1-1-2011

The potential role of innate immunity in the pathogenesis of post-operative adhesions

Jennell White
Wayne State University,

THE POTENTIAL ROLE OF INNATE IMMUNITY IN THE PATHOGENESIS OF POST-OPERATIVE ADHESIONS

by

JENNELL WHITE

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2011

MAJOR: PHYSIOLOGY

Approved by:

_____

Advisor Date

_____

_____

_____

_____

DEDICATION

To my husband and parents for their love and support. To my daughter for serving as a driving force down the home stretch.

ACKNOWLEDGEMENTS

First and foremost I offer my sincerest gratitude to my advisor, Dr. Ghassan Saed, who has extended his knowledge throughout my graduate program. I attribute my growth as a research scientist to his mentoring aesthetic. His involvement throughout this process has definitely strengthened me mentally and professionally.

To my committee members: Dr. Jiang Zhong has always taken the time out to perfect my skill set. His technical strengths and willingness to train me has truly been an important tool in successfully completing my research project. Jiang is a team player and has always made me feel as part of the team. Dr. Michael Diamond has been very insightful throughout my research program. He has always taken the time to revise manuscripts and presentations while offering a clinical view to my research studies. Dr. Joseph Dunbar awarded me my first funding opportunity as a graduate student. This will never be forgotten. As director of the IMSD program he conducted informative meetings which touch on key elements within the research field such as writing manuscripts, presenting at scientific meetings, and seeking additional funding opportunities. He established a platform for students to interact and exchange ideas and explore opportunities pertaining to graduate and post-graduate studies. Dr. Robert Pauley has always been willing to provide needed constructive criticism at very critical check points within my program. He is very thorough and has been a tremendous asset to my committee. Dr. James Rillema stepped up to the challenge when I needed him most. By agreeing to commit his time at the end of my program he allowed me to smoothly transition to the next level.

I whole-heartedly extend my gratitude to everyone for playing an integral role in my doctoral training. (Support: NIH 5 R25 GM58905-08 IMSD Prog; NIGMS 2 R25 GM58905-09 Graduate; NIH 3 R01 GM069941-02S1 J. Whi; NIGMS 5 R25 GM58905-11 Graduate: Kamran S Moghissi MD Endowed Chair; Robert Sokol, OB/GYN, ICR)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF DIAGRAMS

LIST OF FIGURES

LIST OF ABBREVIATIONS

2OG: 2-oxoglutarate

ADEPT: adhesion reduction solution

ALK5: activin receptor-like kinases

Bax: Bcl-2–associated X protein

Bcl-2: B-cell lymphoma

BMP: bone morphogenetic proteins

BTM: basic transcriptional machinery

Co-Smads: common mediator smad

DMEM: Dulbecco's modified Eagle's medium

E: ubiquitin ligase

E1: ubiquitin-activating enzyme

E2: ubiquitin-conjugating enzyme

ECM : extracellular matrix

FBS: Fetal bovine serum

FIH-1: factor inhibiting HIF-1

FYVE domain: Fab 1 (yeast orthologue of PIKfyve), YOTB, Vac 1 (vesicle transport protein), and EEA1

GLUT1/3: glucose transporter

GSH: glutathione

H_2O_2: hydrogen peroxide

HIF: hypoxia-inducible factor

HRE: hypoxia response elements

HRP: horse-radish peroxidase

IFN-γ: interferon gamma

LAP: latency associated peptide

LPS: lipopolysaccharide

LTBP: latent TGF-β-binding protein

MMPs: matrix metalloproteinases

MT: microtubules

O_2: molecular oxygen

$O_2^{\bullet -}$: superoxide

p300/CBP: transcriptional co-activator

PAI: plasminogen activator inhibitor

PHD: prolyl hydroxylases

Real-time RT-PCR: real-time reverse transcription polymerase chain reaction

RI-III: receptor 1-3

ROS: reactive oxygen species

RPM: revolutions per minute

R-Smad: receptor Smad

R-Smads: receptor-associated Smads

SARA: Smad anchor for receptor activation

siRNA: small interfering ribonucleic acid

SOD: superoxide dismutase

TIMPs: tissue inhibitor of matrix metalloproteinases

Ub: ubiquitin

Smad: name originates from a fusion between Drosophila mothers against decapentaplegic (Mad) and *C. elegans* Sma (small pathways)

TGF: transforming growth factor

tPA: tissue plasminogen activator

VEGF: vascular endothelial growth factor

VHL: Von Hippel-Lindau

PREFACE

Aims of the Study

Until recently minimal insight involving the role of macrophages in peritoneal wound healing has been achieved. The **objective of this study** is to determine the role that macrophages play in the development of post-operative adhesions. The **central hypothesis** is that surgical-induced hypoxia triggers a pro-inflammatory signal, leading to the activation and recruitment of resident and infiltrating macrophages to traumatized tissue. This hypothesis has been formulated on the basis of strong preliminary and published data, which suggest that hypoxia plays a major role in the development of the adhesion phenotype, an integral part to post-operative adhesion development (1, 11-16, 28-34). This phenotype is characterized by a reduction in plasminogen activator activity, increased extracellular matrix deposition, increased cytokine production, and reduced apoptosis (1, 11-16). Furthermore, we have shown that exposure of normal peritoneal fibroblasts to hypoxia, irreversibly induces the adhesion phenotype (1, 28-34). The rationale for this study is that hypoxia, through the induction of HIF-1α and TGF-β1 expression, induces the expression of TGF-β1, type I collagen and VEGF, not only in fibroblasts but also in macrophages.

We examined the hypothesis by pursuing three specific aims:

1. **Determine whether macrophages express adhesion phenotype markers TGF-β1, type I collagen and VEGF, and whether this expression is induced by hypoxia.**
2. **Determine whether the adhesion phenotype is induced in macrophages through a HIF-1α dependent mechanism following hypoxia exposure.**
3. **Determine whether human macrophages stimulate normal peritoneal fibroblasts to acquire the adhesion phenotype following hypoxia exposure.**

Collectively, the outcome from this study will support the role of macrophages in the development of the adhesion phenotype and thus may involve an important role of innate immunity in the pathogenesis of post-operative adhesion development.

CHAPTER 1

Introduction

Post-operative Adhesions

Post-operative adhesion development remains a very frequent occurrence that is often unrecognized by physicians due to the limited ability to conduct early second-look surgeries. We and others have had the opportunity to perform such studies and found that nearly 85% of patients examined developed adhesions (1, 2). Adhesions are abnormal fibrous connections that develop between tissues and organs as a result of inflammation, but most frequently as a sequel to surgical trauma. Consequently, adhesions are the major contributors to small bowel obstruction, infertility, and severe pelvic and abdominal pain (1-3). Managing adhesions and their related complications affects the health-care system drastically. The prolongation of hospital stay, readmissions, surgical intervention, and potential litigation impose an enormous economic burden (4). Within the United States alone, adhesion-related issues, cost the healthcare system over $2.24 billion annually (1, 2). Therefore, adhesions are considered the most frequent and costly problem associated with surgery (3).

In spite of the current use of less traumatic surgical methods, including microsurgical and laproscopic techniques, a high incidence of adhesion development persists. Laparoscopic lysis of adhesions is optional for patients experiencing chronic adhesion-related pelvic pain, though adhesions have been shown to reform at one or more sites in almost all patients who undergo adhesiolysis, and at over two-thirds of the actual sites where adhesiolysis was performed (5-7). Having awareness of the many reasons to address the problem of adhesions, and the large number of surgical procedures performed each day in the United States, our understanding of peritoneal healing is extremely limited. However, one thing stands out: some patients heal adhesion-free, while others develop severe scarring from seemingly equivalent procedures. The

basis for this difference is completely unknown. Our understanding of these findings has focused our attention to better understanding molecular mechanisms responsible for adhesion development. Improved understanding of these mechanisms may allow clinicians to inhibit or selectively limit the development of post-operative adhesions.

Healing of the Peritoneum

In order to better understand mechanisms involved in the pathophysiology of peritoneal wound healing and postoperative adhesion development, we must first examine the anatomy of the normal peritoneum. The peritoneum is a serous membrane that lines the abdominal cavity and consists of mesothelial cells whose primary function is to ensure free movement of abdominal structures. In doing so, mesothelial cells produce a lubricating fluid to minimize friction within the peritoneal cavity. Trauma to the peritoneum resulting from infection, inflammatory disease, and/or surgical procedures, disrupts this activity and promotes adhesion development. Subsequently, healing in the presence/absence of adhesions is determined by four sequential, yet overlapping phases; these include blood homeostasis, inflammation, cell proliferation, and tissue remodeling (1, 8). Mesothelial cells, fibroblasts, inflammatory, and immune cells are integral to these processes (3). Substances released from these cells regulate fibrinolytic activity, tissue remodeling, angiogenesis, and extracellular matrix turnover, processes that are central to the development of post-operative adhesions (1).

While many phases of peritoneal wound healing resemble dermal wound healing, two fundamental differences exist. First, in contrast to dermal injuries, which heal from the edges toward the middle (such that time of healing is dependent on the surface area of the lesion), peritoneal injuries are thought to heal by differentiation of underlying progenitor cells (such that healing occurs throughout the lesion simultaneously) (1, 9). Second, peritoneal wounds are continuously exposed to factors present in the peritoneal fluid, such as growth factors and

cytokines (1, 10). These substances are released from various cells that are resident to the wound; therefore, an autocrine/paracrine feedback is an important component of peritoneal healing. For normal peritoneal healing to occur, optimal concentrations of these signaling molecules must be present. Therefore, inhibition or over-stimulation may lead to impairment (nonhealing) or excessive scar tissue (adhesions).

The Adhesion Phenotype

To date, approaches to limit adhesion development have primarily focused on minimizing surgical trauma such as meticulous attention to operative techniques, delicate purposeful tissue handling, and uncompromising hemostasis. Another approach utilizes internal barriers, such as Seprafilm (Genzyme, Cambridge, MA), Interceed (Gynecare, Somerville, NJ), and Adhesion reduction Solution (ADEPT; Baxter BioSurgery, Deerfield, IL), to separate surgically injured tissue from non-injured tissue surfaces during the initial postoperative timing period while remesothelialization is occurring, a process that is usually expected to take 3 to 5 days (4). Over time these approaches have not been completely effective in reducing adhesion development therefore additional research focusing on mechanisms involved in peritoneal wound healing is critical. Delineating mechanisms that are paramount to peritoneal healing may provide a more successful approach for adhesion prevention. Our laboratory has a long-standing interest in studying molecular mechanisms involved in the pathogenesis of postoperative adhesions. We have established primary cultures of fibroblasts isolated from normal peritoneum and adhesion tissues (from the same patients) and characterized differences between these two cell lines at the molecular level (1, 11). Adhesion fibroblasts, when compared to normal peritoneal fibroblasts, exhibit a reduction in plasminogen activator activity (lower tPA and higher PAI-I), increased angiogenesis (increased VEGF), increased extracellular matrix deposition (higher type I collagen and fibronectin), increased cytokine production (higher TGF-β1), and reduced apoptosis (higher

Bcl and lower Bax), collectively known as the "adhesion phenotype" (1, 11-16). Additionally, we have established an animal model as well as an *ex vivo* model to further investigate molecular mechanisms *in vivo*.

Hypoxia and Oxidative Stress

Recently, we have gathered an immense amount of compelling evidence to support the hypothesis that surgical-induced hypoxia is the initiating factor leading to post-operative adhesion development. In support of this hypothesis, we have shown that hypoxia irreversibly induces the adhesion phenotype in normal peritoneal fibroblasts (1, 17-23). However, the mechanism by which hypoxia acts is not fully understood. During the first five minutes of hypoxia, there is a significant production of free radicals, either through an increase in reactive oxygen species (ROS) or by decreasing ROS scavengers (24-28). This increase in ROS has significant consequences, such as stimulation of cellular proliferation, promotion of mutations and genetic instability, and ultimately, alteration in both the phenotype and genotype (24, 29-30).

We have shown that hypoxia-generated superoxide ($O_2^{\bullet -}$), a potent reactive oxygen species (ROS), induces the development of the adhesion phenotype (31). Under normoxic conditions, ROS are maintained at optimal levels by specific scavenging systems (31-33). This process is achieved by various enzyme systems that neutralize toxic oxidants, such as ROS. Superoxide dismutase (SOD), an anti-oxidizing enzyme, catalyzes the conversion of superoxide ($O_2^{\bullet -}$) to hydrogen peroxide (H_2O_2), which subsequently leads to the conversion of water by catalase or glutathione (GSH) peroxidase coupled with glutathione reductase. We have shown that scavenging $O_2^{\bullet -}$, by superoxide dismutase (SOD), restores adhesion markers in adhesion fibroblasts to levels observed in normal peritoneal fibroblasts (31). Also, scavenging $O_2^{\bullet -}$ by SOD during hypoxia exposure inhibits the development of the adhesion phenotype in normal peritoneal fibroblasts. Similarly, a role for ROS has been established in adhesion formation

which is derived from the observation that ROS scavengers reduce adhesion formation following open surgery in different animal models (30). It has been shown that SOD reduces adhesion formation induced by vascular obstruction/reperfusion of an ileal segment in rats (30). Based on these observations and others we have concluded that hypoxia, through the production of ROS, plays a significant role in the pathogenesis of postoperative adhesion development. More specifically, acute oxidative stress in the peritoneum induces mesothelial cell loss or dysfunction, peritoneal fibrosis, and more importantly intra-abdominal adhesion formation (34).

Homeostatic Response to Hypoxia: HIF-1α

Hypoxia initiates transcription of a number of genes that help to sustain O_2 supply to tissues and to enhance cell survival during severe O_2 deprivation. One of these classic cellular adaptations to ensure O_2 availability is the manifestation of hypoxia-inducible factor-1alpha (HIF-1α) (35). Hypoxia-inducible factor (HIF) is a major transcriptional regulator of cell metabolism and stress caused by oxygen deficiency (hypoxia) (35). The significance of HIF was recently demonstrated by a significant decrease in mRNA expression of vascular endothelial growth factor (VEGF) and glycolytic enzymes seen during hypoxia in HIF-1α-deficient murine embryonic stem cells (36-38). At the tissue level VEGF stimulates the formation of new capillaries and at the cellular level glycolytic enzymes and glucose transporter 1/3 (GLUT1 and GLUT3) gene products are enhanced (36-38).

The HIF protein is composed of two subunits, an oxygen-sensitive HIF-1α subunit and a constitutively expressed HIF-1β subunit. Therefore, HIF-1α expression, and thus the formation of the HIF-1α-HIF-1β complex, is largely dependent on O_2 availability (Diagram 1). Activity of the HIF-1α/HRE pathway, under optimal oxygen conditions, is substantially suppressed by the activity of prolyl hydroxylases (PHD) which modify HIF-1α at specific prolyl and asparingyl

amino acid residues (35-37, 39-41). This results in polyubiquitination of HIF-1α by von Hippel-Lindau tumor suppressor protein (VHL) (35-37). Ultimately, the HIF-1α subunit undergoes proteasomal degradation and is unable to form the heteromeric, HIF complex. Post-translational modifications (hydroxylation) are inhibited during hypoxia (35). Unmodified HIF-1α is then able to migrate to the nucleus and bind HIF-1β (35). The HIF-1α/HIF-1β complex stabilizes and goes on to regulate HIF-dependent gene expression. Upon complex formation HIF controls transcription by associating with hypoxia response elements (HREs) in the promoter region of a wide array of genes (35-37). HIF-controlled pathways influence metabolism, angiogenesis, cell differentiation, and apoptosis, and also have implications for normal physiology and development (35-37).

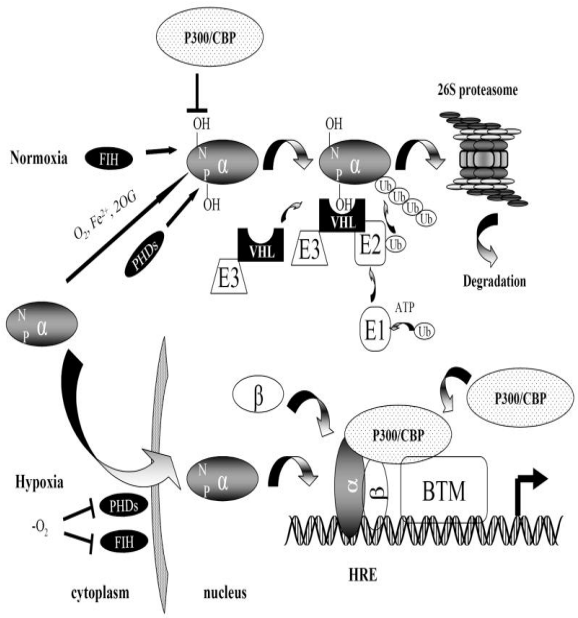

Diagram 1: Stability and Transcriptional Activity of HIF-α. In the presence of O_2 and cofactors, Fe^{+2} and 2-OG, PHDs hydroxylate HIF-1α. Modified HIF-1α recognized by the E3 complex that is followed by E2/E1-mediated ubiquitylation and degradation in the 26S proteasome. FIH-1-mediated N-hydroxylation prevents recruitment of p300/CBP transcriptional coactivators. In the absence of O_2, PHDs and FIH-1 are inactivated. Inmodified HIF-1α translocates to the nucleus, dimerizes with HIF-β, recruits p300/CBP, and induces the expression of its target genes via binding to the HRE. BTM, basic transcriptional machinery; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; FIH-1, factor inhibiting HIF-1; HRE, hypoxia-response element; 2OG, 2-oxoglutarate; PHDs, prolyl hydroxylases; Ub, ubiquitin; VHL, von Hippel-Lindau protein. (93)

ROS have been proposed to participate in the signal transduction process mediating the stabilization of HIF-1α during hypoxia (42). Under hypoxic conditions, mitochondria participate in a ROS burst (42). When the partial pressure of oxygen is reduced, electrons bind to molecular

oxygen, forming superoxide ($O_2^{\bullet -}$) (42). Superoxide is then converted to H_2O_2 by SOD. The resulting H_2O_2 efflux into the cytosol exerts an inhibitory effect on PHD activity, allowing HIF1α to accumulate, dimerize with HIF1β, and translocate into the nucleus where it modulates the expression of genes that favor survival under hypoxic conditions (42).

The Role of Cytokines: TGF-β1

In addition to HIF-1α, transforming growth factor beta (TGF-β) also plays a predominant role in peritoneal wound healing. Under normal conditions TGF-β regulates inflammation, angiogenesis, and extracellular matrix (ECM) turnover (43-47). Excessive TGF-β exposure exaggerates these processes thus promoting the development of adhesions (43). We have shown that hypoxia significantly increases the expression of TGF-β isoforms and their receptors in multiple cell types (28, 30, 48-50). Hypoxia-induced TGF-β1 expression has been shown to control the stability of HIF-1α through the regulation of prolyl hydroxylase (52). In addition, TGF-β1 can influence the accumulation of HIF-1α in normoxic conditions which further exacerbates TGF-β1's ability to promote adhesions even following reoxygenation (52).

Transforming growth factor β (TGF-β) exists in three isoforms (TGF-β1, TGF-β2 and TGF-β3). Practically all cells have receptors for the TGF-βs, and at least one of the isoforms is produced in all tissues (53-57). Unlike other cytokines, TGF-β is secreted in a latent form. TGF-β and the non-covalently bound latency-associated peptide (LAP; derived from the N-terminal of the TGF-β precursor), must be released for activation. Therefore, several possible modes of activation exist. It is thought that proteolysis by plasmin and other proteases is an important mechanism (53-57). LAP-TGF-β is usually secreted as a large latent complex consisting of LAP-TGF-β covalently bound to a latent TGF-β-binding protein (LTBP). LTBPs serve as structural components of the ECM and modulators of TGF-β availability.

TGF-β reecptors have three components: type I (RI, or ALK5); type II (RII) and type III (RIII, or β-glycan). RIII binds TGF-β (all isoforms) and recruits TGF-β to RII, which then phosphorylates RI to form a heterotetrameric serine/threonine kinase complex. In turn, RI phosphorylates Smad2 and Smad3 (receptor-associated Smads (R-Smads)), and the latter form a heteromeric complex with Smad4, which translocates to the nucleus, binds to DNA and regulates transcription. In contrast to Smads 2-4, Smad7 inhibits TGF-β signaling.

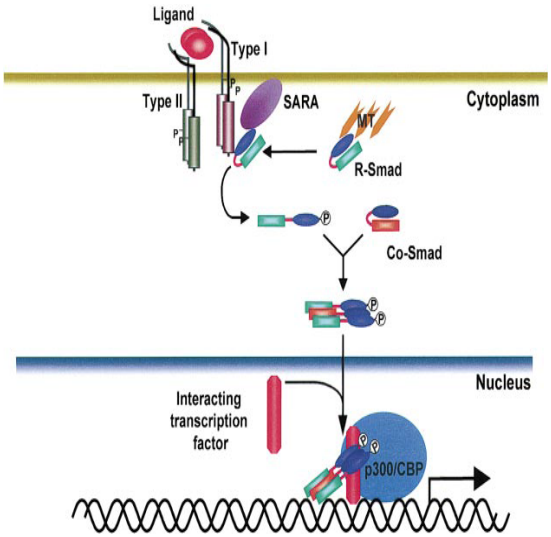

Diagram 2: Activation of R- and Co-Smads. Upon ligand-induced heteromeric complex formation and activation of type I and type II receptors, R-Smads are phosphorylated and form heteromeric complexes with Co-Smads that translocate into the nucleus, where they control the expression of target genes in a cell type specific manner. Nonactivated Smads can be retained in cytoplasm through association with microtubules (MT). The recruitment of Smad2 to the TGF-β receptor complex has been shown to involve a FYVE domain containing protein termed Smad anchor for receptor activation (SARA). Transcriptional modulation by Smads is achieved through complex formation with, e.g. transcriptional coactivators like p300/CBP and interacting transcription factors. R- and Co-Smads appear to form preferentially trimers consisting of one Co-Smad and two R-Smads. However, Smad complexes with other stoichiometry cannot be excluded (94).

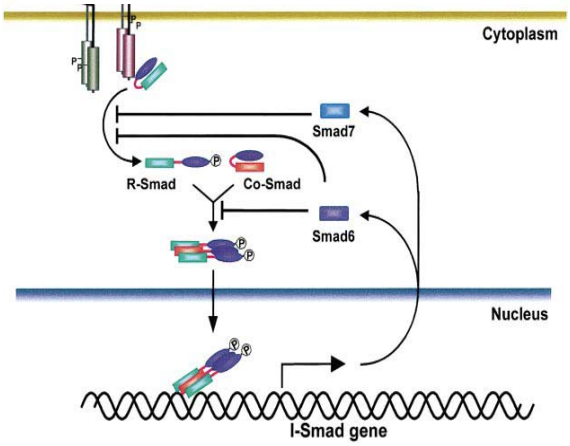

Diagram 3: Mechanism of Action of I-Smads Negative regulation of TGF-β/Smad signaling can occur through I-Smads that prevent activation of R-Smads or compete with Smad4 for heteromeric complex formation with activated R-Smads. I-Smads are induced by TGF-β family members and they may thus participate in a negative feedback loop (94).

The Role of Angiogenesis: VEGF

Stimulation of cells with TGF-β can result in the activation or inhibition of several genes including VEGF, a major angiogenic growth factor (53-57). Angiogenesis is involved in the restructuring process following tissue injury by regulating regeneration of capillaries, from pre-existing vasculature, to compensate for the loss of nutrients and oxygen (1, 8-10, 58-60). Capillaries are needed in all tissues for exchange of nutrients and metabolites. Changes in metabolic activity, as a result of tissue trauma, leads to proportional changes in angiogenesis and hence, proportional changes in capillarity (58-60). Oxygen plays a pivotal role in this process. More specifically, hypoxia potentiates angiogenesis, in part, by increasing both HIF-1α and TGF-β1 levels subsequently leading to the up-regulation of VEGF (1, 45, 61-66). Additionally, VEGF stimulates endothelial cell proliferation and increases vascular permeability.

Most control systems of the body, including angiogenesis, act by a negative feedback mechanism. When oxygenation is inadequate, the tissues become hypoxic, and this hypoxic signal induces or suppresses various proangiogenic or antiangiogenic substances (69). The increase in neovascularization promotes oxygen delivery to the tissue thereby increasing both capillary surface area and maximum blood flow to the tissues, and decreasing diffusion distances between capillaries (69). When oxygen levels normalize, proangiogenic and antiangiogenic factors return to normal levels and development of the vasculature is minimized, thereby closing the feedback loop (69).

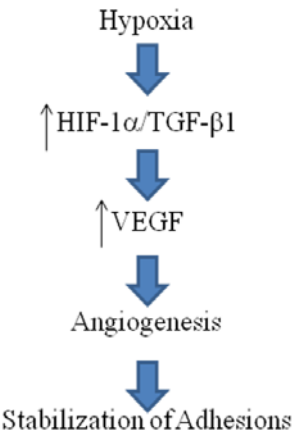

Diagram 4: Regulation of Angiogenesis during Hypoxia. HIF-1α and TGF-β1 activates VEGF to stimulate angiogenesis during hypoxia. Over expression of VEGF favors adhesion formation.

Excessive vascularization promotes adhesion development therefore, therapeutic applications targeting angiogenic pathways, specifically VEGF, have potential to reduce peritoneal adhesions (67, 68). For example, Bevacizumab has been shown to decrease adhesion formation following laparotomy in rats by blocking VEGF receptor occupancy (68). Binding of VEGF to its respective receptor results in receptor dimerization and ligand-dependent receptor tyrosine kinase phosphorylation, thereby activating intracellular signaling pathways involved in endothelial cell proliferation, migration, survival, sprouting, and tube formation, as well as up-regulation of molecules involved in degradation of the extracellular matrix.

The Role of the Extracellular Matrix: Type I Collagen

The earliest event in tissue repair involves deposition of fibrin-rich exudates, also referred to as provisional matrix (1). This process is critical to the initiation of peritoneal wound healing, though its resolution is also essential to healing without adhesions. Fibrin functions to restore injured tissues, and under normal circumstances are only temporary (29, 70-72). Lack of fibrin degradation allows the provisional matrix to become more organized which results in vascularization (increased VEGF) and collagen deposition. The organization of these fibrin bands over time and their transformation into mature fibrous adhesions is what allows adhesions to persist. The fibrinolytic pathway is responsible for degrading these filmy, fibrinous adhesions though following surgery this pathway is limited due to decreased blood supply (29, 70-72). Plasminogen activators convert plasminogen to plasmin, and are inhibited by plasminogen activator inhibitor (PAI). Plasmin degrades fibrin and also activates collagenases which go on to degrade type I collagen. We have shown that hypoxia, in addition to TGF-β1, decreases the fibrinolytic pathway by increasing PAI expression while decreasing PA expression (29, 70-72).

In addition to the PA/plasmin system the outcome of normal wound healing or impairment, such as adhesion development, largely depends on extracellular matrix turnover.

Type I collagen is the most abundant extracellular matrix protein. Several studies have shown

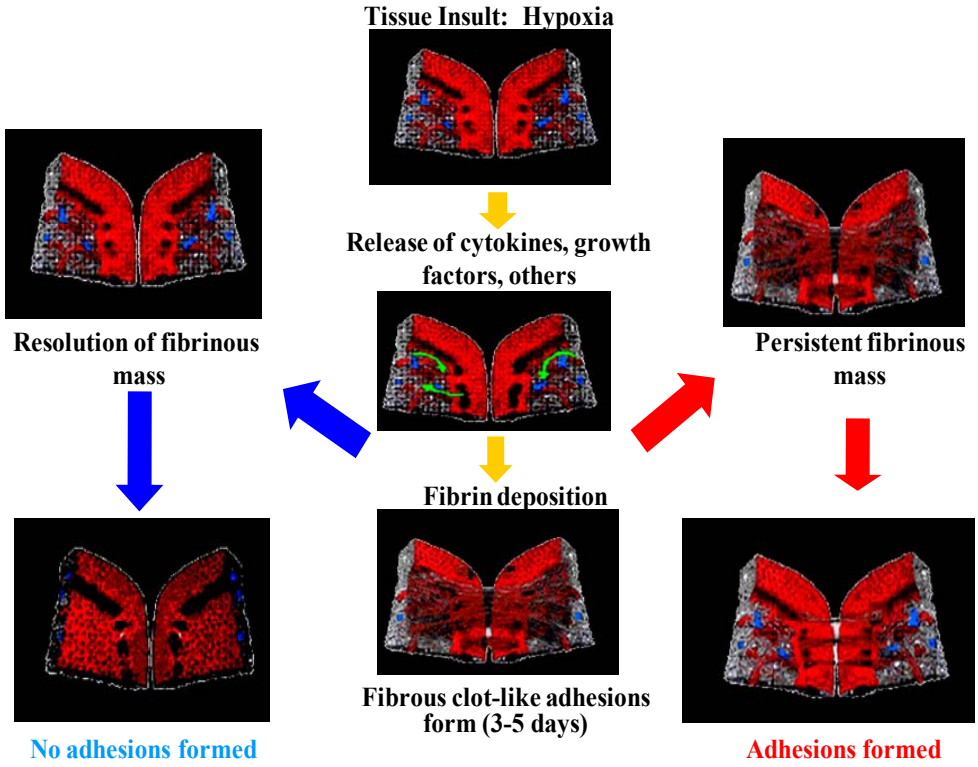

Diagram 5: Normal vs. pathologic conditions of peritoneal wound healing. Surgery causes damage to the mesothelium and results in a hypoxic microenvironment. Hypoxia leads to accumulation of a proteinaceous exudate at the injured site. These factors regulate fibrin deposition and are responsible for driving the recruitment of multiple cell types, such as fibroblasts, mesothelial cells, and macrophages, to the injured site. The earliest event in tissue repair involves deposition of fibrin-rich exudates, also referred to as provisional matrix. This process is critical to the initiation of peritoneal wound healing, but its resolution is also essential to healing without adhesions. Fibrin functions to restore injured tissues and under normal circumstances is only temporary. The fibrinolytic pathway is responsible for degrading these filmy, fibrinous adhesions though following surgery this pathway is limited due to decreased blood supply. Lack of fibrin degradation allows the provisional matrix to become more organized. The organization of these fibrin bands over time and their transformation into mature fibrous adhesions is what allows adhesions to persist.

that over-expression of type I collagen leads to adhesion formation (28). Hypoxia and hypoxia-induced TGF-β1 expression enhance type I collagen expression and therefore promote conditions favorable for adhesion development (30, 73-76). Matrix metalloproteinases (MMPs) degrade various components of the ECM, including type I collagen, and are induced in tissues undergoing extensive remodeling, as seen during inflammation and wound healing. The

proteolytic activity of MMPs is inhibited by tissue inhibitor of matrix metalloproteinases (TIMPs), which also influences cell growth, angiogenesis, and tumor invasion. Therefore the balance between the expression of MMPs and TIMPs is critical to normal wound healing. In general, hypoxia inhibits the expression of MMPs and increases TIMPs, thus decreasing matrix degradation and increasing fibrosis (73-76). Furthermore, hypoxia-induced TGF-β1 expression decreases MMPs which furthers minimizes the proteolytic ability of MMPs to maintain over-expressed ECM factors, such as type I collagen, following tissue injury (30, 73-76).

Factors involved in the fibrinolytic pathway and ECM turnover have been popular targets in reducing adhesion development (70, 74-75, 77). For example, Interceed, an adhesion barrier, reduces the incidence, extent, and severity of adhesions by physically separating tissue layers during the healing process. We have shown that Interceed increases the expression of PA and the PA/PAI ratio thereby promoting dissolution of fibrin which highlights Interceed's role as a barrier as well as having biologic effects (70). On the other hand, Tisseel, a fibrin sealant, facilitates initiation of coagulation at the wound site and thereby restores tissue stability by ensuring hemostasis. We have shown that Tisseel enhances the expression of MMPs while reducing TIMP expression (74-75). Collectively, these observations demonstrate the ability of internal tissue barriers to successfully reduce post-operative adhesion development thus implicating the importance of factors involved in tissue remodeling.

A Proposed Role of Macrophages in Adhesion Development

Until recently minimal insight involving the role of macrophages in peritoneal wound healing has been explored. Macrophages are evolutionarily ancient cells deriving from the primordial amoeba that evolved in the hypoxic atmosphere and adapted to normoxic conditions. Consequently, macrophages are highly versatile cells that are able to function under adverse, environmental conditions, such as hypoxia. Extensive monocyte extravasation is an early event

in the onset of inflammation and wound healing (78-81). Chemo-attractants, released in the environment as a result of inflammation, establish chemo-tactic gradients that are responsible for driving the recruitment of macrophages within avasular and necrotic, hypoxic areas of diseased tissue (80-81). Macrophages travel toward injured tissue as monocytes released from systemic circulation and subsequently undergo a process of differentiation and activation. Hypoxia is known to induce marked changes in the secretory activity of macrophages thereby eliciting cellular processes including, angiogenesis, ECM turnover, inflammation, and phagocytosis (78-79). The objective of this study is to determine the role of macrophages in the development of post-operative adhesions.

Materials and Methods

Cell cultures

Macrophages: Macrophages, from a human macrophage cell line, EL-1 (American Type Culture Collection Global Bioresource Center Manassas, Virginia), was cultured in Iscove's Modified Dulbecco Medium supplemented with 10% fetal bovine (FBS) and 2% penicillin and streptomycin. The next day macrophages were centrifuged for 5 min at 1800 RPM. Old media was discarded and macrophages were re-suspended in 10 mL of PBS buffer and re-centrifuged. Macrophages were transferred into a fresh 100 mm cell culture dish with pre-warmed media and placed in a 37°C incubator (95% air and 5% CO_2). Once confluence was reached dishes were sub-cultured (1:3 split ratio). Studies were conducted using passages 3-5 to maintain comparability to ensure normal phenotype of cells was not compromised. Based on previous experience with these cells, variability is observed following splitting of cells more than 5 times.

Fibroblasts: Collection and isolation of fibroblasts have been previously described (1). At the initiation of surgery adhesion tissue and normal parietal peritoneal tissue from the anterior abdominal wall lateral to the midline incision were excised from patients undergoing laparotomy

for pelvic pain, following entry into the abdominal cavity (1). Normal peritoneum was at minimum three inches from any adhesions. Subjects did not have an active pelvic or abdominal infection and were not pregnant. All patients gave informed written consent to tissue collection, which was conducted under a protocol approved by the Wayne State University Institutional Review Board.

Harvested tissue samples from five women were immediately placed in standard media [Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 2% penicillin and streptomycin]. Tissues were cut into small pieces in a sterile culture dish and transferred into another fresh T-25 flask with 3 ml of dispase solution (2.4 U/ml; GIBCO BRL, Invitrogen Corporation, Life Technologies, Carlsbad, California). The flasks were incubated overnight at 37°C in an environ-shaker (LAB-LINE Instruments, Barnstead International, Dubuque, Iowa). The samples were then centrifuged for 5 min at 1400 *g*, transferred into a fresh T-25 flask with pre-warmed DMEM and placed in a 37°C incubator (95% air and 5% CO_2); outgrowth of fibroblasts generally took 2 weeks. Once confluence was reached, the cells were transferred to 90 mm tissue culture dishes and cultured in standard media with 10% FBS. Thereafter, the confluent dishes were sub-cultured by trypsinization (1:3 split ratio). Studies were conducted using passages 3-5 to maintain comparability to ensure normal phenotype of cells was not compromised. Based on previous experience with these cells, variability is observed following splitting of cells more than 5 times.

Lipofectamine-siRNA Transfection

Macrophages (2.5 X 10^5) were seeded in a 6-well dish (Becton Dickinson, Franklin Lakes, NJ) transfected with 5 μL of Lipofectamine RNAiMAX Reagent (Invitrogen, Carlsbad, CA; catalogue no. 13778-075) complexed with 150 picomoles of a specific siRNA (HIF-

1α/TGF-β1) in standard medium without antibiotics. Macrophages were then placed in a standard tissue incubator (37°C; 95% air and 5% CO_2) for 24-hrs.

siRNA Design, Synthesis, and Labeling

Small interfering RNAs were designed after determination of target sequences by aligning specific sequences (HIF-1α/TGF-β1) to an Ambion Web-based algorithm. The nucleotide duplex siRNA molecules with 2-dTdT overhangs, were resuspended in nuclease-free water according to the instructions of the manufacturer (Ambion). To ensure a stringent control, a scrambled control sequence was obtained from Ambion (Silencer Negative Control No. 1 siRNA; catalogue no. 4611).

TGF-β1 treatment

TGF β 1 (2μg) human recombinant was purchased from Sigma-Aldrich and dissolved in phosphate buffered saline containing 2 mg/mL bovine serum albumin to reach a concentration of 20 μg/mL. Working concentrations (0, 375, 750, 1500 ng TGF-β1/mL media) were prepared by further diluting the stock solution.

Hypoxia Treatment

Hypoxic experiments were performed in an airtight modular incubator chamber. The chamber was deoxygenated by positive infusion of 2% O_2 in a CO_2-nitrogen gas mixture for 15 minutes. Cultures were then placed in a standard humidified tissue incubator. There were no statistically significant differences in viability by crystal violet or trypan blue exclusion (data not shown). Parallel cultures were placed in normoxic conditions for all experiments. Cells were harvested after 24 hrs. All experiments were performed in triplicate.

Real-Time RT-PCR for HIF-1α, VEGF, TGF-β1, and type I collagen

The real-time RT-PCR technique was used to detect and compare mRNA levels for each

experiment. The advantage of the real-time RT-PCR method over the conventional PCR method is that it allows the determination of the absolute copy number of mRNA.

RNA isolation

Total RNA was extracted from normal peritoneal fibroblasts and macrophages using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the protocol provided by the manufacturer.

Reverse transcription

A 20*u*L cDNA reaction volume was prepared with the use of QuantiTect Reverse Transcription Kit (Qiagen), as described by the manufacturer's protocol.

Real-time RT-PCR analysis

Optimal oligonucleotide primers for real-time RT-PCR amplification of reverse-transcribed cDNA were selected with the aid of a commercial software program (Beacon Designer 7.0; Premier Biosoft, Palo Alto, CA). Human oligonucleotide primers, which amplify variable portions of the protein coding regions, are summarized in Table 1 below.

Table 1: Human Oligonucleotide Primers

Locus	**Sense (5'-3')**	**Antisense (3'-5')**	**Product length**
TGF-β1	AGTGGTTATCTTTTGATGTC	GTAGTGAACCCGTTGATG	142
VEGF	GGAAACCAGCAGAAAGAGG	AAGCAGGTCACTCACTTTG	132
Type I Collagen	CCTGTAAACTCCCTCCATCC	AAGTCCATGTGAAATTGTCTCC	133
HIF-1α	AGCCGAGGAAGAACTATGAAC	ACTGAGGTTGGTTACTGTTGG	100

Real-time RT-PCR was performed with a QuantiTect SYBR Green RT-PCR Kit (Qiagen) and a Cepheid 1.2f Detection System (Cepheid, Sunnyvale, CA). Reactions are 25-μL volumes including 12.5 μL of 2× QuantiTect SYBR Green RT-PCR Master Mix, 1 μL of cDNA template, and 0.2 μmol/L of each target-specific primer that were designed to amplify a portion

of each gene. To quantify each target transcript, a standard curve was constructed with a 10-fold dilution series of a standard plasmid. The three-step PCR protocol applied for HIF-1α, VEGF, TGF-β1, and type I collagen reactions consists of 45 cycles of 95°C for 15 seconds, 60°C (HIF-1α), 57°C (VEGF), 52°C (TGF-β1), 58°C (type I collagen) for 30 seconds, and 72°C for 30 seconds. After PCR, a melting curve analysis will be performed to demonstrate the specificity of the PCR product as a single peak.

Immuno-precipitation and Western Blot Analysis

An antibody for HIF-1α, VEGF, TGF-β1, and type I collagen was incubated with nuclear/cytoplasmic protein extracted from normal peritoneal fibroblasts and macrophages. The antibody/antigen complex was pulled out of the total protein sample using protein A/G-coupled agarose beads. The sample was combined with Tris-Glycine SDS Sample Buffer (Invitrogen LC2676) and heated to 85ºC for 5 minutes to unfold the protein. Proteins were separated on a 4-20% Tris-Glycine gel (Invitrogen) at 130V for 180 minutes against a molecular weight ladder. Proteins were transferred from the gel to a nitrocellulose membrane using an electroblotting apparatus at 40V for 2 hrs.

Non-specific protein binding was blocked by incubating the membrane for 30 min in 5% non-fat milk solution. Membranes were washed 1X with wash buffer. Primary antibody diluted in 1% non-fat milk solution for HIF-1α, TGF-β1, VEGF, type I collagen, and tubulin primary antibodies were incubated for 24 hrs at 4ºC. Membranes were washed 4X in wash buffer and incubated for 1 hr at room temperature, with horse-radish peroxidase (HRP)-conjugated secondary antibody, in 2% non-fat milk. Membranes were submerged in a chemiluminescence reagent (Millipore) for 5 min and developed. Protein bands were scanned and analyzed by NIH image J 3.0.

Statistical Analysis

Real-time RT-PCR and Western blot data was analyzed using paired t-tests. Statistically significant differences for comparison of means were established at $P<0.05$. All statistical analyses were performed using prism 4.0 for Windows.

CHAPTER 2

Hypoxia Induces the Adhesion Phenotype Markers in Human Macrophages

Introduction

Emerging evidence suggests that macrophage dysfunction is an integral component of the pathogenesis of wound healing (78-81). As a result, increased understanding of this multifunctional cell is an attractive therapeutic target to limit or prevent post-operative adhesion development. The ***objective*** of this study is to determine whether human macrophages express adhesion phenotype markers TGF-β1, type I collagen and VEGF, and whether this expression is induced by hypoxia exposure.

It is well known that macrophages accumulate in poorly perfused, hypoxic sites, where comparable damage has occurred. Inflammation is a prerequisite for healing and there is compelling evidence linking TGF-β production to wound macrophages and adhesion formation (82). As mentioned previously, TGF-β1 plays a role in chemotactically recruiting multiple cell types to the site of injury, including macrophages and fibroblasts, regulating angiogenesis, as well as extracellular matrix turnover (43-47). Therefore, macrophages may mediate multiple wound healing processes through their production in TGF-β1 alone. Additionally, macrophages are well documented to produce abundant amounts of VEGF and therefore are considered to have pro-angiogenic capabilities following tissue trauma (82-84). We ***hypothesize*** that *h*ypoxia will induce significant changes in macrophage expression of the adhesion pehonotype markers. The ***rationale*** of this study is derived from extensive data from our laboratory and others indicating that hypoxia exposure irreversibly induces the development of the adhesion phenotype in fibroblasts (1, 28-34). The ***significance*** of this aim is that macrophages represent a role not only for protecting tissues from invading pathogens and toxicants but also for initiating and promoting the development of post-operative adhesions. Understanding the role of macrophages

in the pathogenesis of post-operative adhesions may lead to the development of better and more efficacious approaches for treating and/or preventing adhesion development.

Design

To accomplish this aim, we measured the levels of select adhesion phenotype markers in macrophages following hypoxia exposure. Macrophages (1.0×10^7 cells) were cultured in 10 mL of standard media [Dulbecco's modified media supplemented with 10% fetal bovine serum (FBS) and 2% of streptomycin and penicillin] under normal ($20\%O_2$) and hypoxic ($2\% O_2$) conditions, for 24 hrs. After 24 hr incubation macrophages and media were centrifuged at 1800 RPM for 5 min. Macrophages were collected from normal and hypoxic cultures, protein and RNA extracted, cDNA synthesized, and analysis performed.

Real-time RT-PCR was utilized to measure mRNA levels for TGF-β1, type I collagen, and VEGF. This method is well established in our laboratory and all primer sets for each gene target have been validated. Additionally, western blot analysis was utilized to correlate TGF-β1 and VEGF protein with mRNA levels. Collectively, this experimental design should yield insight into the expression of the adhesion phenotype markers, by macrophages, under normal and hypoxic conditions.

Results

Macrophages manifest increased expression of the adhesion phenotype markers following exposure to hypoxia:

Total RNA isolated from human macrophages cultured under normal and hypoxic conditions were subjected to real time RT/PCR to determine whether macrophages express baseline levels of select adhesion phenotype markers including TGF-β1, type I collagen, and VEGF and whether hypoxia regulates this expression. As shown in Figures 1-3, macrophages express TGF-β1, VEGF, and type I collagen and hypoxia resulted in a significant increase in this

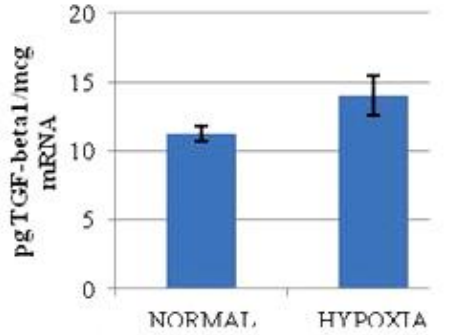

Figure 1: **Comparison of TGF-β1 mRNA levels in human macrophages before and after hypoxia exposure**. Results are representative of the mean of three independent experiments.

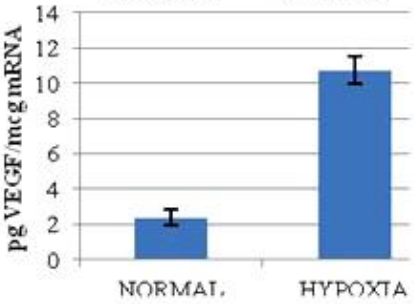

Figure 2: **Comparison of VEGF mRNA levels in human macrophages before and after hypoxia exposure**. Results are representative of the mean of three independent experiments.

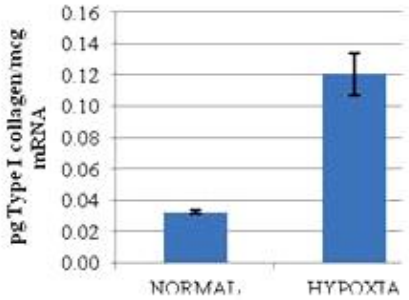

Figure 3: **Comparison of type I collagen mRNA levels in human macrophages before and after hypoxia exposure**. Results are representative of the mean of three independent experiments.

expression when compared to normoxic conditions [from 11.23 to 14.01 pg TGF-β1/μg mRNA, from 2.38 to 10.7 pg VEGF/μg mRNA, and from 0.03 to 0.12 pg type I collagen/μg mRNA, respectively; each $p<0.05$].

Additionally, as shown in Figures 4-5, hypoxia resulted in a significant increase in TGF-β1 and VEGF protein levels [from 27.7 to 39.1 and from 69.0 to 192.9, respectively; $p < 0.05$], when compared to normoxic conditions.

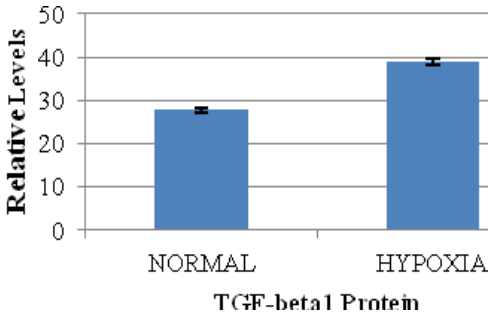

Figure 4: **Comparison of TGF-β1 protein levels in human macrophages before and after hypoxia exposure**. Results are representative of the mean of three independent experiments.

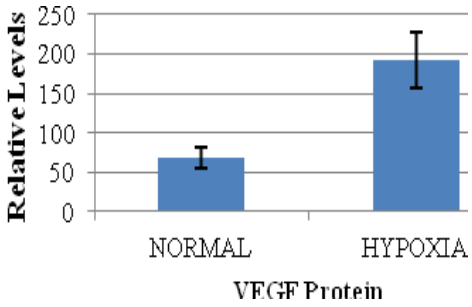

Figure 5: **Comparison of VEGF protein levels in human macrophages before and after hypoxia exposure**. Results are representative of the mean of three independent experiments.

Conclusion

The role of macrophages in post-operative adhesion development is not well defined. During the healing of the peritoneum, macrophage secretory activity is rarely the sole source for factors involved in peritoneal healing. Many other cell types within the peritoneum, including other immune cells, fibroblasts, and mesothelial cells, are also involved in this process. In this study we have shown that macrophages may contribute to the development of adhesions by expressing the adhesion phenotype markers TGF-β1, type I collagen and VEGF. Similar to normal peritoneal fibroblasts, hypoxia exposure induces a significant increase in TGF-β1, type I collagen and VEGF in human macrophages (1, 28-34). Future studies encompassing additional factors secreted in macrophages, following surgical trauma, would be a valuable tool to fully elucidating the role of macrophages in the pathogenesis of post-operative adhesions.

CHAPTER 3

Silencing TGF-β1 and HIF-1α Gene Expression Significantly Decreased the Adhesion Phenotype Markers in Macrophages

Introduction

In the previous study we have shown that macrophages express basal levels of select adhesion phenotype markers including TGF-β1, type I collagen, and VEGF. Additionally we have shown that hypoxia further induces the expression of these markers in macrophages. The ***objective*** of this aim is to determine whether TGF-β1, type I collagen, and VEGF expression is mediated by a HIF-1α and/or TGF-β1 dependent mechanism following hypoxia exposure. We ***hypothesize*** that surgical-induced hypoxia triggers a pro-inflammatory signal, leading to the activation and recruitment of resident and infiltrating macrophages to traumatized tissue, which may contribute to the development of the adhesion phenotype, a process we believe is mediated by HIF-1α and TGF-β1. The ***rationale*** of this study was derived from extensive data in our laboratory and others indicating that HIF-1α mediates multiple stages of peritoneal healing following hypoxia exposure, and that TGF-β1 regulates multiple stages of peritoneal healing. The ***significance*** of this aim is to provide insight into mechanisms involving peritoneal healing in macrophages. Understanding the nature of these mechanisms in macrophages will further highlight their role in peritoneal healing and also demonstrate specific mechanisms involved in the pathogenesis of postoperative adhesions which may lead to the development of better and more efficacious approaches for treating and/or preventing adhesions.

Design

To accomplish this aim real-time RT-PCR and small interfering RNA (siRNA) technology were utilized to assess HIF-1α, TGF-β1, type I collagen, and VEGF gene expression

in response to hypoxia in human macrophages. Macrophages (2.5 X 10^5) were seeded in a 6-well dish (Becton Dickinson, Franklin Lakes, NJ) transfected with 5 μL of Lipofectamine RNAiMAX Reagent (Invitrogen, Carlsbad, CA; catalogue no. 13778-075) complexed with 150 picomoles of a specific siRNA (HIF-1α/TGF-β1) in standard medium without antibiotics. Macrophages were then placed in a standard tissue incubator (37°C; 95% air and 5% CO_2) for 24-hrs. Following the initial incubation period macrophages were placed under normal (20% O_2) and hypoxic (2% O_2) conditions for an additional 24 hrs. Macrophages were collected from normal and hypoxic cultures, protein and RNA extracted, cDNA synthesized, and analysis performed.

Total RNA from each group was subjected to real-time RT/PCR to measure HIF-1α, TGF-β1, type I collagen, and VEGF mRNA levels. Additionally, western blot analysis was utilized to correlate HIF-1α, TGF-β1, and VEGF protein with mRNA levels. Collectively, this experimental design should yield insight into mechanisms involved in the expression of the adhesion phenotype markers, by macrophages, under normal and hypoxic conditions.

Results

Silencing HIF-1α gene expression significantly decreased TGF-β1, VEGF, and type I collagen expression in human macrophages under hypoxic conditions:

Total RNA isolated from human macrophages cultured under normal and hypoxic conditions were subjected to real time RT/PCR to determine the effects of hypoxia on HIF-1α, TGF-β1, type I collagen, and VEGF expression. As shown in Figures 6-9, hypoxia resulted in a significant increase in HIF-1α, TGF-β1, VEGF, and type I collagen expression when compared to normoxic conditions [from 0.32 to 0.42 pg HIF-1α/μg mRNA, from 11.23 to 14.01 pg TGF-

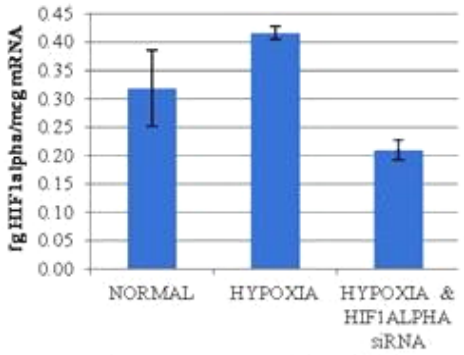

Figure 6: **Comparison of HIF-1α mRNA levels in human macrophages following hypoxia exposure with and without HIF-1α knock down**. Results are representative of the mean of three independent experiments.

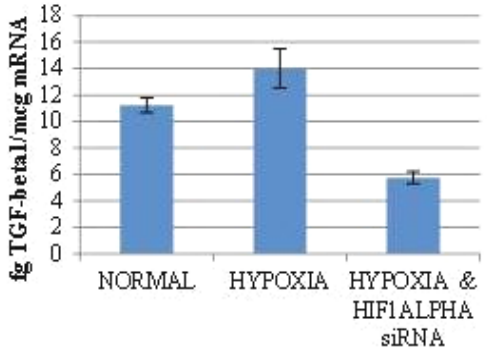

Figure 7: **Comparison of HIF-1β mRNA levels in human macrophages following hypoxia exposure with and without HIF-1β knock down**. Results are representative of the mean of three independent experiments.

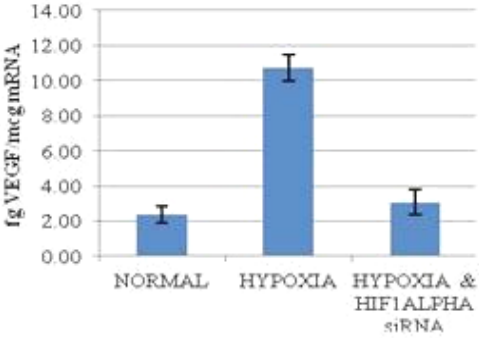

Figure 8: **Comparison of VEGF mRNA levels in human macrophages following hypoxia exposure with and without HIF-1α knock down**. Results are representative of the mean of three independent experiments.

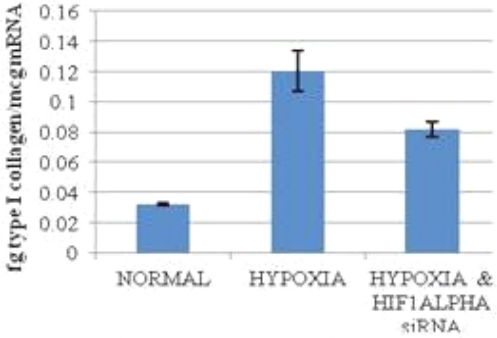

Figure 9: Comparison of type 1 collagen RNA levels in human macrophages following hypoxia exposure with and without HIF-1α knock down. Results are representative of the mean of three independent experiments.

β1/μg mRNA, from 2.38 to 10.7 pg VEGF/μg mRNA, and from 0.03 to 0.12 pg type I collagen/μg mRNA, respectively; each $p<0.05$]. In contrast, silencing HIF-1α under hypoxic conditions resulted in a significant decrease in HIF-1α, TGF-β1, type I collagen, and VEGF when compared to hypoxia alone without silencing HIF-1α as shown in Figures 6-9 [from 0.42 to 0.21 pg HIF-1α/μg mRNA, from 14.01 to 5.77 pg TGF-β1/μg mRNA, from 10.74 to 3.07 pg VEGF/μg mRNA, and from 0.12 to 0.08 pg type I collagen/μg mRNA, respectively; each $p<0.05$].

Additionally, total protein isolated from human macrophages cultured under normal and hypoxic conditions were subjected to Western blot analysis to determine the effects of hypoxia on TGF-β1 and VEGF protein levels. Hypoxia resulted in a significant increase in TGF-β1 and VEGF [from 27.7 to 39.1 and from 69.0 to 192.9, respectively; $p < 0.05$] when compared to normoxic conditions, as shown in Figures 10-11. In contrast, silencing HIF-1α gene expression resulted in a significant decrease in TGF-β1 and VEGF [from 39.07 to 23.10 and from 192.87 to 93.90, respectively; $p < 0.05$] when compared to hypoxic conditions, as shown in Figures 10-11.

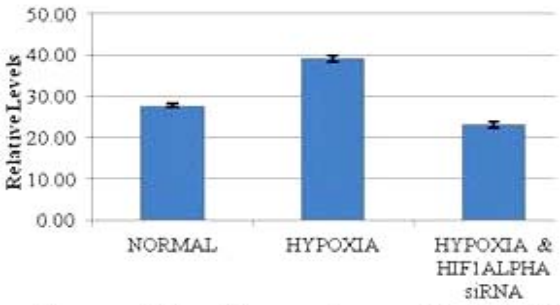

Figure 10: Comparison of TGF-1β protein levels in human macrophages following hypoxia exposure with and without HIF-1α knock down. Results are representative of the mean of three independent experiments.

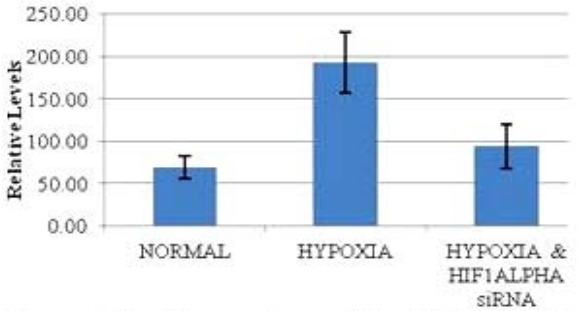

Figure 11: Comparison of VEGF levels in human macrophages following hypoxia exposure with and without HIF-1α knock down. Results are representative of the mean of three independent experiments.

Silencing TGF-β1 gene expression significantly decreased type I collagen, and VEGF levels in human macrophages under hypoxic conditions:

As shown in Figures 12-14, hypoxia resulted in a significant increase in TGF-β1, VEGF, and type I collagen expression when compared to normoxic conditions [from 47.53 to 130.30 fg TGF-β1/μg mRNA, from 1258.30 to 1655.01 fg VEGF/μg mRNA, and from 23.68 to 53.31 fg type I collagen/μg mRNA, respectively; $p<0.05$]. In contrast, hypoxia with TGF-β1 knockdown resulted in a reversal of the effects of hypoxia, with a significant decrease in TGF-β1, VEGF, and type I collagen when compared to hypoxia alone [from 130.30 to 38.23 fg TGF-β1/μg

mRNA, from 1655.01 to 1157.72 fg VEGF/µg mRNA, and from 53.31 to 15.12 fg type I collagen/µg mRNA, respectively; $p<0.05$).

Conclusion

To compensate for low oxygen conditions (hypoxia) cells activate a number of adaptive responses to stabilize O_2 levels to maintain metabolic, bioenergetic, and redox demands. HIF-1α is a major transcriptional regulator of hypoxic stress (85-86). When O_2 levels are adequate PHD modifies HIF-1α which leads to polyubiquitination by VHL (35-37). Thus, modified HIF-1α is a target for proteosomal destruction by an E3 ubiquitin ligase. Under hypoxic stress, PHD activity is diminished, thereby stabilizing HIF-1α (85–86).

Insight into mechanisms regulating HIF protein has lead to clinical applications in ischemic disease (35-38). The most prominent involve PHD inhibitors though HIF-1α activators/inhibitors are becoming increasingly popular (85). In some cases, HIF-1α activation promotes disease progression, while in others,

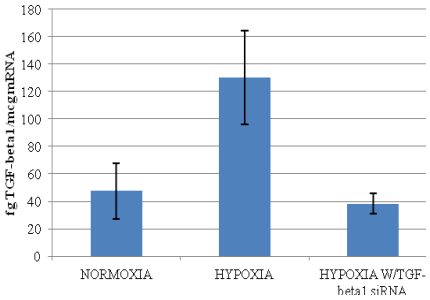

Figure 12: **Comparison of TGF-β1 mRNA levels in human macrophages following hypoxia exposure with and without TGF-β1 knock down**. Results are representative of the mean of three independent experiments.

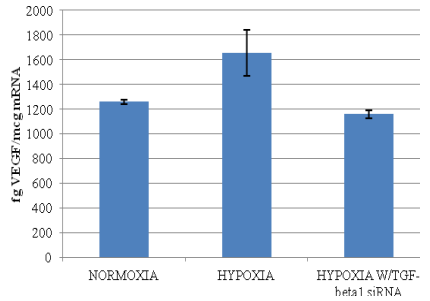

Figure 13: **Comparison of VEGF mRNA levels in human macrophages following hypoxia exposure with and without TGF-β1 knock down**. Results are representative of the mean of three independent experiments.

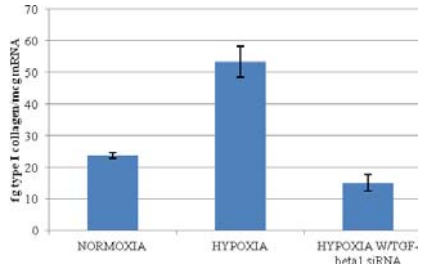

Figure 14: **Comparison of type I collagen mRNA levels in human macrophages following hypoxia exposure with and without TGF-β1 knock down**. Results are representative of the mean of three independent experiments.

HIF inhibitory responses are a part of disease recovery (85). Overall, these findings support a role for HIF-dependent therapies in preventing adhesion development.

In addition to HIF-1α, the TGF-β system regulates a plethora of biological processes that may be involved in peritoneal healing. It has been shown that alterations in TGF-β expression contribute to a broad range of pathologies, including fibrosis and adhesion development (87). The fibrotic reaction is characterized by an increased production of ECM components, such as type I collagen, as well as, proliferation, migration and accumulation of various cells (87). Due to TGF-β promiscuity the TGF-β signaling pathway has been an attractive target for therapy in a number of diseases. The therapeutic approach is dependent on the disease and may involve the inhibition/activation of the pathway. Inhibitory treatments include ligand traps, such as ligand-specific neutralizing antibodies, soluble ligand receptors, antisense-dependent silencing of ligands and chemical inhibitors that block kinase activity of TGF-β receptors (87). Conversely, increased ligand-dependent signaling may be beneficial for therapeutic purposes. For example, recombinant human bone morphogenetic proteins (BMPs) activate the bone regenerative properties of the BMP pathway (87). Additionally, several preclinical studies have assessed the efficacy of endogenous/synthetic Smad inhibitors, Smad sequestration, or targeting degradation in several diseases *in vitro* and *in vivo* (87). A comprehensive understanding of TGF-β signaling and regulation in additional cell types, including macrophages, will further provide targets for treatment of diseases caused by malfunctioning of the TGF-β signaling pathway.

In the past, increased understanding of the cellular and molecular events promoting adhesion formation has led to the identification of many biologically active molecules with the potential of regulating events that are central to normal peritoneal and adhesion formation (4). In this study we have shown that hypoxia exposure resulted in a significant increase in HIF-1α,

TGF-β1, type I collagen, and VEGF expression in human macrophages. Silencing HIF-1α gene expression under hypoxic conditions resulted in a significant decrease in TGF-β1, type I collagen, and VEGF levels. Additionally, silencing TGF-β1 gene expression under hypoxic conditions resulted in a significant decrease in both type I collagen, and VEGF levels. Collectively, results from this study has shown that HIF-1α and TGF–β1 down-regulation will indeed minimize the expression of TGF-β1, type I collagen, and VEGF which provides additional insight into mechanisms capable of reducing adhesions *in vivo*. This study validates HIF-1α, as well as TGF–β1, as a potential target in post-operative adhesion prevention and directs us towards evaluating similar mechanisms involved in *in vivo* animal models.

CHAPTER 4

Macrophages Induce the Adhesion Phenotype in Normal Peritoneal Fibroblasts

Introduction

The inflammatory response activates the innate immune system which stimulates the recruitment of macrophages from circulation (82-84). Macrophages influence peritoneal healing by regulating specific factors involved in repair mechanisms. Hypoxia is known to induce marked changes in these processes by increasing the secretory activity of macrophages, which elicits angiogenesis, ECM turnover, inflammation, and phagocytosis (78-79, 82-84).

In addition to macrophages, the role of fibroblasts has also been illustrated in wound healing. More specifically, fibroblasts secrete ECM proteins resulting in wound contraction and scar formation (88-89). We have shown that hypoxia alters the phenotype of fibroblasts by altering an array of wound healing processes including a reduction in plasminogen activator activity (lower tPA and higher PAI-I), increased angiogenesis (VEGF), increased extracellular matrix deposition (higher type I collagen and fibronectin), increased cytokine production (higher TGF-β1), and reduced apoptosis (higher Bcl and lower Bax), collectively referred to as the "adhesion phenotype" (1, 11-16).

Coupled with hypoxia, TGF-β1 is believed to coordinate macrophage and fibroblast role in wound healing by its ability to chemotactically recruit fibroblasts and macrophages to the site of injury, regulate angiogenesis, as well as, tissue remodeling tissue remodeling (43-47). We have shown that hypoxia increases the expression of TGF−β1 in multiple cell types which further supports the role for this cytokine in wound healing (28, 30, 48-50).

The ***objective*** of this study is to determine whether macrophages, exposed to hypoxia, stimulates primary cultures of fibroblasts to acquire the adhesion phenotype. We ***hypothesized*** that surgical-induced hypoxia triggers macrophages to release factors, more specifically TGF-β1,

with the capability of inducing the adhesion phenotype in normal peritoneal fibroblasts by modulating factors involved in wound healing. The ***rationale*** of this study was derived from extensive data in our laboratory and others indicating that macrophages express TGF-β1 and that hypoxia induces this expression. The ***significance*** of this aim will elucidate the cooperative aspects of cell-to-cell interactions, comprised of macrophage and fibroblasts, that are representative of *in vivo* microenvironments.

Design

To accomplish this aim media collected from human macrophages cultured under hypoxic conditions (2% O2) were used to treat normal peritoneal fibroblasts. Macrophages (1.0 X 10^7 cells) were cultured in 10 mL of standard media [Dulbecco's modified media supplemented with 10% fetal bovine serum (FBS) and 2% of streptomycin and penicillin] under normal (20%O_2) and hypoxic (2% O_2) conditions, for 24 hrs. After 24 hr incubation macrophages and media were centrifuged at 1800 RPM for 5 min. Media (10 mL) collected from normal and hypoxic macrophage cultures were used to culture normal peritoneal fibroblasts (1.0 x 10^7 cells) under normal oxygen (20% O_2) conditions in a standard tissue incubator for 24 hrs. Additionally, human peritoneal fibroblasts (5 X 10^6) were cultured in a 6-well dish with 0, 12.5, 25, and 50 ng TGF-β1 and then placed in a humidified tissue incubator for 24 hours.

Total RNA from each group was subjected to real-time RT/PCR to measure HIF-TGF-β1, type I collagen, and VEGF mRNA levels. Additionally, western blot analysis was utilized to correlate TGF-β1, type I collagen, and VEGF protein with mRNA levels. Collectively, this experimental design should yield insight into the role of fibroblasts and macrophages in peritoneal wound healing, thus emphasizing the synergistic interaction of different cell types involved in adhesion development.

Results

***Hypoxia treatment resulted in a significant increase in TGF-β1 expression in human macrophages*:**

As shown in Figures 1 & 4, hypoxia resulted in a significant increase in TGF-β1 mRNA and protein levels [26.8%, respectively; $p < 0.05$)], when compared to normoxic conditions.

***Hypoxia treatment resulted in a significant increase in TGF-β1, VEGF, and type I collagen mRNA levels in normal peritoneal fibroblasts*:**

As shown in Figure 15, hypoxia resulted in a significant increase in TGF-β1 [26.8%; $p < 0.05$)], VEGF [86.4%; $p < 0.05$)], and type I Collagen [35.1%; $p < 0.05$)] when compared to normoxic conditions.

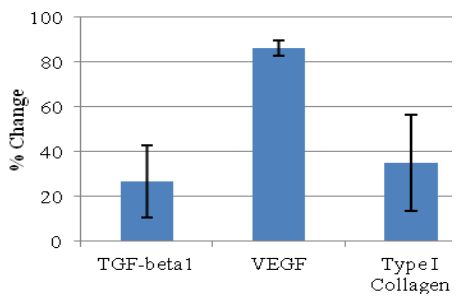

Figure 15: **Hypoxia treatment resulted in a significant increase in TGF-β1, VEGF, and type I collagen mRNA levels in human peritoneal fibroblasts**: Comparison of TGF-β1, VEGF, and type I collagen mRNA levels in normal peritoneal fibroblasts before and after hypoxia (2% O_2) exposure. Results are representative of the mean of three independent patients.

***Hypoxia treatment resulted in a significant increase in TGF-β1, VEGF, and type I collagen protein levels in normal peritoneal fibroblasts*:**

As shown in Figure 16, hypoxia resulted in an increase in TGF-β1 (28.6%), VEGF (39.0%), and type I collagen (9.8%) protein levels.

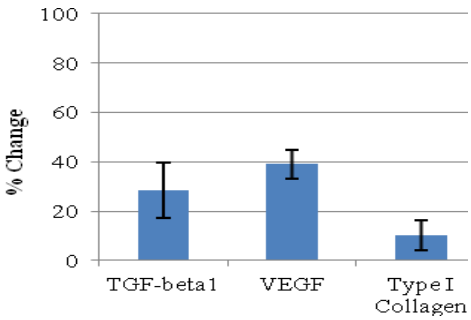

Figure 16: **Hypoxia treatment resulted in an increase in TGF-β1, VEGF, and type I collagen protein levels in human peritoneal fibroblasts**: Comparison of TGF-β1, VEGF, and type I collagen protein levels in normal peritoneal fibroblasts before and after hypoxia (2% O_2) exposure. Results are representative of the mean of three independent patients.

Macrophage secretion significantly increased TGF-β1, VEGF, type I collagen mRNA levels in normal peritoneal fibroblasts under hypoxic conditions:

As shown in Figure 17 normal peritoneal fibroblasts cultured with media collected from macrophages following hypoxia exposure resulted in a significant increase in TGF-β1 [11.2%; $p< 0.05$)], VEGF [51.2%; $p < 0.05$)], and type I Collagen [28.8%; $p < 0.05$)] mRNA levels when compared to normal peritoneal fibroblasts cultured with media collected from macrophages cultured under normal conditions.

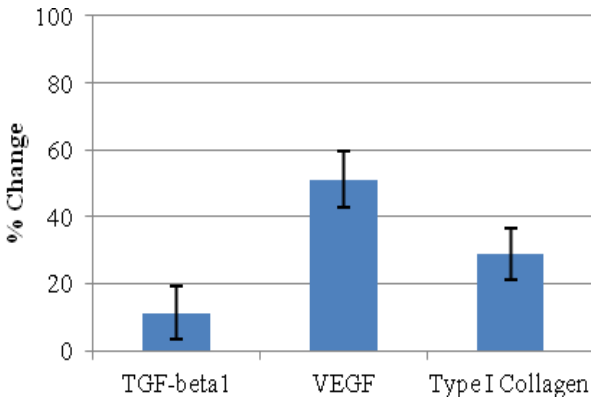

Figure 17: **Macrophage secretion, following hypoxia exposure, significantly increased TGF-β1, VEGF, type I collagen mRNA levels in human peritoneal fibroblasts**: Comparison of TGF- β1, VEGF, and type I collagen mRNA levels in normal peritoneal fibroblasts following incubation with media collected from macrophages cultured under normal and hypoxic conditions. Results are representative of the mean of three independent patients.

***Hypoxia treatment resulted in a significant increase in TGF-β1, VEGF, and type I collagen protein levels in normal peritoneal fibroblasts*:**

As shown in Figure 18, hypoxic-macrophage media resulted in an increase in TGF-β1 (47.0 %), VEGF (3.1%), and type I collagen (78.2%) protein levels.

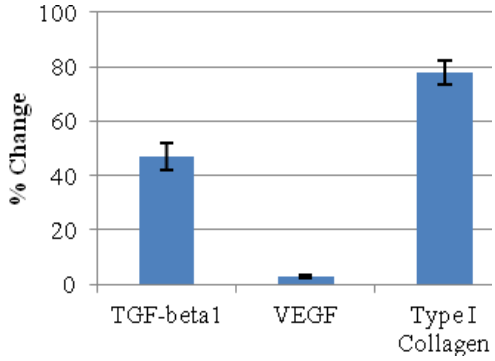

Figure 18: **Macrophage secretion, following hypoxia exposure, significantly increased TGF-β1, VEGF, type I collagen protein levels in human peritoneal fibroblasts**: Comparison of TGF-β1, VEGF, & type I collagen protein levels in normal peritoneal fibroblasts following incubation with media collected from macrophages cultured under normal and hypoxic conditions.

TGF-β1 regulation is dose-dependent:

Normal peritoneal fibroblasts exposed to varying concentrations of TGF-β1 exhibited a dose-dependent response in TGF-β1, VEGF, and type-I collagen mRNA levels (Figure 19A-C). At a low TGF-β1 concentration (12.5 ng TGF-β1/mL media), TGF-β1 [(19A) increased from 0.9 to 1.4 pg TGF–β1/μg mRNA; $p < 0.05$)], VEGF [(19B) increased from 0.6 to 1.6 pg VEGF/μg mRNA; $p<0.05$)], and type I Collagen [(19C) increased from 0.3 to 0.6 pg type I Collagen/μg mRNA; $p < 0.05$)] were significantly increased when compared to baseline TGF-β1 levels. In contrast, at higher TGF-β1 concentrations (25 and 50 ng TGF-β1/mL media), TGF-β1 [(19C) declined from 1.4 to 1.3 to 1.1 pg TGF–β1/μg mRNA; $p < 0.05$)], VEGF [(19C) declined from 1.6 to 0.9 to 0.2 pg VEGF/μg mRNA; $p < 0.05$)], and type I Collagen [(19-C) declined from 0.6 to 0.5 to 0.3 pg type I Collagen/μg mRNA; $p < 0.05$)] mRNA levels were significantly reduced when compared to peak (12.5 ng TGF-β1/mL media) TGF-β1 levels.

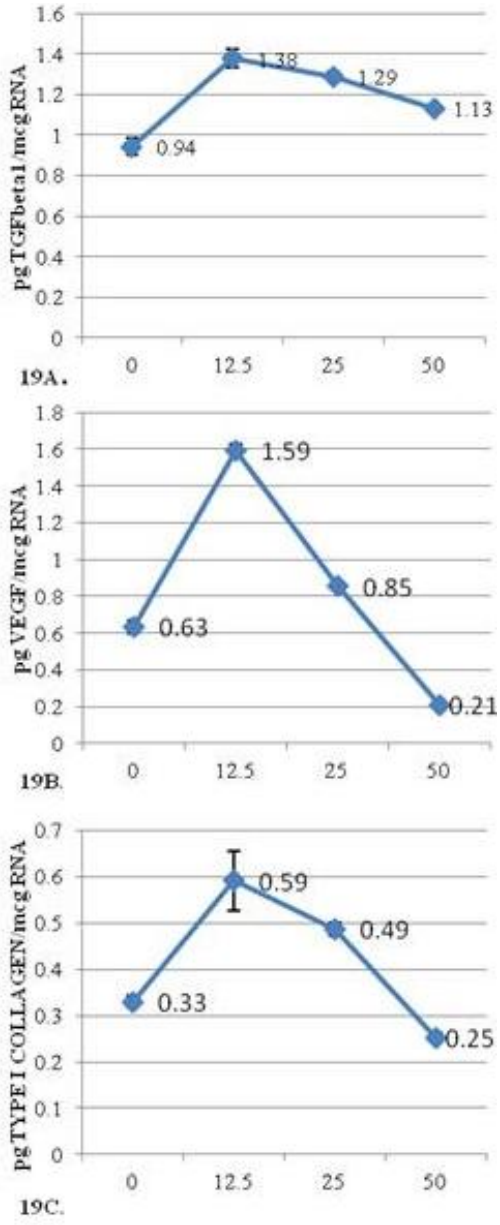

Figure 19: **TGF-β1 regulation is dose-dependent**: Comparison of TGF-β1 (19A), VEGF (19B), & type I collagen (19C) mRNA levels in human peritoneal fibroblasts in response to varying TGF-β1 concentrations (0, 12.5, 25, & 50 ng TGF-β1/mL media). Representative of the mean of three independent experiments.

Conclusion

In the present study normal peritoneal fibroblasts were cultured under normal and hypoxic conditions. In addition, media collected from macrophage cultures, with and without hypoxia exposure, was utilized to culture normal human peritoneal fibroblasts. Base-line levels for adhesion markers varied from patient to patient. Our results indicate similar trends for each experimental condition. Consistent with previous findings we have shown that TGF-β1, VEGF, and type I collagen is significantly increased in normal peritoneal fibroblasts following hypoxia exposure (1). Additionally, this study indicates that macrophage conditioned media encompass specific factors promoting TGF-β1, VEGF, and type I collagen expression in normal peritoneal fibroblasts, thus implicating macrophages as a major contributor to the development of the adhesion phenotype. In this study we suggest that hypoxia-induced TGF-β1 expression mediates the induction of the adhesion phenotype in normal peritoneal fibroblasts.

This hypothesis has been formulated on the basis of strong preliminary and published data, which supports the role of TGF-β1 in inflammation, angiogenesis, and extracellular matrix (ECM) turnover under normal conditions (43-47). Excessive TGF- exposure exaggerates these processes thus promoting the development of adhesions (43). We have shown that TGF-β1 regulates VEGF and type I collagen as well as its own expression TGF-β1 regulation occurs by a dose-dependent mechanism (19, 45, 90, 91, 92). For example, we have shown that a low concentration (12.5 ng TGF-β1/mL media) of TGF-β1 resulted in increased expression of VEGF and type I collagen, as well as, TGF β 1 itself. However, higher concentrations of TGF-β1 (25 ng and 50 ng TGF-β1/mL media) resulted in decreased expression of VEGF and type I collagen, as well as TGF-β1. These findings indicate that TGF-β1 secreted by macrophages, in response to hypoxia exposure, is partly responsible for the induction of the adhesion phenotype markers, VEGF and type I collagen, as well as its own expression in normal peritoneal fibroblasts.

Therefore, this study not only confirms the biphasic response of TGF-β1 in normal peritoneal fibroblasts but also emphasize its major role in the development of the adhesion phenotype involving both autocrine and paracrine mechanisms (91).

Collectively, results from this study support the role of fibroblasts and macrophages in peritoneal wound healing, thus emphasizing the synergistic effect of multiple cells involved in adhesion development.

CHAPTER 5

Discussion

To date, minimal insight involving the role of macrophages in post-operative adhesion development exists. This study is novel because it highlights a role for the innate immune system in the pathogenesis of peritoneal wound healing and adhesion development. We have shown that macrophages express basal levels of adhesion phenotype markers: TGF-β1, VEGF, and type I collagen. Hypoxia enhances the expression of TGF-β1, VEGF, and type I collagen through a HIF-1α and TGF-β1 dependent mechanism. Additionally, macrophages exposed to hypoxia release specific factors that are responsible for inducing the adhesion phenotype in normal peritoneal fibroblasts. More specifically, hypoxia-induced TGF-β1 expression, in macrophages, mediates this response through a dose-dependent mechanism.

The innate immune system is activated immediately following injury. Subsequent release of inflammatory mediators drives recruitment of inflammatory cells from circulation. This rapid response triggers activation and recruitment of resident and infiltrating macrophages to the site of injury. Hypoxia exaggerates the inflammatory response. Specifically, hypoxia stimulates numerous cell types, including macrophages, to produce inflammatory mediators, such as TGF-β, that are paramount to inflammation (82). HIF-1α, a major regulator of oxygen deficiency, mediates TGF-β1 expression under hypoxic conditions. Lack of TGF-β signaling substantially diminishes the inflammatory response (83). Hence, TGF-β-deficient SMAD3 knockouts recruit macrophages poorly into skin wounds (83). Likewise, we have shown that human macrophages express TGF-β1 and hypoxia enhances this expression through a HIF-1α mediated mechanism. Therefore, results from this study support the role of macrophages in wound healing partially by their ability to express TGF-β1, a major inflammatory cytokine.

Hypoxia, a result of surgical trauma, also triggers angiogenesis to re-establish oxygen availability. Neovascularization during granulation tissue formation in wounds is controlled by a variety of cytokines and growth factors, including TGF-β1 and VEGF. VEGF plays a central role in neovascularization. TGF-β1 induces VEGF expression (45). Smad3 and HIF-1α cooperate with TGF-β to induce VEGF transcription in humans (45). As mentioned previously, excessive vascularization promotes adhesion viability and therefore therapeutic applications targeting angiogenic pathways, specifically VEGF, have been explored. There is compelling evidence linking macrophages to angiogenesis (45, 82-83). More specifically, in a study utilizing macrophage-deficient mice abnormal vascularization was observed in macrophage-depleted wound models (82). Also, treatment of macrophages with anti-VEGF antibody neutralized the angiogenic activity of human wound fluids *in vitro* (82). In support of these findings, our current study reveals that macrophages express basal levels of VEGF and that hypoxia induces VEGF expression through a HIF-1α and TGF-β1-dependent mechanism. Additionally we have shown that hypoxia-induced TGF-β1 expression in macrophages enhances angiogenic pathways in normal peritoneal fibroblasts.

In addition to macrophages role in angiogenesis we have also shown that macrophages play an important role in extracellular matrix turnover, a critical process in wound healing. Type I collagen is the most abundant ECM protein. Collagen synthesis is critically dependent on the availability of molecular oxygen in tissue culture, animal, and human wound healing experiments. Thus, hypoxia promotes collagen deposition. As mentioned previously, the balance between the expression of MMPs and TIMPs is critical to normal wound healing. Hypoxia inhibits the expression of MMPs and increases TIMPs, thus decreasing matrix degradation and increasing fibrosis. Therefore, increased expression of type I collagen combined

with decreased proteolytic activity within the peritoneal cavity, under hypoxic conditions, perpetuates adhesion development. In addition to hypoxia TGF-β1 is known to increase type I collagen expression and decrease MMP expression which furthers promotes adhesion development. Similarly, our current study, utilizing macrophage cultures, supports these findings. We have shown that macrophages express basal levels of type I collagen and that hypoxia induces type I collagen expression through a HIF-1α and TGF-β1-dependent mechanism. Additionally we have shown that hypoxia-induced TGF-β1 expression in macrophages enhances collagen deposition by normal peritoneal fibroblasts.

Indeed results from our study highlight an important role of macrophages in the pathogenesis of peritoneal wound healing. We have shown that peritoneal macrophages regulate key processes involved in the pathophysiology of post-operative adhesion development including inflammation, angiogenesis, and ECM turnover through their production of TGF-β1, VEGF, and type I collagen, respectively. Additionally, we have shown that macrophages exposed to hypoxia elicit conditions favorable for adhesion development by enhancing the expression of specific adhesion phenotype markers: TGF-β1, VEGF, and type I collagen. HIF-1α and TGF-β1 mediate hypoxia effects in macrophages. Additionally, we have proven a synergy that exists between macrophages and normal peritoneal fibroblasts that promotes the development of the adhesion phenotype.

Further understanding the nature of additional factors and the relative contributions of macrophages activated by other mechanisms, such as infection and predisposing diseases, may lead to the development of better and more efficacious approaches for treating and/or preventing adhesion development. Surgical trauma, infection, and predisposing diseases, such as endometriosis, exaggerate the inflammatory response and subsequently lead to macrophage

recruitment. As we have shown, macrophage responses to hypoxia are central to these events. Relatively few studies have shown the effects of hypoxia on macrophages in the presence of co-stimuli, such as LPS and IFN-γ, which may provide further insight in macrophage activity under these conditions. Future studies in our laboratory would examine the role of these stimuli and others that are present following tissue trauma and disease including glucose depletion, accumulation of lactic acid and other metabolic byproducts, and low pH. Examination of macrophage activity under these conditions may provide a better picture. Once mechanisms are better understood it would be possible to block their activity or manipulate their involvement in peritoneal wound healing. Additional co-culture models could be explored to better understand the synergistic/antagonistic effects that exist amongst different cell populations, which is more representative of what occurs *in vivo*. Also, translating these *in vitro* studies into an animal model would be advantageous and confirm our current findings.

REFERENCES

[1] Saed, G.M. and M.P. Diamond, Molecular characterization of postoperative adhesions: the adhesion phenotype. J Am Assoc Gynecol Laparosc, 2004. 11(3): p. 307-14.

[2] The Practice Committee of the American Society of Reproductive Medicine. Pathogenesis, consequences, and control of peritoneal adhesions in gynecologic surgery. FertilSteril, 2008. 90(5 Suppl): p. S144-9.

[3] diZerga, Gere. Peritoneal Surgery. New York: Springer, 2000.

[4] Diamond, M. P., S. D. Wexner, et al. (2010). "Adhesion prevention and reduction: current status and future recommendations of a multinational interdisciplinary consensus conference." Surg Innov **17**(3): 183-188.

[5] Steege JF, Stout AL. Resolution of chronic pelvic pain after laparoscopic lysis of adhesions. Am J Obstet Gynecol. 1991;165(2):278-81; discussion 81-3.

[6] Postoperative adhesion development after operative laparoscopy: evaluation at early second-look procedures. Operative Laparoscopy Study Group. Fertil Steril. 1991;55(4):700-4.

[7] Awonuga AO, Saed GM, Diamond MP. Laparoscopy in gynecologic surgery: adhesion development, prevention, and use of adjunctive therapies. Clin Obstet Gynecol. 2009;52(3):412-22.

[8] Yager, D.R., R.A. Kulina, and L.A. Gilman, Wound fluids: a window into the wound environment?Int J Low Extrem Wounds, 2007. 6(4): p. 262-72.

[9] Bittinger F, Schepp C, Brochhausen C, et al: Remodeling of peritoneal-like structures by mesothelial cells: Its role in peritoneal healing. J Surg Res 1999, 82:28-33

[10] diZerga GS: Biochemical events in peritoneal tissue repair. Eur J Surg Suppl 1997, 577:10-6

[11] Saed, G.M., W. Zhang, and M.P. Diamond, Molecular characterization of fibroblasts isolated from human peritoneum and adhesions. FertilSteril, 2001. 75(4): p. 763-8.

[12] Jiang ZL, Zhu X, Diamond MP, Abu-Soud HM, Saed GM. Nitric oxide synthase isoforms expression in fibroblasts isolated from human normal peritoneum and adhesion tissues. Fertility and sterility. 2008.

[13] Saed GM, Munkarah AR, Diamond MP. Cyclooxygenase-2 is expressed in human fibroblasts isolated from intraperitoneal adhesions but not from normal peritoneal tissues. Fertil Steril. 2003;79(6):1404-8.

[14] Saed GM, Diamond MP. Differential expression of α smooth muscle cell actin in human fibroblasts isolated from intraperitoneal adhesions and normal peritoneal tissues. Fertil Steril. 2004;82 Suppl 3:1188-92.

[15] Alpay Z, Saed GM, Diamond MP. Postoperative adhesions: from formation to prevention. Semin Reprod Med. 2008;26(4):313-21.

[16] Rout UK, Saed GM, Diamond MP. Expression pattern and regulation of genes differ between fibroblasts of adhesion and normal human peritoneum. Reprod Biol Endocrinol. 2005;3(1):1.

[17] Saed, G.M. and M.P. Diamond, Hypoxia-induced irreversible up-regulation of type I collagen and transforming growth factor-β1 in human peritoneal fibroblasts.FertilSteril, 2002. 78(1): p. 144-7.

[18] Saed, G.M. and M.P. Diamond, Modulation of the expression of tissue plasminogen activator and its inhibitor by hypoxia in human peritoneal and adhesion fibroblasts.FertilSteril, 2003. 79(1): p. 164-8.

[19] Saed, G.M., Transforming growth factors β1, β2 and β3 and their receptors are differentially expressed in human peritoneal fibroblasts in response to hypoxia.

American Journal of Reproductive Immunology, 2002. 48(6): p. 387-393.

[20] Saed GM, Jiang Z, Fletcher NM, Diamond MP. Modulation of the BCL-2/BAX ratio by interferon-gamma and hypoxia in human peritoneal and adhesion fibroblasts. Fertility and sterility. 2007.

[21] Saed GM, Diamond MP. Effects of interferon-gamma reverse hypoxia-stimulated extracellular matrix expression in human peritoneal and adhesion fibroblasts. Fertil Steril. 2006;85 Suppl 1:1300-5.

[22] Saed GM, Munkarah AR, Abu-Soud HM, Diamond MP. Hypoxia upregulates cyclooxygenase-2 and prostaglandin E(2) levels in human peritoneal fibroblasts. Fertil Steril. 2005;83 Suppl 1:1216-9.

[23] Jiang ZL, Fletcher NM, Diamond MP, Abu-Soud HM, Saed GM. Hypoxia regulates iNOS expression in human normal peritoneal and adhesion fibroblasts through nuclear factor kappa B activation mechanism. Fertility and sterility. 2009;91(2):616-21.

[24] Fletcher NM, Jiang ZL, Diamond MP, Abu-Soud HM, Saed GM. Hypoxia-generated superoxide induces the development of the adhesion phenotype. Free Radic Biol Med. 2008;45(4):530-6. PMCID: 2574925.

[25] Inoue M, Sato EF, Nishikawa M, Park AM, Kira Y, Imada I, et al. Mitochondrial generation of reactive oxygen species and its role in aerobic life. Curr Med Chem. 2003;10(23):2495-505.

[26] Saed GM, Jiang ZL, Fletcher NM, Al Arab A, Diamond MP, Abu-Soud HM. Exposure to polychlorinated biphenyls enhances lipid peroxidation in human normal peritoneal and adhesion fibroblasts: a potential role for myeloperoxidase. Free Radic Biol Med. 2010;48(6):845-50. PMCID: 2834263.

[27] Reed KL, Heydrick SJ, Aarons CB, Prushik S, Gower AC, Stucchi AF, et al. A

neurokinin-1 receptor antagonist that reduces intra-abdominal adhesion formation decreases oxidative stress in the peritoneum. Am J Physiol Gastrointest Liver Physiol. 2007;293(3):G544-51.

[28] Pelicano H, Carney D, Huang P. ROS stress in cancer cells and therapeutic implications. Drug Resist Updat. 2004;7(2):97-110.

[29] Ara C, Kirimlioglu H, Karabulut AB, Coban S, Hascalik S, Celik O, et al. Protective effect of melatonin against oxidative stress on adhesion formation in the rat cecum and uterine horn model. Life Sci. 2005;77(12):1341-50.

[30] Souza A, Rogers M, Wang C, Yuen P, Ng P. Comparison of peritoneal oxidative stress during laparoscopy and laparotomy. J Am Assoc Gynecol Laparosc. 2003;10:65-74.

[31] Taskin O, Buhur A, Birincioglu M, Burak F, Atmaca R, Yilmaz I, et al. The effects of duration of CO2 insufflation and irrigation on peritoneal microcirculation assessed by free radical scavengers and total glutathion levels during operative laparoscopy. J Am Assoc Gynecol Laparosc. 1998;5(2):129-33.

[32] Taskin O, Sadik S, Onoglu A, Gokdeniz R, Yilmaz I, Burak F, et al. Adhesion formation after microlaparoscopic and laparoscopic ovarian coagulation for polycystic ovary disease. J Am Assoc Gynecol Laparosc. 1999;6(2):159-63.

[33] Gibellini L, Pinti M, Nasi M, De Biasi S, Roat E, Bertoncelli L, et al. Interfering with ROS Metabolism in Cancer Cells: The Potential Role of Quercetin. Cancers. 2010(2).

[34] Binda MM, Molinas CR, Koninckx PR. Reactive oxygen species and adhesion formation: clinical implications in adhesion prevention. Hum Reprod. 2003;18(12):2503-7.

[35] Choi, K.S., et al., Hypoxia-induced angiogenesis during carcinogenesis. J Biochem Mol Biol, 2003. 36(1): p. 120-7.

[36] Salceda, S., Beck, I., Srinivas, V., and Caro, J. (1997) Kidney Int. 51, 556–559

[37] Wang, G. L., Jiang, B.-H., and Semenza, G. L. (1995) Biochem. Biophys. Res.Commun. 212, 550–556

[38] Chandel, N. S., D. S. McClintock, et al. (2000). "Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1α during hypoxia: a mechanism of O_2 sensing." J Biol Chem 275(33): 25130-25138.

[39] Metzen E, Zhou J, Jelkmann W, Fandrey J, Brune B. Nitric oxide impairs normoxic degradation of HIF-1α by inhibition of prolyl hydroxylases. Mol Biol Cell 2003;14:3470–81.

[40] Bell EL, Emerling BM, Chandel NS. Mitochondrial regulation of oxygen sensing. Mitochondrion 2005;5:322–32.

[41] Wallace DC. Mitochondria and cancer: Warburg addressed. Cold Spring Harb Symp Quant Biol 2005;70:363–74.

[42] Fruehauf, J. P. and F. L. Meyskens, Jr. (2007). "Reactive oxygen species: a breath of life or death?" Clin Cancer Res 13(3): 789-794.

[43] Cutroneo, K. R. (2007). "TGF-β-induced fibrosis and SMAD signaling: oligo decoys as natural therapeutics for inhibition of tissue fibrosis and scarring." Wound Repair Regen 15 Suppl 1: S54-60.

[44] Prud'homme, G. J. (2007). "Pathobiology of transforming growth factor β in cancer, fibrosis and immunologic disease, and therapeutic considerations." Lab Invest 87(11): 1077-1091.

[45] Jeon, S. H., B. C. Chae, et al. (2007). "Mechanisms underlying TGF-β1-induced expression of VEGF and Flk-1 in mouse macrophages and their implications for angiogenesis." J Leukoc Biol 81(2): 557-566.

[46] Freeman, M. L., G. M. Saed, et al. (2003). "Expression of transforming growth factor β

isoform mRNA in injured peritoneum that healed with adhesions and without adhesions and in uninjured peritoneum." Fertil Steril 80 Suppl 2: 708-713.

[47] Yoshinaga, K., H. Obata, et al. (2008). "Perturbation of transforming growth factor (TGF)-β1 association with latent TGF-β binding protein yields inflammation and tumors." Proc Natl Acad Sci U S A 105(48): 18758-18763.

[48] White, J. C., Z. L. Jiang, et al. (2011). "Macrophages induce the adhesion phenotype in normal peritoneal fibroblasts." Fertil Steril 96(3): 758-763 e753.

[49] Jiang, Y., A. Dai, et al. (2007). "Hypoxia induces transforming growth factor-β1 gene expression in the pulmonary artery of rats via hypoxia-inducible factor-1α." Acta Biochim Biophys Sin (Shanghai) 39(1): 73-80.

[50] Oberringer, M., C. Meins, et al. (2008). "*In vitro* wounding: effects of hypoxia and transforming growth factor β1 on proliferation, migration and myofibroblastic differentiation in an endothelial cell-fibroblast co-culture model." J Mol Histol 39(1):37-47.

[51] Moore, L. D., T. Isayeva, et al. (2008). "Silencing of transforming growth factor-β1 in situ by RNA interference for breast cancer: implications for proliferation and migration in vitro and metastasis *in vivo*." Clin Cancer Res 14(15): 4961-4970.

[52] McMahon, S., M. Charbonneau, et al. (2006). "Transforming growth factor β1 induces hypoxia-inducible factor-1 stabilization through selective inhibition of PHD2 expression." J Biol Chem 281(34): 24171-24181.

[53] Santibanez, J. F., M. Quintanilla, et al. (2011). "TGF-β/TGF-β receptor system and its role in physiological and pathological conditions." Clin Sci (Lond) 121(6): 233-251.

[54] Verrecchia, F. and A. Mauviel (2002). "Transforming growth factor-β signaling through the Smad pathway: role in extracellular matrix gene expression and regulation." J Invest

Dermatol 118(2): 211-215.

[55] Vilar, J. M., R. Jansen, et al. (2006). "Signal processing in the TGF-β superfamily ligand-receptor network." PLoS Comput Biol 2(1): e3.

[56] Trompezinski, S., I. Pernet, et al. (2000). "Transforming growth factor-β1 and ultraviolet A1 radiation increase production of vascular endothelial growth factor but not endothelin-1 in human dermal fibroblasts." Br J Dermatol 143(3): 539-545.

[57] Hayashida, T., M. Decaestecker, et al. (2003). "Cross-talk between ERK MAP kinase and Smad signaling pathways enhances TGF-β-dependent responses in human mesangial cells." FASEB J 17(11): 1576-1578.

[58] Hoeben, A., B. Landuyt, et al. (2004). "Vascular endothelial growth factor and angiogenesis." Pharmacol Rev 56(4): 549-580.

[59] Cahill, R. A., J. H. Wang, et al. (2006). "Mast cells facilitate local VEGF release as an early event in the pathogenesis of postoperative peritoneal adhesions." Surgery 140(1): 108-112.

[60] Rout, U. K., K. Oommen, et al. (2000). "Altered expressions of VEGF mRNA splice variants during progression of uterine-peritoneal adhesions in the rat." Am J Reprod Immunol 43(5): 299-304.

[61] Giatromanolaki, A., M. I. Koukourakis, et al. (2006). "Phosphorylated KDR expression in endometrial cancer cells relates to HIF1α/VEGF pathway and unfavourable prognosis." Mod Pathol 19(5): 701-707.

[62] Kafousi, M., T. Vrekoussis, et al. (2011). "Immunohistochemical Study of the Angiogenetic Network of VEGF, HIF1α, VEGFR-2 and Endothelial Nitric Oxide Synthase (eNOS) in Human Breast Cancer." Pathol Oncol Res.

[63] Lund, E. L., A. Hog, et al. (2004). "Differential regulation of VEGF, HIF1α and

angiopoietin-1, -2 and -4 by hypoxia and ionizing radiation in human glioblastoma." Int J Cancer 108(6): 833-838.

[64] Christiaansen, C. E., Y. Sun, et al. (2011). "Alterations in expression of HIF-1α, HIF-2α, and VEGF by idiopathic overactive bladder urothelial cells during stretch suggest role for hypoxia." Urology 77(5): 1266 e1267-1211.

[65] Lee, B. W., M. Lee, et al. (2011). "Effect of hypoxia-inducible VEGF gene expression on revascularization and graft function in mouse islet transplantation." Transpl Int 24(3): 307-314.

[66] Oladipupo, S., S. Hu, et al. (2011). "VEGF is essential for hypoxia-inducible factor-mediated neovascularization but dispensable for endothelial sprouting." Proc Natl Acad Sci U S A 108(32): 13264-13269.

[67] Tabernero, J. (2007). "The role of VEGF and EGFR inhibition: implications for combining anti-VEGF and anti-EGFR agents." Mol Cancer Res 5(3): 203-220.

[68] Basbug, M., N. Bulbuller, et al. (2011). "The effect of antivascular endothelial growth factor on the development of adhesion formation in laparotomized rats: experimental study." Gastroenterol Res Pract 2011: 578691.

[69] Henze, A. T. and T. Acker (2010). "Feedback regulators of hypoxia-inducible factors and their role in cancer biology." Cell Cycle 9(14): 2749-2763.

[70] Gago, L. A., G. Saed, et al. (2006). "Effect of oxidized regenerated cellulose (Interceed) on the expression of tissue plasminogen activator and plasminogen activator inhibitor-1 in human peritoneal fibroblasts and mesothelial cells." Fertil Steril 86(4 Suppl):1223-1227.

[71] Li, W. Y., S. S. Chong, et al. (2003). "Plasminogen activator/plasmin system: a major player in wound healing?" Wound Repair Regen 11(4): 239-247.

[72] Brokelman, W. J., L. Holmdahl, et al. (2009). "Decreased peritoneal tissue plasminogen activator during prolonged laparoscopic surgery." J Surg Res 151(1): 89-93.

[73] Chegini, N., Y. Zhao, et al. (2002). "Differential expression of matrix metalloproteinase and tissue inhibitor of MMP in serosal tissue of intraperitoneal organs and adhesions." BJOG 109(9): 1041-1049.

[74] Diamond, M. P., M. Kruger, et al. (2004). "Effect of Tisseel on expression of tissue plasminogen activator and plasminogen activator inhibitor-1." Fertil Steril 81(6): 1657-1664.

[75] Saed, G. M., M. Kruger, et al. (2004). "Expression of transforming growth factor-β and extracellular matrix by human peritoneal mesothelial cells and by fibroblasts from normal peritoneum and adhesions: effect of Tisseel." Wound Repair Regen 12(5): 557-564.

[76] Chegini, N., K. Kotseos, et al. (2001). "Matrix metalloproteinase (MMP-1) and tissue inhibitor of MMP in peritoneal fluids and sera and correlation with peritoneal adhesions." Fertil Steril 76(6): 1207-1211.

[77] Imai, A. and N. Suzuki (2010). "Topical non-barrier agents for postoperative adhesion prevention in animal models." Eur J Obstet Gynecol Reprod Biol 149(2): 131-135.

[78] Lewis, C. and C. Murdoch (2005). "Macrophage responses to hypoxia: implications for tumor progression and anti-cancer therapies." Am J Pathol 167(3): 627-635.

[79] Lewis, J. S., J. A. Lee, et al. (1999). "Macrophage responses to hypoxia: relevance to disease mechanisms." J Leukoc Biol 66(6): 889-900.

[80] Senturk, L. M., E. Seli, et al. (1999). "Monocyte chemotactic protein-1 expression in human corpus luteum." Mol Hum Reprod 5(8): 697-702.

[81] Bosco, M. C., M. Puppo, et al. (2008). "Monocytes and dendritic cells in a hypoxic environment: Spotlights on chemotaxis and migration." Immunobiology 213(9-10): 733-

749.

[82] Brancato, S. K. and J. E. Albina (2011). "Wound macrophages as key regulators of repair: origin, phenotype, and function." Am J Pathol 178(1): 19-25.

[83] Koh, T. J. and L. A. DiPietro (2011). "Inflammation and wound healing: the role of the macrophage." Expert Rev Mol Med 13:e23.

[84] Laskin, D. L., V. R. Sunil, et al. (2011). "Macrophages and tissue injury: agents of defense or destruction?" Annu Rev Pharmacol Toxicol 51: 267-288.

[85] Majmundar, A. J., W. J. Wong, et al. (2010). "Hypoxia-inducible factors and the response to hypoxic stress." Mol Cell 40(2):294-309.

[86] Bilton, R. L. and G. W. Booker (2003). "The subtle side to hypoxia inducible factor (HIFα) regulation." Eur J Biochem 270(5):791-798.

[87] Santibanez, J. F., M. Quintanilla, et al. (2011). "TGF-β/TGF-β receptor system and its role in physiological and pathological conditions." Clin Sci (Lond) 121(6):233-251.

[88] Zeng, Q. and W. Chen (2010). "The functional behavior of a macrophage/fibroblast co-culture model derived from normal and diabetic mice with a marine gelatin-oxidized alginate hydrogel." Biomaterials 31(22):5772-5781.

[89] Steinhauser, M. L., S. L. Kunkel, et al. (1998). "Macrophage/fibroblast coculture induces macrophage inflammatory protein-1α production mediated by intercellular adhesion molecule-1 and oxygen radicals." J Leukoc Biol 64(5):636-641.

[90] Liu X, Li P, Liu P, Xiong R, Zhang E, Chen X, Gu D, et al., The essential role for c-Ski in mediating TGF-β1-induced bi-directional effects on skin fibroblast proliferation through a feedback loop. Biochem J, 2008. 409(1): p. 289-97.

[91] Ghosh, A.K., Factors involved in the regulation of type I collagen gene expression: implication in fibrosis. Exp Biol Med (Maywood), 2002. 227(5): p. 301-14.

[92] Chegini, N., “TGF-β system: the principal profibrotic mediator of peritoneal adhesion formation.” Semin Reprod Med, 2008. 26(4): 298-312.

[93] Kaluz, S., M. Kaluzova, et al. (2008). "Regulation of gene expression by hypoxia: integration of the HIF-transduced hypoxic signal at the hypoxia-responsive element." Clin Chim Acta 395(1-2): 6-13.

[94] Itoh, S., F. Itoh, et al. (2000). "Signaling of transforming growth factor-β family members through Smad proteins." Eur J Biochem 267(24): 6954-6967.

ABSTRACT

THE POTENIAL ROLE OF INNATE IMMUNITY IN THE PATHOGENESIS OF POST-OPERATIVE ADHESIONS

by

JENNELL WHITE

December 2011

Advisor: Ghassan Saed

Major: Physiology

Degree: Doctor of Philosophy

Post-operative adhesion development occurs in the vast majority of patients following abdominal surgery and is a natural occurrence of peritoneal-wound healing. These fibrous bands may form within the first 5-7 days post-surgery and have the ability to cause a distortion in the normal anatomical positioning of abdominal organs. Consequently, adhesions are major contributors to small bowel obstruction, infertility, and severe pelvic and abdominal pain. Physiological processes responsible for adhesion formation remain obscure though it is believed to involve cell migration, proliferation, and differentiation of several cell types including mesothelial cells, fibroblasts, and inflammatory and immune cells. Substances released from these cells regulate fibrinolytic activity, tissue remodeling, angiogenesis, and extracellular matrix turnover, processes that are known to be central to the development of adhesions. Our ***long-term goal*** is to prevent or selectively limit the development of post-operative adhesions. *The* ***objective of this study*** is to determine the role that macrophages play in the development of post-operative adhesions. Our ***central hypothesis*** states that surgical-induced hypoxia triggers a pro-inflammatory signal, leading to the activation and recruitment of resident and infiltrating macrophages, which contribute to the development of the adhesion phenotype. This hypothesis

has been formulated on the basis of strong preliminary and published data, which suggest that hypoxia plays a major role in the development of the adhesion phenotype. Indeed, we have shown that exposure of normal peritoneal fibroblasts to hypoxia, irreversibly, induces the adhesion phenotype. This phenotype is characterized by a reduction in plasminogen activator activity, increased extracellular matrix deposition, increased cytokine production, and reduced apoptosis. In this study we have shown that macrophages express basal levels of adhesion phenotype markers: TGF-β1, VEGF, and type I collagen. Hypoxia enhances the expression of TGF-β1, VEGF, and type I collagen through a HIF-1α and TGF-β1 dependent mechanism. Additionally, macrophages exposed to hypoxia release specific factors that are responsible for inducing the adhesion phenotype in normal peritoneal fibroblasts. More specifically, hypoxia-induced TGF-β1 expression in macrophages mediates this response through a dose-dependent mechanism. Collectively, results from this study highlight a role for the innate immune system in the pathogenesis of peritoneal wound healing and adhesion development.

AUTOBIOGRAPHICAL STATEMENT

JENNELL WHITE

EDUCATION

Ph.D.(RPS Conc.):	Wayne State University, Detroit, MI (8/06-Present) Degree: Physiology
M.Sc.:	Wayne State University, Detroit, MI (2003-2005)
B.Sc.:	Xavier University of Louisiana, New Orleans, LA (1999-2003)

EXPERIENCE

08/06-current	Graduate Research Assistant, OB/GYN, Wayne State University, Detroit, MI
01/06-current	Medical Technologist, Microbiology, St. John Hospital, Detroit, MI
06/01-12/05	Lab Technician, Microbiology, St. John Hospital, Detroit, MI
08/04-12/04	Graduate Research, Microbiology, Wayne State University, Detroit, MI
08/02-05/03	Undergraduate Research, Biochemistry, Xavier University, New Orleans, LA
06/00-08/00	Research Assistant, Biochemistry Karmanos Cancer Institute, Wayne State University, Detroit, MI
06/98-08/98	Research Assistant, Cancer Biology, Karmanos Cancer Institute, Wayne State University, Detroit, MI

FUNDING

- NIH 5 R25 GM58905-08 IMSD Prog
- NIGMS 2 R25 GM58905-09 Graduat
- NIH 3 R01 GM069941-02S1 J. Whi
- NIGMS 5 R25 GM58905-11 Graduat
- Kamran S Moghissi Md End Chair
- Robert Sokol, OB/GYN, ICR

PUBLICATIONS

1. **White, J.C**., Jiang, Z.L. Diamond, M.P., Saed, G.M. (2011). "Macrophages induce the adhesion phenotype in normal peritoneal fibroblasts." Fertil Steril. (in press)

ABSTRACTS

1. "Hypoxia Induces the Expression of Transforming Growth Factor β1 in Human Macrophages through a HIF-1α dependent mechanism." Graduate Student Research Day, Detroit, MI, August, 2008.
2. "Hypoxia Induces the Expression of Transforming Growth Factor β1 in Human Macrophages through a HIF-1α dependent mechanism." The American Society for Reproductive Medicine**,** Atlanta, GA, October 2009.
3. "TGF-β1 Inhibits the Adhesion Phenotype Under Hypoxic Conditions: A Negative Feedback Mechanism." Society for Gynecologic Investigation, Orlando, FL, March, 2010.