a multifunctional regulator present at sites of acute vascular injury *in vivo*. α-Thrombin produced concentration-dependent increases in tenascin mRNA levels that were maximal (14-fold) by 6 hours after agonist addition. Stimulation of SMC tenascin gene expression was dependent upon the enzyme active site of α-thrombin and was mimicked by a peptide from the tethered ligand sequence of the cloned thrombin receptor. Both transcription and translation-dependent mechanisms are required during the first two hours after α-thrombin addition for increases in tenascin mRNA levels to occur. Western blot analysis showed that α-thrombin produced a large increase in tenascin protein secretion into conditioned medium. The major forms of tenascin produced were polypeptides of 205, 220 and 280-kD in both control and thrombin-treated SMC. Immunohistochemical staining 24 hours after α-thrombin addition showed an increase in incorporation of tenascin into the extracellular matrix. A comparison of SMC of the medial vs neointimal phenotype showed the latter had increased basal and maximal thrombin-induced levels of tenascin gene expression *in vitro*. These findings suggest that activation of tenascin synthesis after acute vascular injury may be necessary for conversion of stationary SMC into a motile and proliferative phenotype and that α-thrombin may be a critical stimulus for these events *in vivo*.

Submission to: J. Biological Chemistry

α-Thrombin Stimulates the Synthesis and Secretion of Tenascin, An Anti-Adhesive Glycoprotein, by Vascular Smooth Muscle Cells

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Key Words: Cell Migration, Anti-Adhesive, Cytotactin, Arterial Wound Repair, Neointima

Running Head: α-Thrombin Stimulates Tenascin Synthesis

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