The role of red blood cells in wound healing

by

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Abstract

Wound healing is a complex & intricate process that involves the coordinated efforts of an enormous number of unique tissues & cell lineages. Regardless of the cause, defects in this well-orchestrated process often lead to dramatic morbidity and treatments that are among the most costly, from an economic perspective. In an effort to understand the wound healing mechanisms and to establish more effective treatments, the roles of many underlying factors and cell types have been extensively studied. In spite of their ubiquitous presence and involvement in coagulation, thrombosis, and inflammation, the role(s) of red blood cells (RBC) in wound healing remains to be elucidated - RBCs are generally thought of as inert bystanders in wound healing.

Recent evidence regarding RBCs novel secondary functions and their ability to actively participate in dynamic biological processes such as inflammation, led us to investigate their role in wound healing. In order to explore RBCs involvement in wound healing, three specific objectives were successfully accomplished in this research project: (1) utilizing Western blot and proteomic analyses the presence of five erythrocytic 14-3-3 isoforms were confirmed, (2) it was established that treatment of dermal fibroblasts with RBC lysate for 24 hours results in significant (p<0.01) upregulation of matrix metalloproteinase (MMP)-1, -2, -3, fibronectin and down regulation of type-I collagen, and (3) utilizing specific inhibitors for three mitogen activated protein kinase (MAPK)
pathways, the extracellular regulated protein kinase 1/2 (ERK 1/2) pathway was found to be involved in the RBC mediated upregulation of MMP-1 in dermal fibroblasts.

Collectively, this project’s findings, demonstrate that RBCs are equipped with some form of anti-fibrogenic factor which enables them to actively participate in the dynamic process of ECM modulation, through the activation of ERK 1/2 pathway. Such observations, could potentially pave the way for designing novel treatments for fibrotic disorders such as hypertrophic scars and keloids, which are frequently caused by thermal injuries, surgical incisions and other deep-dermal injuries.
Preface

Dr. Aziz Ghahary was the principal investigator of the research project, in this thesis. In addition, he supervised all experimental designs and analysis and critically reviewed all data included in this thesis. Amir Akbari has been responsible for the identification and design, performing the research, data analysis, and manuscript preparation for all the work described in this thesis with the exception of:

Chapter 3: Dr. Kilani and Amir Akbari equally contributed to the acquisition of data presented in this manuscript, as well as its preparation. The work described in this thesis has been conducted with the approval of the University of British Columbia Biohazards Committee under the certificate number H05-0103.
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<table>
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<th>Full Form</th>
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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BFU-E</td>
<td>Burst Forming Unit-Erythroid</td>
</tr>
<tr>
<td>BM</td>
<td>Bone Marrow</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CFU-E</td>
<td>Colony Forming Unit-Erythroid</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediamine Tetraacetic Acid</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>ERK 1/2</td>
<td>Extracellular Activated Protein Kinase 1/2</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like Growth Factor</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>KGF</td>
<td>Keratinocyte Growth Factor</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte Chemoattractant Protein-1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered-Saline</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cell</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
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<tr>
<td>TIMP</td>
<td>Tissue Inhibitor of Matrix Metalloproteinase</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand Factor</td>
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Dedication

I dedicate this work to my brothers Ashkan & Ardalan; sister & brother-in-law Elaheh & Victor; parents Soussan & Majid; and to my dear friend Amy who meticulously assisted me in editing this thesis.
Chapter 1. Introduction

History of wound healing research

From the early Egyptians who repaired wounds utilizing primitive suture materials, made with “insect claws”, to Greeks who utilized natural remedies, such as wine and honey, to suppress infections, every generation has brought forth innovations for progress in the field of wound healing. The evolution of wound healing as a science has been fuelled by great minds such as John Hunter who described angiogenesis, and Claude Bernard who pioneered basic laboratory methods for clinical manipulation and advancements in wound healing. It was through the work of such people that wound healing studies evolved in cell culture and animal models.

In the 1940s, the devastating casualties and injuries of World War I inspired scientists like Alexis Carrel to revolutionize wound healing research by conducting in vitro studies as well as the first ever microsurgery. Carrel’s efforts not only resulted in the prestigious Nobel prize, but more importantly inspired brilliant future leaders whose efforts have resulted in an exponential growth in novel wound healing discoveries and extraordinary laboratory tools, such as electron microscopy, Western blot, Northern blot, PCR, and gene arrays.

Perhaps one of the most important lessons that could be learned from the evolution of wound healing as a science is that simple observations can potentially alter the course
of medical history for the better and failure to objectively assess new, unproven, ideas could impede progress.

**Biology of wound healing**

As a prominent wound healing researcher, Dr. Cohen once said, “To treat a clinical wound today without having a basic understanding of the biological principles of wound repair is like trying to sail across the ocean without a compass.” Cutaneous wound healing involves the coordinated interaction of many unique tissues and cell types such as fibroblasts, keratinocytes, blood cells, cytokines, growth factors, extracellular matrix (ECM), and several types of parenchymal cells. It consists of three dynamic phases - inflammation, proliferation, and remodeling. The following sections describe each stage of wound healing in detail.

**Inflammation**

The first step in the inflammatory phase is hemostatis, the cessation of bleeding or hemorrhaging, which initiates within moments following injury. A blood clot consisting of mainly red blood cells (RBC) and aggregates of platelets embedded in a mesh of cross-linked fibrin fibers, forms the hemostatic plug (Singer and Clark, 1999). This process requires the interaction of platelet membrane receptors with adhesive molecules, von Willebrand factor (vWF), and fibrinogen (Hawiger, 1987).
In addition to protecting blood vessels and reducing bleeding, the hemostatic plug serves as a provisional ECM for cell migration and infiltration (Heinlin et al., 2010). Additionally, the clot also provides a reservoir of essential mediators of wound healing, such as platelet-derived growth factor, that attract and activate macrophages and fibroblasts (Singer and Clark, 1999).

The combination of mediators and growth factors within the hemostatic plug initiates the healing process by recruiting circulating inflammatory cells to the wound site. Defects in clot formation, such as that observed in factor XI (fibrin-stabilizing factor) deficient disorder, leads to impaired wound healing and sometimes non-healing wounds (Duckert, 1973).

Neutrophils are one of the first immune cell types to migrate towards the wound bed (Singer and Clark, 1999). The combination of prostaglandins and chemotactic substances, such as tumor necrosis factor alpha (TNF-α), platelet factors, transforming growth factor beta (TGF-β) and interleukin-1 (IL-1), produced in the clot stimulate neutrophil migration (Heinlin et al., 2010), Fig 1.1. The primary function of neutrophils is to cleanse the wound area of foreign particles and bacteria (Singer and Clark, 1999). Additionally, neutrophils possess more than 700 functional sites for assembly of the prothrombinase complex, and therefore play a crucial role in generating thrombin (Duckert, 1973). In response to specific signals, such as monocyte chemoattractant protein-1 (MCP-1), monocytes, the second type of immune cells involved in the inflammatory step of wound repair, migrate to the wound site and differentiate into
macrophages (Worley, 2004b). Macrophages actively dispose of neutrophils and the remaining cellular debris through phagocytosis.

Both monocytes and macrophages release vital growth factors, such as platelet-derived growth factor, and insulin like growth factor, that are necessary for the initiation and proliferation of new tissue in wounds (Worley, 2004a). The pivotal role of monocytes and macrophages is further highlighted by wound healing studies that involve macrophage and monocyte depleted mice. Absence of macrophages and monocytes results in a prolonged inflammatory phase, which leads to defective wound healing (Singer and Clark, 1999).

Figure 1.1: Schematic diagram of a cutaneous wound three days after injury (Epstein et al., 1999).
Proliferation

In order for new cells to migrate and proliferate between the collagenous dermis and the fibrin eschar, Fig 1.2, the blood clot must first be degraded (Singer and Clark, 1999). This is performed by keratinocytes, which dissolve the fibrin layer by secreting fibrinolytic enzymes such as plasmin (Martin, 1997). Several days after the injury fibroblasts and endothelial cells invade the wound space and initiate the proliferative stage, Fig 1.3. Fibroblasts and endothelial cells proliferate from the surrounding tissue and intact venules, respectively (Singer and Clark, 1999).

In order for the proliferating fibroblasts to become functional and express the appropriate integrin receptors, they must first be activated from their quiescent state by various cytokines (Heinlin et al., 2010). One such cytokine is TGF-β, which is produced by platelets, macrophages, and T lymphocytes (Heinlin et al., 2010). TGF-β plays a crucial role in regulating the function of fibroblasts in ECM remodeling. It enhances fibroblast ECM production through increasing the transcription levels of genes for ECM proteins such as collagen and fibronectin and by decreasing the secretion of proteases responsible for ECM degradation (Huang et al., 2002). Collagen type III and I are the major structural proteins during this stage and are central to the integrity of the newly synthesized ECM (Nagase and Woessner, 1999a). Once sufficient collagen has been produced and deposited, fibroblasts, signaled by various signals, decrease collagen production.
Figure 1.2: Photomicrograph demonstrating reepithelialization of a cutaneous wound in a pig (Epstein et al., 1999). Epidermal cells (E) are seen dissecting under the fibrin clot across the wound. The advancing edge of the epidermal cells is shown with arrows. The white oval is an artifact of preparation.
Figure 1.3: A Cutaneous wound five days after injury (Epstein et al., 1999). Blood vessels are seen sprouting into the fibrin clot as epidermal cells resurface the wound. Proteinases thought to be necessary for cell movement are shown. The abbreviation u-PA denotes urokinase-type plasminogen activator; MMP-1, 2, 3, and 13 matrix metalloproteinases 1, 2, 3, and 13 (collagenase 1, gelatinase A, stromelysin 1, and collagenase 3, respectively); and t-PA tissue plasminogen activator.

Remodeling

During the final phase of wound healing, the remodeling phase, inflammatory cells such as macrophages continue their roles and assist in the migratory effort of mesenchymal cells to deep layers of the wound. At the initial stages of the remodeling phase, the tensile strength of the newly formed epithelialized tissue is significantly less
than that of the uninjured tissue (Levenson et al., 1965). This newly formed tissue is enhanced, mainly through changes in type, amount and organization of collagen fibers, which were randomly deposited during the proliferative phase.

The remodeling phase begins when the rate of collagen synthesis and degradation equilibrate (Schiller et al., 2004). At this time, ECM degrading enzymes, such as matrix metalloproteinases (MMPs), cleave the collagen fibers at specific sites and break them down to their characteristic three-quarter and one-quarter pieces (Page-McCaw et al., 2007). A hallmark of the remodeling phase is the replacement of the initially predominate type III collagen with type I collagen, which results in increased tensile strength of the wound (Page-McCaw et al., 2007).

The extracellular matrix

The concept of ECM, during the 1950s, was relatively simple, mainly consisting of a “colloidal ground substance” and collagen fibers. The evolutionary origin of ECM, in its simplest form, has traditionally been attributed to multi-cellular organisms’ need to establish a link with their extracellular environment and to respond to extracellular stimuli. Over the past sixty years, tremendous progress in the field of cellular and molecular biology has allowed for a greater understanding of the complex nature and important biological roles of ECM. Currently, there are more than 20 gene products recognized as collagen and the concept of the “simple colloidal” ground substance has
been upgraded to a complex family of glycoproteins and proteoglycans with hundreds of unique members (Aouacheria et al., 2006).

Skin’s remarkable tensile strength, which on a weight basis is comparable to that of steel, is due to ECM’s components such as collagen and elastin. The main form of collagen in the dermal matrix is type I collagen, which is composed of three protein chains arranged in a triple helical conformation. By self-assembling into a head-to-tail and staggered side-to-side arrangement, collagen molecules adjacently bind to each other and function as skin’s “re-bars”.

In addition to having extraordinary tensile strength, skin needs to be highly resilient as well. Skin’s ability to recoil after stretching, is largely owed to a network of elastic fibers in its ECM. The main components of these elastic fibers are cross-linked elastin molecules, which are extensively covered by glycoproteins such as fibrillin.

Due to its highly dynamic nature, ECM has a profound influence on many vital biological activities such as cell proliferation, differentiation, adhesion, migration, structural support, tissue morphogenesis and apoptosis (Bosman and Stamenkovic, 2003; Gelse et al., 2003). As such, factors that affect ECM composition or remodeling have great implications in both health and disease. In wound healing, for instance, defects in ECM and its remodeling are associated with failure of reepithelialization, and prolonged inflammation (Cook et al., 2000).
Matrix metalloproteinases

Timely breakdown of ECM components is essential for its reorganization, which is required in many vital biological processes. In normal physiological conditions, the majority of ECM components are highly resistant to proteolysis and their degradation can only be carried out by specialized enzymes such as MMPs.

MMPs are involved in ECM catabolism, which is often seen during embryonic development, bone remodeling and growth, wound healing, as well as in other forms of tissue remodeling (Okada et al., 1997). The complex interaction between MMPs, growth factors, and cytokines, results in a well-organized and efficient maintenance of ECM. As such, defects in the regulation of MMPs have been correlated with numerous disorders including: arthritis, emphysema, and wound healing (Okada et al., 1997).

The MMP protein family consists of 23 unique zinc-dependent proteolytic enzymes. Based on their primary structure, cellular localization and substrate specificity, these enzymes are divided into five subclasses. Key variations and unique characteristics within each subclass further results in differentiation of these enzymes. For example, in the collagenase subclass, MMP-8 is stored in neutrophil granules and can be activated in seconds after being released while MMP-1 must first be transcribed and then secreted (resulting in a 12 hour gap between its transcription and activation) (Page-McCaw et al., 2007). Regardless of their subclass, all MMPs contain zinc ions at their catalytic site, and
are: secreted as inactive pro-enzymes, able to degrade at least one component of the ECM, and degraded by tissue inhibitors of MMPs (TIMP).

Structurally, all MMPs share several key domains, which are essential to their functions and regulation, Fig 1.4. The first key domain is the signal-peptide domain that has to be cleaved before secretion and is not generally present in the inactive pro-enzyme. The second domain is the pro-peptide domain. Its cleavage results in the activation of the MMP enzyme. The catalytic domain is another key domain that serves as the zinc-binding region. Finally, the last key domain is the hemopexin domain that modulates individual substrate specificity of MMPs.

A key feature that enables MMPs to serve their vital functions in ECM modulation is that their activity and expression can be “finely-tuned” through diverse regulatory processes including, RNA transcription, protein translation, intracellular as well as extracellular localization, and pro-enzyme activation (Page-McCaw et al., 2007). Furthermore, MMPs can be regulated through the actions of tissue TIMPs. By binding to the inactive MMP, tissue inhibitors form stable complexes that are biologically less active in ECM degradation.

Figure 1.4: Main catalytic domains of matrix metalloproteinases (Modified from Kahari et al., 1997).
Chapter 2. Biology of red blood cells

Erythrocytes are among the most specialized cell types in the human biological system. Their primary role is to transport oxygen and carbon dioxide throughout the body. Under intricate humoral and cellular regulation, the erythroid precursor cells continually produce the appropriate number of functional mature erythrocytes. In the mature state erythrocytes can be viewed as containers for hemoglobin, which lack a nucleus and other important cellular machineries such as mitochondria. Nevertheless, their marvelous structure and biology makes erythrocytes ideally suited for their oxygen-transport function and enables them to function and survive in the surrounding environment. The following sections review the general characteristics and functions of RBCs as a background for discussing their possible roles in wound healing.

History of red blood cell research

The first person credited with the discovery of RBC is Jan Swammerdam, who studied amphibian blood using an early microscope in 1658. Several years later, Anton van Leeuwenhoek formed a more detailed description of RBC’s structure and characteristics. Nearly 300 years later in 1959, Max Perutz finally unraveled the structure of hemoglobin utilizing X-ray crystallography. Several centuries since their initial discovery, RBCs have been the subject of research in diverse fields such as molecular biology, genetics, biophysics and engineering.
The erythroid marrow

Blood cells develop from immature precursor cells through the process of hematopoiesis. Hematopoietic stem cells possess the ability to migrate within an organism to regions that support their survival and allow for appropriate differentiation. In humans, they originate in the yolk-sac mesenchyme and sequentially migrate to liver, spleen and the bone marrow (BM) (Muller-Sieburg et al., 2002). The unique structure of the BM allows for a special environment which is well suited for proliferation and maturation of hematopoietic cells.

While the actual factors that affect homing to different hematopoietic sites are still unknown, studies involving lower vertebrates indicate that seasonal changes can cause a shift in the hematopoietic sites possibly due to alterations in the levels of circulating steroids (Claver DVM and Quaglia., 2009). Thus, it is plausible that migration of hematopoietic stem cells during the developmental stages is also affected by alterations in the circulatory steroid levels.

Erythropoiesis

Erythropoiesis is a dynamic process which is affected by developmental and physiological changes within an organism. RBCs are made and destroyed in massive numbers on an hourly basis. As such, erythropoiesis is regulated by an extremely complex and organized system which involves the interactions of kidneys, heart, BM, liver, spleen as well as the vascular system. Equipped with oxygen sensors, kidneys
detect changes in oxygen tension caused by variations in hemoglobin oxygen saturation levels, RBC mass and blood flow rate. In response to hypoxic conditions, kidneys production of erythropoietin (EPO) increases. In addition to EPO, factors including insulin, and growth hormones provide positive feedback for RBC production.

EPO interacts with the RBC precursor cells in the BM known as burst forming unit-erythroid and colony forming unit-erythroid (BFU-E and CFU-E, respectively) to stimulate their proliferation and differentiation (Iavarone et al., 2004). The pluripotent stem cell differentiates into the committed stem cell, which in turn differentiates to the earliest form of “committed” erythroid precursor known as the BFU-E. After a few days, the BFU-E differentiates to the late erythroid precursor the CFU-E, which in turn differentiates to the first morphologically identifiable stage of the erythroid cells, the pronormoblast (Muller-Sieburg et al., 2002). The pronormoblast is the first of six stages ending in the mature RBC.

**The mature erythrocyte**

In humans, erythrocytes are the most common type of blood cells, contributing to more than one quarter of the total number of blood cells. Upon maturation, RBCs lose their nucleus and all organelles to contain mostly hemoglobin, electrolytes and water. Consequently, mature erythrocytes rely solely on anaerobic lactic acid fermentation as a source of energy (van Wijk and van Solinge, 2005). In fact, almost every erythrocytic enzymopathies that is involved in this pathway has dramatic implications on RBC
survival and functions, such as shortened cellular survival and hemolytic anemia (van Wijk and van Solinge, 2005).

Following maturation, the average RBC has a diameter of 6-8 µm and a thickness of approximately 2 µm with a characteristic shape of a biconcave disk with a flattened and depressed centre (Canham and Burton, 1968). This unique shape is vital to RBC’s survival and functions. RBC’s shape has been evolutionarily designed to maximize its respiratory exchange efficiency as well as its ability to pass through the circulatory system with ease- RBC is capable of travelling through capillaries as small as one-third its diameter. Similarly, it has been demonstrated that another possible role of the biconcave design is to minimize the required energy for RBC membrane bending while travelling through microvasculature (Canham, 1970).

In spite of their excellent adaptations to the circulatory environments, RBCs eventually “wear out” and are destroyed within 120 days of being in circulation. The precise mechanism through which RBCs are signaled for removal remains unknown. However, several promising theories have recently been emerging, including: the accumulation of autologous IgG on RBC membrane (Paleari et al., 2004), and modifications of protein band 3 by proteolytic cleavage (Hamasaki et al., 1997). Both of these membrane alterations lead to removal of RBCs by macrophages and degradation through the reticuloendothelial system, which encompasses spleen, liver as well as the BM. Following degradation, the majority of the breakdown byproducts are recycled. Hemoglobin constituents, for instance, are successively broken down to Fe3+ and
biliverdin and transported to the liver for additional processing. The appropriate timing of RBC programmed cell death, called eryptosis, is crucial to the organism’s survival-eryptosis is abnormally up-regulated in various life threatening disorders such as hemolytic-uremic syndrome (Foller et al., 2008).

**Secondary functions of red blood cells**

While previously the function of RBCs was considered limited to gas exchange, advances in cellular biology, functional genomics, and proteomics have enabled scientists to unravel other novel biological functions of RBCs. In spite of lacking cellular organelles and protein machineries, RBCs are capable of responding to a variety of extracellular stimuli. In response to variations in pH, oxygen concentration and osmotic pressure, RBCs are able to produce and secret adenosine triphosphate (ATP) (Leitch and Carruthers, 2007). Extracellular ATP secreted by RBCs bind to special receptors on endothelial cells which in turn respond by secreting nitric oxide, a potent vasodilator (Olearczyk et al., 2004). Defects in RBC ATP synthesis are linked to disorders such as cystic fibrosis (Sprague et al., 1998), pulmonary hypertension (Sprague et al., 2001), and even diabetes (Rabini et al., 1997). Additionally, it has been demonstrated that extracellular ATP can play a role in down regulation of cancerous tumors (Pellegatti et al., 2008) and take part in the inflammatory response in wound healing (Chiang et al., 2007). Another mechanism through which RBCs can regulate the vascular tone is through production of hydrogen sulfide in response to high blood pressure (Wagner, 2009).
In addition to being involved in regulation of the vascular tone, recent evidence suggests that RBCs may also play a role in the immune response. One such function is RBC’s ability to act as a sink for Interleukin 8 (IL-8) (Darbonne et al., 1991). During an inflammatory response IL-8, which can be secreted from a myriad of cell types including activated monocytes, T-lymphocytes and fibroblasts, induces the chemotaxis of neutrophils to the area of inflammation resulting in recruitment of other potent pro-inflammatory mediators (Darbonne et al., 1991). Being equipped with specialized IL-8 binding proteins, RBC binds to and deactivates IL-8, preventing it from causing an over-amplification in the inflammatory response (Darbonne et al., 1991). Additionally, RBCs are equipped with a variety of functional cytokines including tumor necrosing factor, IL-1, and IL-6, all of which play major roles in the inflammatory response following trauma or infection (Kristiansson et al., 1996).

Another immune function of RBCs is their ability to associate with antigen-antibody-complement complexes, through membrane receptors that recognize C3b or C4b complement elements. By binding to C3b elements, RBCs facilitate the movement of the complement system to macrophages of the reticuloendothelial system for removal (Medof et al., 1983).

**Hypothesis**

Wound healing proceeds through a very orderly and efficient but yet complex manner. Regardless of the cause, defects in this well-orchestrated process often lead to
dramatic morbidity and treatments that are among the most costly, from an economic perspective. In an effort to better understand the wound healing mechanisms and to establish more effective treatments, the roles of many underlying factors and cell types including, fibroblasts, keratinocytes, platelets, platelets-derived growth factors, MMPs and immune cells, have been closely studied and elucidated. In spite of their ubiquitous presence and involvement in coagulation, thrombosis, inflammation and initial stages of wound healing as well as their ability to interact with many cell types including immune cells, the role(s) of RBCs remain unclear in wound healing.

Here, we hypothesize that RBCs are capable of playing an active role in wound healing through interaction with dermal fibroblasts. To address such hypothesis several aims were pursued as described below.

**Specific aims**

1. The first aim was to investigate the presence of 14-3-3 isoforms in RBCs through Western blot and proteomic analyses.

2. In an effort to investigate RBCs ability to potentially influence wound healing we pursued the second aim of this study, which was to investigate the role(s) of RBC proteins in wound healing by examining their effects on dermal fibroblasts: (i) survival by utilizing cytotoxicity assays, (ii) expression levels of intracellular MMP-1, -2, -3, fibronectin and type-I collagen protein levels through Western blot analysis.
3. The third aim was to identify the elements that are involved in the underlying signaling pathway by examining the three distinct MAPK pathways that are involved in fibroblast MMP regulation.
Chapter 3. Identification of 14-3-3 isoforms in red blood cells

Introduction

The emergence of new tools and methods, such as technological advances in functional genomics and state of the art microarray methods, have allowed for a greater understanding of RBC biology. The elucidated biological functions of RBC are no longer limited to its traditional role as oxygen and carbon dioxide transport. Wide ranges of many other novel functions are now attributed to RBC. In 1993, Neote and his colleagues demonstrated that RBC possesses multi-specific receptors that associate with important inflammatory cytokines, consequently revealing RBC as active participants of the inflammatory process (Gelse et al., 2003). Additionally, coagulation of blood in cultured endothelial cell-coated tubes has revealed that activation of factor IX on the erythrocyte surface results in activation of the intrinsic coagulation system, highlighting RBC’s active role in thrombosis and coagulation (Kawakami et al., 1995). It has also been shown that RBC participates in ECM remodeling, during processes such as fibrotic lung disorders, by regulating the activity of essential ECM proteins like MMPs (Fredriksson et al., 2006).

This wealth of knowledge, about RBC’s composition and its complex membrane organization, has primarily been attained through biochemical approaches and advances in proteomic techniques. To date, more than 300 membrane and 200 cytosolic RBC proteins have been identified (Goodman et al., 2007). The specific functions of many of these proteins with respect to RBC’s biological roles have yet to be determined. One such
protein belongs to a family of conserved regulatory proteins called 14-3-3. This protein family was first discovered in 1967, by Moore & Perez in cerebrospinal fluid. It consists of seven isoforms named with Greek letters for their elution profile on reversed phase high pressure-liquid chromatography. All seven isoforms are usually found in their dimeric forms with molecular mass of approximately 30 kDa (Obsilova et al., 2008). Throughout the last forty years numerous re-discoveries of 14-3-3 isoforms in different cell types and organs have resulted in their evolution from ‘novel’ proteins to ubiquitously expressed regulatory proteins that have more than 50 unique signaling proteins as their ligands. The 14-3-3 proteins have been implicated in a diverse array of regulatory processes such as mutagenic signal transduction (van Heusden, 2009), apoptotic cell death (Obsilova et al., 2008), and cell cycle control (Aitken, 1996).

In 2006, two isoforms of 14-3-3 proteins, ε, and ζ, were found in RBC (Pasini et al., 2006). In spite of this novel discovery, minimal work has been performed in investigating the role of 14-3-3 proteins in RBC. Our discovery of a secreted form of 14-3-3 σ with anti-fibrogenic characteristics, in primary dermal fibroblasts (Ghahary et al., 2004; Ghahary et al., 2005), and previous reports of RBC’s role in ECM remodeling during certain fibrotic disorders, led us to investigate the presence of all seven 14-3-3 isoforms in RBC.
Materials and methods

Red blood cell samples

In accordance with University of British Columbia ethic approval, human peripheral whole blood, from healthy donors, was collected in vacutainer tubes containing EDTA (K2 EDTA 10.8 mg purchased from BD®) and used within 24 h. The RBCs were sedimented from 10 ml human blood at 13000 RPM for 10 min at 4 °C and washed with 10 ml of phosphate-buffered saline (PBS; 10 mM NaPO₄, pH 7.6, 150 mM NaCl) for four times. Each time, the upper 1–2 mm layer of packed cells was aspirated along with liquid phase to remove white blood cells. RBCs were finally re-suspended in 10 ml of PBS (original volume), aliquoted into 10-ml centrifugal tubes (2–3 ml of packed cells per tube) and stored at 4 °C for further use. Red blood cells were then sedimented again from each tube and diluted ten times using RBC lysis buffer (Bioscience, Inc. San Diego, CA, and USA), vortexed, followed by centrifugation at 13000 PRM for 10 minutes. The supernatants, which contained the RBC lysate, were kept frozen at -20°C for later use.

Identification of 14-3-3 isoforms in RBC by western blot analysis

In order to test for the presence of 14-3-3 isoforms in RBC, 15 µl of RBC lysate was run on a 10% SDS-polyarylamide gel. The erythrocytic proteins were then transferred onto nitrocellulose membrane utilizing the iBlot system (Invitrogen). Non-specific bindings were blocked with PBS-T containing 5% fat free milk for 1 hr. The
membranes were then incubated with rabbit anti 14-3-3 primary antibodies (1: 3000 dilution in PBS containing 5% Skim milk) specific to each 14-3-3 isoform (gift from Dr. Aitkin), and were kept at 4°C overnight. Immuno-reactive proteins were then visualized using ECL Western blotting detection system (Amersham Biosciences, Buckinghamshire, UK). For the purpose of statistical analysis, the following experiment was repeated with three different RBC samples donated by three different individuals.

Identification of 14-3-3 isoforms in RBC by proteomic analysis

In order to re-confirm the presence of 14-3-3 isoforms in RBC, erythrocytic proteins were subjected to proteomic analysis as follow: (1) RBC lysate (30 µl) proteins were size fractioned by a 10% of SDS-polyarylamide gel and the gel was stained with 0.25% Comassie Blue, (3) proteins bands which were visually detected within the 22-30 kDa range were excised and subjected to trypsin digestion, and (4) digested samples were then identified by API Q Star pulsar i Hybrid LC-MS-MS at the University of British-Columbia MSL/LMB Proteomics core Facility. The peptide sequences were identified by using the Mascot search engine and Analyst software (Applied Biosystems, Foster City, CA) against the non-redundant NCBI database.
Results

Identification of 14-3-3 isoforms in RBC lysate

After excising proteins that have a molecular weight in the 22-30 kDa range, from polyacrylamide gel, for three samples of RBC lysate (15 µL), Western blot analysis revealed the presence of all seven 14-3-3 isoforms, Fig 3.1. Unlike the data generated by the Western blot experiments, proteomic analysis revealed the presence of only five 14-3-3 isoforms (ε, σ, ζ, β, and τ), table 3.1.

![Image of Western blot analysis](image-url)

**Figure 3.1: Identification of 14-3-3 isoforms in RBC lysate by western blot analysis.** RBC lysate (15 µL) from three individuals (A, B, C) were tested by Western blot analysis for the presence of seven isoforms of 14-3-3 proteins using specific rabbit polyclonal antibodies against each isoform.
Table 3.1: Identification of 14-3-3 isoforms in RBC lysate using proteomic analysis

<table>
<thead>
<tr>
<th>14-3-3 Isoform</th>
<th>Beta (β)</th>
<th>Epsilon (ε)</th>
<th>Gamma (γ)</th>
<th>Eta (η)</th>
<th>Theta (θ)</th>
<th>Sigma (σ)</th>
<th>Zeta (ζ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Discussion

14-3-3 proteins are expressed in most eukaryotic cells and play important roles in a wide range of vital regulatory processes, including signal transduction, cell survival, metabolism control, cell cycle progression and DNA replication (Gelperin et al., 1995; Aitken, 1996; Ajjappala et al., 2009). In spite of the ubiquitous distribution of the 14-3-3 protein family, the expression of the σ isoform is restricted to epithelial cells (Hermeking, 2003). Surprisingly, in the current study, we found all seven isoforms to be present in RBC. From these seven isoforms ε, σ, ζ, β, and τ were identified by both Western blot analysis and proteomics. This discovery makes RBC the only hematopoietic cell to contain 14-3-3 σ. In human platelets, five isoforms (β, γ, ε, ζ, and η) have previously been detected by Western blotting (Mangin et al., 2009). In platelets, from these five isoforms β, γ and ζ are found to be expressed at a relatively higher level compared to the η and ε isoforms (Wheeler-Jones et al., 1996).

Mammalian RBCs have many unique characteristics, which could help explain the possible roles of erythrocytic 14-3-3s. Upon maturation, for instance, RBCs lose their nuclei and other cell machineries and consequently are unable to divide and have limited repair capabilities (Koury et al., 1987). As a result of this distinctive maturation process the mature form of RBC, which remains in circulation for 120 days, contains many proteins that are remnants from earlier developmental stages where gene expression and protein production were active (Koury et al., 1987). RBC’s 14-3-3 protein family might be one such remnants protein. Interestingly, in a recent study, published during the
writing process of this paper, Patrick and his colleagues showed that the ζ isoform act as a mediator for the proerythroid differentiation (Patrick et al., 2010). Mice lacking miR-451, a micro RNA that represses 14-3-3ζ in RBC, showed dramatic reduction in hematocrit, and erythroid differentiation compared to healthy mice.

Moreover, 14-3-3 proteins may participate in the metabolism processes and oxidative stress response in both the mature and the progenitor form of RBC. The maintenance of many of RBC’s vital functions depends on the anaerobic conversion of carbohydrates by the Embden-Meyerhof pathway (Rose and O’connell, 1964). Several key enzymes, which are involved in carbohydrate metabolism, such as trehalose-6-phosphate synthase, glyceraldehydes-3-phosphate dehydrogenase and ATP, synthase have been found to be regulated by 14-3-3 proteins (Moorhead et al., 1999).

14-3-3 isoforms might also be involved in regulating RBC’s ability to respond to extracellular stimuli. Regulations of cellular responses to extracellular conditions, such as oxidative stress, are crucial for RBC’s survival and function. A recent study conducted by Marinkovic and his colleagues demonstrated that the forkhead box O3 (FoxO3) is specifically required for induction of proteins that regulate the in vivo oxidative stress response in murine erythrocytes (Marinkovic et al., 2007; Yalcin et al., 2008). This process is required for binding of phosphorylated FoxO3 with some isoforms of 14-3-3. The binding of FoxO3 and 14-3-3 protein inhibits nuclear accumulation of FoxO3 and activates the FoxO3 anti-oxidant process (Yu et al., 2010), indicating 14-3-3 proteins role in regulating cell stability. However, different isoforms may play different role in this
aspect. Another group found that the bicistronic microRNA-451 ablation in mice cause 14-3-3ζ accumulation which relocates FoxO3 from nucleus to cytoplasm (Patrick et al., 2010). This change leads to a mild erythrocyte instability and increases its susceptibility to damage after exposure to oxidative stress. Moreover, suppression of 14-3-3ζ by shRNA can protects miR-144/451+ ε erythrocytes against peroxide-induced destruction (Patrick et al., 2010).

In addition to their intracellular roles as adaptor molecules, several studies have previously found some isoforms of 14-3-3 proteins to be function as paracrine factors (Kilani et al., 2008; Kuzelova et al., 2009; Benz et al., 2010). In one of our previous studies, for instance, we found a releasable form of 14-3-3 σ in keratinocytes conditioned media (Ghahary et al, 2004). This factor as well as η (Kilani et al., 2007) and β (Katz and Taichman, 1999) were found to increase the expression of MMP-1, in dermal fibroblasts, by binding to CD13 on the cell surface (Ghaffari et al., 2010). Although the current study did not identify the actual erythrocytic factors responsible for this effect, it is possible that 14-3-3 σ, η and β are involved in this anti-fibrogenic effect. Such hypothesis is further supported by a previous study that utilized a co-culture cell system to illustrate that releasable factors from RBC stimulate the secretion of MMPs from human lung fibroblasts (Margulis et al., 2009). Even days after initial trauma has ceased, vessels continue to leak essential mediators of wound healing, a phenomena known as vascular hyperpermeability (Brown et al., 1995). Therefore, it is possible that some anti-fibrogenic factors, such as 14-3-3 σ, might either be secreted from intact RBCs or be released in
circulation during RBC breakdown and subsequently play an important role in regulation of ECM remodeling.

A notable discrepancy that we observed was the difference between the results obtained from Western blot analysis and the proteomics analysis. The Western blot data revealed all seven isoforms whereas the proteomics analysis, for identical samples, only revealed five isoforms. One possible explanation for these observations could be that the detected 14-3-3 isoforms, in proteomic analysis, where more abundant than the other isoforms, in our samples, and therefore resulted in an oversaturated signal that masked the other isoforms from detection. Given that all seven 14-3-3 isoforms are very similar in size, sequence and charge such explanation is highly warranted.

In conclusion, this study shows that all seven isoforms of 14-3-3 are present in RBC and that some factors in RBC influence ECM protein expression and ECM remodeling. This is particularly important because 14-3-3 σ, η and β which were previously confirmed as a stimulatory factor of MMP-1 in dermal fibroblasts, were detected in RBC. Ultimately, the presence of different 14-3-3 isoforms in erythrocytes may reveal a novel biological function for RBC as well as 14-3-3 proteins in inflammatory conditions.
Chapter 4. Red blood cells mediate the expression of extracellular matrix proteins via ERK 1/2 MAPK activation

Introduction

Wound healing requires a sequence of highly synchronized events in which various cell types and tissue systems interact to restore tissue integrity. It consists of three dynamic phases – inflammation, proliferation, and remodeling. During the inflammatory phase, a blood clot is established and it functions as a temporary scaffold for cell migration and proliferation (Singer and Clark, 1999). The roles of many factors and cell types that are involved in this process, such as platelets, platelets-derived growth factors, and macrophages, have been closely studied and elucidated. However, the role(s) of RBCs which are among the most predominate cell types, during the initial phases of wound healing, remain unclear – in spite of their ubiquitous nature in coagulation, thrombosis and inflammation, RBCs have traditionally been regarded as inert bystanders in wound healing.

One circumstance, during which RBCs might play a crucial role in wound healing, is trauma-induced hemorrhagic wounds. A defining feature of such wounds is prolonged bleeding accompanied by increased RBC presence in the wound site. Previous studies have demonstrated that severe bleeding results in dramatically impaired wound healing (McGinn, 1976; Beule et al., 2010). Upon comparing experimental wounds induced in patients with concurrent excessive bleeding and normal volunteers, for instance, collagen
accumulation was found to be significantly reduced in the former group (Angele et al., 1999). There have also been reports of increased wound dehiscence in patients undergoing surgery for bleeding ulcers, compared to patients with non-bleeding ulcers, of the upper gastrointestinal injury (Guiney et al., 1966). Additionally, the effects of excessive bleeding on wound healing is further highlighted by studies involving defects in homeostasis. For example, unlike healthy mice, hemophilic mice develop severe subcutaneous hematomas that require an extensive amount of time to resolve (Hoffman et al., 2006). Similarly, wounds of hemophilic mice show abnormal histology, and reduced as well as delayed macrophage infiltration during the inflammatory and proliferative stages of wound healing (Hoffman et al., 2006).

In normal wound healing, reepithelialization of the wound begins within hours after inflammation (Heinlin et al., 2010). In order for new epidermal cells to migrate and proliferate between the collagenous dermis and the fibrin eschar, the provisional scaffold (hemostatic plug) must be degraded (Singer and Clark, 1999). Such process is controlled by the activity of MMPs which are also involved in the modulation of the ECM.

ECM regulation relies heavily on the fine balance between ECM degradation and regeneration. While the proinflammatory cytokines and signals appear to reduce the synthesis of collagen, the anti-inflammatory cytokines such as (TGF)-β enhance collagen synthesis (Agaiby and Dyson, 1999). Several studies have shown that delayed wound healing, as seen in chronic wounds, is due to high concentrations of pro-inflammatory signals that induce abnormally elevated levels of different groups of proteases such as
MMPs (Agaiby and Dyson, 1999; Agren, 1999; Chubinskaya et al., 1999; Parks, 1999; Gillard et al., 2004; Mirastschijski et al., 2010).

Similar to chronic wounds, in hemorrhagic wounds, due to unknown reasons, the fine balance between the release of pro-inflammatory and anti-inflammatory cytokines is disturbed (Angele et al., 1999). This defect in ECM assembly and its remodeling is associated with failure of re-epithelialization, and abnormally prolonged inflammation (Cook et al., 2000). While many studies have focused on characterization of cytokines and growth factors, released by cells such as immune cells and platelets, no group has directly studied the possible role(s) of RBCs in wound healing. RBCs constitute more than 95% of the cellular mass of the blood; therefore, it can cogently be hypothesized that they may interact with other cells during the inflammatory phase and impact the outcome of wound healing. In fact, RBCs are capable of interacting with several types of cells such as macrophages and neutrophils (Chin-Yee et al., 1998) both of which are involved in the regulation and production of various MMPs in wound healing (Singer and Clark, 1999). Additionally, by actively releasing RBC-derived microvesicles, mature RBCs have been shown to actively participate in key signaling processes (Kriebardis et al., 2008), further highlighting RBCs involvement in important cellular processes. To test our hypothesis, we pursued the first aim of this study, which was to investigate the interaction of RBC with dermal fibroblast by evaluating their effects on fibroblast: (i) survival, (ii) expression levels of MMP-1, 2, 3, fibronectin and type-I collagen. The second aim was to identify the elements that are involved in the underlying signaling pathway.
Materials and methods

Red blood cell samples

In accordance with University of British Columbia ethic approval, human peripheral whole blood, from healthy donors, was collected in vacutainer tubes containing EDTA (K2 EDTA 10.8 mg purchased from BD®) and used within 24 h. The RBCs were sedimented from 10 ml human blood at 13000 RPM for 10 min at 4 °C and washed with 10 ml of phosphate-buffered saline (PBS; 10 mM NaPO₄, pH 7.6, 150 mM NaCl) for four times. Each time, the upper 1–2 mm layer of packed cells was aspirated along with liquid phase to remove white blood cells. RBCs were finally resuspended in 10 ml of PBS (original volume), aliquoted into 10-ml centrifugal tubes (2–3 ml of packed cells per tube) and stored at 4 °C for further use. Red blood cells were then sedimented again from each tube and diluted ten times using RBC lysis buffer (Bioscience, Inc. San Diego, CA, and USA), vortexed, followed by centrifugation at 13000 PRM for 10 minutes. The supernatants, which contained the RBC lysate, were kept frozen at -20°C for later use.

Fibroblast culture

Following informed consent, foreskin samples were obtained from patients undergoing elective surgery under local anesthesia. Written informed consent was obtained from each participant, and the study was approved by the University of British Columbia Hospital Human Ethics Committee and conducted according to the Declaration
of Helsinki Principles. Human primary dermal fibroblasts were harvested as described previously (Ghaffari et al., 2006). In brief, biopsies were collected individually and washed three times in sterile Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Grand Island, New York) supplemented with antibiotic-antimycotic preparation (100 µg/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B) (Gibco). Specimens were dissected free of fat and minced into small pieces less than 2.0 mm in diameter, washed six times with DMEM, and distributed into 60 X 15-mm petri dishes. Upon reaching confluence, the cells were released by trypsinization, split for subculture at a ratio of 1:6, and reseeded onto 75-cm² flasks. Fibroblasts at passages 3-7 were used in all experiments conducted in this study.

**Treatments of fibroblasts**

Different strains of human primary dermal fibroblasts were seeded on 25 cm² flasks (1x10⁶/flask) over night using DMEM containing 10% FBS. Cells were treated with either 0, 15, 30 or 50 µL of RBC lysate (1:10 diluted in RBC lysis buffer) as described above in 1 ml of DMEM containing 2% FBS for 24 hrs.

For the inhibition of ERK 1/2, p38, and JNK MAPK, 10 mM of specific inhibitors for each kinase (PD, SB, and SP, respectively) were added 1 h before stimulation. MEK1/2 inhibitor PD, p38 inhibitor SB, and JNK inhibitor SP were purchased from Calbiochem (San Diego, California). In order to reduce the background phosphorylation
levels of MAPK from the serum, fibroblast culture medium was replaced by serum-free medium, 24 hours prior to treatment with RBC lysate. Utilizing Western blot analysis, total and phosphorylated forms of ERK 1/2 levels were analyzed.

**LDH cytotoxicity assay**

In order to assess whether or not the treatment of RBC lysate has any effect on fibroblast survival, media from test and control cells were collected and subjected to lactate dehydrogenase (LDH) measurement. LDH is a stable cytoplasmic enzyme, which is present in all cells. When the plasma membrane is damaged, LDH is rapidly released into the culture supernatant. LDH assay is a colorimetric alternative to the traditionally used radioactive cytotoxicity assays such as [51Cr]. The cell-free culture supernatant is collected and incubated with the reaction mixture to determine LDH activity and quantify cell death.

The LDH assay was performed in accordance with the LDH cytotoxicity detection kit protocol (Clontech, Mountain View, CA, USA) for adherent cells. In brief, triplicates of primary dermal fibroblasts were seeded in 25 cm² flasks (1x10⁶/flask) over night using 10% FBS. Each flask received 5 ml of DMEM containing 2% FBS and was either kept as untreated (control) or treated with 15, 30, or 50 µL of RBC Lysate for 0, 1, or 2 days. Additionally, in order to control for the contribution of LDH from the media, lysed RBCs, as well as from untreated cells, the following controls were conducted in
triplicates: (1) background control: measured the LDH activity present in the assay medium only, the background absorbance was subtracted from all other absorbance measurements, (2) low control: measured the level of spontaneous LDH release from untreated cells, (3) high control: measured the maximum LDH activity that can be released from the 100% dead cells (in response to Triton X-100), (4) substance controls: measured the LDH activity contained in each RBC lysate treatment, each substance absorbance value was subtracted from the corresponding treated samples (i.e. the absorbance value for 15 µl of RBC lysate was subtracted from the absorbance value of cells treated with 15 µl of RBC lysate). 200 µL of media from each flask was transferred to a 96-well polystyrene plate and spun at 300 × g for 10 min. Next, 100 µL of supernatant was transferred to a new plate in which we added 100 µL of reaction mixture to each well, and incubated at room temperature in the dark for 30 min. The plate was read at 490 nm on a SpectraMax Plus 384 Plate Reader (Molecular Devices). The percent cytotoxicity was calculated using the formula below:

\[
\% \text{ Cytotoxicity} = \frac{\text{Triplicate absorbance} - \text{Low control}}{\text{High Control} - \text{Low Control}} \times 100\%
\]

Protein extraction and western blot analysis

For Western blot analysis, cells were harvested by trypsin-EDTA and lysed in lysis buffer (20-mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% Glycerol, 100 mM PMSF, protease inhibitor cocktail) and kept frozen at least for
one hour and then thawed and sonicated for 4 seconds followed by centrifugation at 14,000 RPM for 10 minutes. Supernatants were transferred to new eppendorf tubes and protein concentration was measured by Bradford method.

Proteins in supernatant were mixed with protein sample loading buffer (final concentration: 60 mM Tris-HCl pH 6.80, 2% SDS, 105 glycerol, 1.5% β-merchanethanol, 0.002% bromphenol blue) and size fractioned by 10% of SDS-polyarylamide gel. After proteins were transferred onto nitrocellulose membrane by iBlot (Invitrogen), non-specific binding were blocked with PBS-T containing 5% fat free milk for 1 hr and membrane was incubated with primary antibody overnight. After incubation with a secondary antibody for 1 hr, protein bands were visualized by an ECL detection system (Santa Cruz Biotechnology, Santa Cruz, CA). Following Abs were used in this study: rabbit anti-human MMP-1 (1:2000 μg/mL) (Epitomics, Burlingame, CA, USA), rabbit anti-human MMP-2 (1:1000 μg/mL) (Epitomics), mouse anti-human MMP-3 (1:1000) (R&D Systems, Minneapolis, MN), Mouse monoclonal anti-human type-I collagen against amino propeptide antibody (1:100) (Developmental Studies Hybridoma Bank, Iowa City, IA) rabbit polyclonal anti-human fibronectin antibody (1:2000) (Santa Cruz Biotechnology, Santa Cruz, CA) or mouse anti-human β-actin (2 μg/mL) (Sigma, saint Luis, MD, USA). For the detection of ERK 1/2 MAPK, rabbit IgG antibodies to total and phosphorylated forms of ERK 1/2 were obtained from Cell Signaling (Beverly, Massachusetts). The membranes were blocked in 5% skim milk powder in PBS 0.1% Tween 20 for 2 hr at room temperature. Membranes were then treated overnight at 4°C
with antibodies to total or phosphorylated forms of the signaling intermediates (1:1000 dilutions) in PBS 0.1% Tween 20 plus 5% BSA.

**Statistical analysis**

Data are presented as a mean ± standard deviation of at least three independent experiments. The differences between two groups were determined by Student’s *t*-test, *P* value of < 0.01 or 0.001 were considered as statistically significant, and very significant respectively.

**Results**

**RBC lysate has no cytotoxic effect on dermal fibroblasts**

To ensure that the RBC mediated change on fibroblast protein expression is not caused or influenced by cell death or membrane damage, an LDH cytotoxicity assay was carried out. Primary dermal fibroblasts were treated with four volumes of RBC lysate (1:10 diluted in RBC lysis buffer), 0 (control), 15, 30, 50 µl in 1 ml of DMEM containing 2% FBS for three different time periods (0, 1 and 2 days). As shown in Fig. 4.1, there were no significant changes between the percent cytotoxicity in treated versus untreated samples. Comparing the cytotoxicity across the three different incubation periods, however, revealed an upward trend.
Figure 4.1: Effects of RBC proteins on primary dermal fibroblast survival. Cells were treated with 0, 15, 30, or 50 µl of RBC lysate, in 1 ml of DMEM containing 2% FBS, for 0, 1, or 2 days. Data represent means ± standard deviations for three separate experiments. Statistical significance (ρ < 0.01) was tested with Student unpaired two-tailed t-test.

RBC lysate increases the expression of MMP-1, -2, -3 in fibroblasts

In order to investigate the effect of RBC on the expression of fibroblast MMPs, cells were treated with either 0 µl (control), 15, 30, or 50 µl of RBC lysate (1:10 diluted in RBC lysis buffer), in 1 ml of DMEM containing 2% FBS for 24 hours. Utilizing Western blot analysis, the expression of intracellular MMP-1,-2 and -3 were examined and quantified by densitometry. The β-actin was also blotted and used as the loading control. All data were normalized to the ratio of MMP / β–actin for the untreated controls.
Treatment with RBC lysate resulted in a significant increase of all three MMP expressions, Fig 4.2. Additionally, increases in RBC lysate volumes (i.e. from 15 to 30 µl) were accompanied by increases in the corresponding MMP levels. The minimum volume of RBC lysate which caused a significant change (p<0.01) in the MMP-1 and MMP-3 levels, was 15 µl, Figs 4.2A and 4.2B, respectively. For a significant change in the levels of MMP-2, on the other hand, the minimum required volume of RBC lysate was observed to be 30 µl, Fig 4.2B.

The highest percent increase was observed in MMP-1 levels at 5.1 fold increase followed by 3.4 and 2.3 fold increase in the levels of MMP-3 and MMP-2 respectively, Figs 4.2B, 4.2D and 4.2C.
Figure 4.2A: RBC lysate increases the expression of MMP-1 in dermal fibroblasts. Cells were treated with 0, 15, 30, or 50 μL of RBC lysate, in 1 ml of DMEM containing 2% FBS for 24 hr. Expression levels of MMP-1 and β-actin (loading control) were assessed by Western blot analysis. For statistical analysis, the experiment was repeated three times with different strains of primary dermal fibroblasts and all data were normalized to the ratio of MMP-1 / β–actin for the untreated controls. Data represent means ± standard deviations for three separate experiments. Statistical significance (ρ < 0.01* or 0.001**) was tested with Student unpaired two tailed t-test.
Figure 4.2B: RBC lysate increases the expression of MMP-2 in dermal fibroblasts. Cells were treated with 0, 15, 30, 50 μL of RBC lysate, in 1 ml of DMEM containing 2% FBS, for 24 hr. Expression levels of MMP-2 and β-actin (loading control) were assessed by Western blot analysis. For statistical analysis, the experiment was repeated three times with different strains of primary dermal fibroblasts and all data were normalized to the ratio of MMP-2 / β–actin for the untreated controls. Data represent means ± standard deviations for three separate experiments. Statistical significance (ρ < 0.01* or 0.001**) was tested with Student unpaired two tailed t-test.
**Figure 4.2C: RBC lysate increases the expression of MMP-3 in dermal fibroblasts.** Cells were treated with 0, 15, 30, or 50 μL of RBC lysate, in 1 ml of DMEM containing 2% FBS, for 24 hr. Expression levels of MMP-2 and β-actin (loading control) were assessed by Western blot analysis. For statistical analysis, the experiment was repeated three times with different strains of primary dermal fibroblasts and all data were normalized to the ratio of MMP-3/β–actin for the untreated controls. Data represent means ± standard deviations for three separate experiments. Statistical significance (p < 0.01* or 0.001***) was tested with Student unpaired two tailed t-test.
Effect of RBC lysate on type-I collagen and fibronectin expression in fibroblasts

The effect of RBC proteins on the expression of type-I collagen and fibronectin proteins in dermal fibroblast was tested by treating cells with either 0 (control), 15, 30, or 50 µl of RBC lysate (1:10 diluted in RBC lysis buffer) in 1 ml of DMEM containing 2% FBS for 24 hours. Utilizing Western blot analysis, the levels of intracellular type I collagen, fibronectin as well as β–actin were examined and quantified by densitometry. All data were normalized to the ratio of the corresponding protein (collagen or fibronectin) to β–actin for the untreated controls. The percent change in the intracellular levels of type I collagen and fibronectin are shown in Figs 4.3A and 4.3B, respectively.

As shown in Fig 4.3A, treatment with 30 µl and 50 µl of RBC lysate resulted in a significant down-regulation of type-I collagen levels at 1.7 and 4.5 fold decrease (P<0.01), respectively. The levels of fibronectin, in contrast, were significantly up regulated by all three volumes of RBC lysate (2.1, 2.6 and 2.7 fold increase (P<0.01) in response to 15 µl, 30 µl and 50 µl of RBC lysate, respectively), Fig 4.3B.

The highest degree of change was observed in the 50 µl treatment group for fibronectin at 2.7 fold increase (P<0.01), Fig 4.3B. The lowest volume of RBC lysate required for causing a significant change (p<0.01) in type I collagen and fibronectin levels were observed to be 30 µl and 15 µl, Figs 4.3A and 4.3B, respectively.
Figure 4.3A: RBC lysate decreases the expression of type-I collagen in dermal fibroblasts. Cells were treated with 0, 15, 30, or 50 μL of RBC lysate, in 1 ml of DMEM containing 2% FBS, for 24 hr. Expression of levels of type-I collagen and β-actin (loading control) were assessed by Western blot analysis. For statistical analysis, the experiment was repeated three times with different strains of primary dermal fibroblasts and all data were normalized to the ratio of type-I collagen / β–actin for the untreated controls. Data represent means ± standard deviations for three separate experiments. Statistical significance (p < 0.01* or 0.001**) was tested with Student unpaired two tailed t-test.
**Figure 4.3B:** RBC lysate increases the expression of fibronectin in dermal fibroblasts. Cells were treated with 0, 15, 30, or 50 μL of RBC lysate, in 1 ml of DMEM containing 2% FBS, for 24 hr. Expression of levels of type-I collagen and β-actin (loading control) were assessed by Western blot analysis. For statistical analysis, the experiment was repeated three times with different strains of primary dermal fibroblasts and all data were normalized to the ratio of type-I collagen / β–actin for the untreated controls. Data represent means ± standard deviations for three separate experiments. Statistical significance (ρ < 0.01* or 0.001**) was tested with Student unpaired two tailed t-test.
Induction of fibroblast MMP-1 expression by RBC is mediated through ERK 1/2

To determine the possible signaling pathway in which RBC lysate increase the expression of MMP-1, we explored the three distinct MAPK pathways in which fibroblast MMP-1 expression is regulated: (1) ERK1/2 (Raf → ERK kinase (MEK)1/2 → ERK1/2); (2) JNK/SAPK (MEK kinase 1–3 → MEK kinase 4 and 7 → JNK/SAPK); and (3) p38 MAPK (MAPK kinase → MAPK kinase 3 and 6 → p38). More specifically, we examined the role of the specific MAPK pathways in the RBC elicited up-regulation of MMP-1 in dermal fibroblasts by blocking ERK 1/2, JNK and p38 MAPK pathways with chemical inhibitors previously shown to block MMP-1 up-regulation by various stimuli.

In this experiment primary dermal fibroblasts were subjected to eight treatment groups: (1) 0 µl of RBC lysate (control), (2) 50 µl RBC lysate (+), (3) P38 inhibitor (SB), (4) JNK inhibitor (SP), (5) ERK 1/2 inhibitor (PD), (6) SB and 50 µl RBC lysate (SB +), (7) SP and 50 µl RBC lysate (SP +), and (8) PD and 50 µl RBC lysate (PD +), for 24 hours. Western blot analysis was used to obtain the expression level of MMP-1 and β-actin. All data were normalized to the ratio of MMP-1 / β-actin for the untreated controls. The percent changes in the levels of MMP-1 were obtained. As seen in Fig 4.4A, up-regulation of fibroblast MMP-1 expression by RBC was potently inhibited by PD98059 (PD), a specific inhibitor of ERK 1/2 activity. In parallel, blocking the activity of P38 and JNK by specific inhibitors SB203580 (SB) and SP600125 (SP), respectively, had no marked effect (P<0.01) on the MMP-1 expression by RBC lysate.
Figure 4.4A: Stimulation of MMP-1 expression by RBC is mediated through ERK 1/2. Fibroblasts in 1 ml of DMEM and 2% FBS were incubated with: either (1) 0 µl of RBC lysate (control), (2) 50 µl RBC lysate (+), (3) P38 inhibitor (SB), (4) JNK inhibitor (SP), (5) ERK 1/2 inhibitor (PD), (6) SB and 50 µl RBC lysate (SB +), (7) SP and 50 µl RBC lysate (SP +), or (8) PD and 50 µl RBC lysate (PD +), in 1 ml of DMEM containing 2% FBS, for 24 hours. Western blot analysis was used to obtain the expression level of MMP-1 and β-actin. For statistical analysis, the experiment was repeated three times with different strains of primary dermal fibroblasts and all data were normalized to the ratio of MMP-1 / β-actin for the untreated controls. Data represent means ± standard deviations for three separate experiments. Statistical significance (p < 0.01* or 0.001**) was tested with Student unpaired two tailed t-test.
RBC lysate stimulates the phosphorylation of ERK 1/2 in fibroblasts

Human dermal fibroblasts were treated with 50 µl of RBC lysate, in 1 ml of DMEM containing 2% FBS, for different periods. For each period, the phosphorylation of ERK 1/2 was determined by Western blot analysis, utilizing antibodies against phosphorylated and total ERK 1/2. Figure 4.4B demonstrates changes in ERK 1/2 phosphorylation at 0, 15, 30, 45 min, 1, 2, 4, 8, 16, and 24 hr in fibroblasts exposed to 50 µl of RBC lysate (1:10 diluted in RBC lysis buffer). Rapid and transient phosphorylation of ERK 1/2 was observed at 2 hr after stimulation and decreased to approximately 40% of the maximal value by 24 hr (p<0.01). Data represent means ± standard deviations for three separate experiments. Statistical significance (ρ < 0.01* or 0.001**) was tested with Student unpaired two tailed t-test.
Figure 4.4B: RBC lysate induces ERK 1/2 phosphorylation in dermal fibroblasts. Fibroblasts were treated with 50 µl RBC lysate, in 1 ml of DMEM containing 2% FBS, for various time intervals as indicated. The levels of phosphorylated ERK 1/2 (p-ERK 1/2) were determined by Western blot analysis using phospho-specific antibodies for ERK 1/2; specific antibodies for total ERK 1/2 (loading control. Data represent means ± standard deviations for three separate experiments. Statistical significance ($\rho < 0.01^*$ or $0.001^{**}$) was tested with Student unpaired two tailed t-test.
Discussion

The main purpose of this study was to study the interaction of RBC proteins with dermal fibroblasts by investigating their effect on various characteristics of fibroblasts. The result of the cytotoxicity assay demonstrated that erythrocytic proteins do not have a negative impact on dermal fibroblast’s survival, *in vitro*, and thereby the observed RBC mediated effects on fibroblast characteristics were not influenced or caused by cell death or membrane damage. Furthermore, the treatment of RBC lysate modulated the expression levels of MMP-1, 2, 3, fibronectin and type-I collagen in dermal fibroblasts. The levels of MMP-1,2,3 and fibronectin were increased, and those of type-I collagen were decreased. Additionally the MMP-1 stimulatory effect of RBC proteins was found to be mediated, at least in part, by ERK 1/2 phosphorylation, in a time dependent manner.

Erythrocyte’s proteome consists of more than 500 membrane and cytosolic protein (Pasini et al., 2010). It is highly probable that many of these, rather “un-identified” proteins are involved in enabling the mature RBC to respond to the needs of the many cells and systems with which it interacts. During cutaneous wound healing, tissue injury results in disruption of blood vessels and an abundance of intact as well as lysed RBCs in the wound site. In this stage, the surrounding fibroblasts are influenced by and respond to a wide range of extracellular signals such as transforming IL-1, and insulin like growth factors (IGF) (Heinlin et al., 2010). As shown in this study, RBCs might also influence fibroblasts by affecting their ECM protein expression. Interestingly, our results are consistent with that of a previous study, which demonstrated that treatment of RBCs,
result in increased levels of released MMP-1, -2, and -3 from lung fibroblasts (Fredriksson et al., 2006). These three MMPs all play essential roles in ECM remodeling as well as wound healing. MMP-1, for instance, is one of the only enzymes capable of degrading native fibrillar collagens of types I, II, III, and V (Herouy et al., 1999). Interestingly, MMP-1 is expressed at dramatically higher levels in chronic ulcers, compared to that seen in normal wounds (Saarialho-Kere et al., 1993). Additionally, MMP-1 levels are detectable as early as 4 hours after injury and wounding (Armstrong and Jude, 2002). This coincides with the inflammatory phase of wound healing, during which tissue injury causes disruption of blood vessels and an over abundance of RBCs in the wound site. Therefore, given the observed stimulatory effect of RBC on fibroblast MMP-1 level, we believe that in vivo RBC might be partly responsible for the induction or increase of dermal fibroblast MMP-1 levels in the early stages of wound healing.

One of the most important roles of fibronectin in wound healing is facilitating the movement of fibroblast and keratinocyte into the wound site (Greiling and Clark, 1997). As defects in fibroblast and keratinocyte migration often lead to impaired wound healing (Xie et al., 2008), their ability to migrate and infiltrate the dense homeostatic blood clot is crucial for wound healing. Through unknown mechanisms, fibronectin in conjunction with growth factors such as platelet-derived growth factors, signals the surrounding fibroblast and keratinocytes to express the appropriate receptors and transmigrate from the periwound stroma into the fibrin clot to initiate the re-epithelialization process
(Greiling and Clark, 1997). Hence, RBC’s up-regulation of fibroblast-produced fibronectin, as seen here, might serve as a catalyst for re-epithelialization.

The reorganization of collagen fibers is essential in wound healing and in order for new collagen fibers to form and be reorganized, disorganized and old collagen fibers must first be removed and degraded. This degradation process takes place via an extracellular or intracellular pathway (Page-McCaw et al., 2007). Extracellularly, collagen breakdown is performed by MMP-1, MMP-13 and MMP-14 (Nagase and Woessner, 1999). MMP-1 cleaves type-I collagen at its Gly-Ile and Gly-Leu helical regions (Gelse et al., 2003). This cleavage results in the generation of 3/4 and 1/4 fragments, which are further digested by MMP-2 and MMP-9 (Nagase and Woessner, 1999). Since the collagen antibodies used in this study only recognize intact type-I collagen, the observed reduction of type-1 collagen levels by RBC proteins could partly be caused by its concurrent upregulation of MMP-1 and MMP-2 levels.

Collagenase (MMP-1) is the principal fibroblast-derived secreted proteinase and variations in its secretion have profound implications on wound healing. In fibroblasts the expression of MMP-1 is mainly regulated by activation of MAPKs (Brauchle et al., 2000). Among the MAPK pathways that we tested here, only ERK 1/2 pathway was found to be involved in RBC mediated upregulation of MMP-1. Since activation of the ERK 1/2 pathway is independently sufficient to enhance MMP-1 expression in fibroblast (Brauchle et al., 2000), it is plausible that the observed increase in MMP-1 expression is achieved through ERK 1/2 activation alone. In fact, our findings show a marked increase
in ERK 1/2 phosphorylation, two hours following RBC treatment. Interestingly, in corneal wound healing, epithelial cells migration has been found to be mainly controlled by ERK 1/2 phosphorylation (Sharma et al., 2003). Therefore, even though the actual process through which RBCs activate ERK 1/2 is unclear, our findings suggest that some erythrocytic factor can directly or indirectly promote ERK 1/2 phosphorylation and lead to an upregulation of MMP-1, which could subsequently contribute to the degradation of the hemostatic plug. Such factor can potentially be either secreted from intact RBCs, in the form of RBC-derived microvesicles, or be released in circulation during RBC’s breakdown, and subsequently play an active role in wound healing. This mechanism could potentially be advantageous early on in wound healing when epithelial cells infiltrate and transmigrate into the provisional ECM to initiate the proliferative phase of wound healing.

Given the established roles of RBC in the formation and maintenance of the primary hemostatic plug, and its ability to interact with essential cells like fibroblasts, as shown here, it can be postulated that RBCs have the capacity to be actively involved in the wound healing process.

In summary, the present study demonstrates that treatment of RBC lysate results in up-regulation of MMP-1, -2, -3, and fibronectin and down-regulation of type-I collagen, in dermal fibroblast. Furthermore, activation of ERK 1/2 MAPK was shown to be involved in the observed RBC mediated changes in fibroblast protein expression. Follow-up studies could examine the exact mechanism of RBC-fibroblast interaction and
investigate the erythrocytic factor that is responsible for RBC’s effect on the expression of ECM proteins in dermal fibroblasts. Results from such studies would ultimately enable us to develop new information regarding unique processes associated with normal and pathological wound healing as well as to better understand RBC’s intricate biology.
Chapter 5. Conclusion

In wound healing, the initial blood clot plays an essential role by acting as a reservoir for vital growth factors, cytokines and cell-secreted constituents, and protects against infections at the wound interface. Therefore, the clot not only functions as an inert scaffold in which granulation tissue is deposited, but also assists in locally “trapping” growth factors that actively signal epidermal, vascular endothelial and fibroblast cells, to initiate the repair process. Given the established roles of RBCs in the formation and maintenance of this primary hemostatic plug, and their ability to interact with and alter the protein expression of essential cells like dermal fibroblasts, it can be postulated that RBCs have the capacity to be actively involved in the wound healing process.

Even though the precise nature of RBC’s role in wound healing is not elucidated in this thesis, the presented results support the hypothesis that RBCs have the capacity to actively participate in wound healing. This is supported through highlighting RBCs ability to: activate the ERK 1/2 pathway which has been shown to result in increased migration of epithelial cells to the wound site (Sharma et al., 2003), increase the expression of fibronectin which facilitates the transmigration of fibroblasts and keratinocytes to the wound site (Greiling and Clark, 1997), and increase the expression of MMP-1,-2 and -3 which lead to an increase in degradation of key ECM components such as type-I collagen.
RBC’s potential role in wound healing is further supported by our discovery of seven erythrocytic 14-3-3 isoforms, some of which have been implicated in modulating the expression levels of several MMP proteins in fibroblasts. Such discovery raises further questions on the regulatory role of RBCs on other ECM proteins as well as the exact nature of their interaction with dermal fibroblasts. Utilizing modern electron microscopy, previous studies have demonstrated that mature RBCs are capable of producing and secreting RBC-derived microvesicles, Fig 5.1, which contain unique signaling components (Anastasios G. Kriebardis, 2008). Thus, one mechanism through which RBCs mediate the induction of ECM proteins in dermal fibroblasts could be through secretion of RBC-derived microvesicles containing 14-3-3 isoforms. Such proposed mechanism would enable intact circulating RBCs to respond to their environment or signals from neighbouring cells and take part in ECM remodeling by secreting MMP stimulatory factors like 14-3-3 proteins. This would add an additional regulatory network that could participate in the crucial fine-tuning of MMP activation in wound healing.
Figure 5.1: Conventional and immunogold electron microscopy of erythrocytic vesicles released after short or prolonged storage (Kriebardis et al., 2008). Panel A: Dense gold particles representing Hb in the main RBC body and the released vesicles (arrows) in a 35-day (d35) RBC preparation (bar, 0.2 mm). Panel B: Hemoglobin (Hb) labeling of vesicles (ves) released from RBCs after 1 day (d1) in storage.

From a therapeutic perspective, RBCs or their purified MMP stimulatory factors can be utilized as an endogenous form of treatment for treating fibrotic disorders such as hypertrophic scars and keloids which are caused by an over abundance of disorganized collagen fibers. Additionally, RBC’s ability to increase the expression of fibronectin, as
shown in this thesis, could be utilized for treating wound healing defects that result from reduced fibroblast or keratinocyte migration to the wound site.

An important limitation of the results included in this thesis is that being obtained from an in vitro model, they do not account for the influence of other cell types and the unique extracellular environment in vivo. In complex processes like wound healing, there are enormous differences between in vivo and culture-specific characteristics. Such discrepancies could dramatically alter results, thereby creating potentially conflicting conclusions. Nevertheless, this thesis paves the way for understanding another dimension of the marvelous and extremely complex biology of erythrocytes and their dynamic role in wound healing.

**Future direction**

As a follow-up to the results and explanations offered in this thesis, certain questions remain unresolved. The following are some suggestions that could advance our understanding of RBC’s interaction with dermal fibroblasts and its role in wound healing:

1) Identify the erythrocytic components which are involved in RBC-fibroblast interaction and protein regulation by treating dermal fibroblasts with either: (i) intact RBCs, (ii) conditioned media obtained from RBC culture, or (iii) RBC ghosts (empty erythrocyte membranes) and test for changes in expression level of ECM proteins in fibroblasts. Results from such study would indentify whether: (i)
intact RBCs are able to interact with dermal fibroblasts and affect their protein expression, (ii) factors released from isolated RBCs have any effect on fibroblast protein expression, and (iii) RBC membrane receptors or components have any effect on fibroblast protein expression.

2) Perform an ECM pathway-specific microarray as well as a large-scale proteomic analysis to investigate the gene transcription and translation profile in fibroblasts stimulated with RBC proteins or components. This would enable us to investigate the regulatory effect of RBCs on fibroblast gene transcription and translation.

3) By inhibiting 14-3-3 activity in RBCs through immune-precipitation assays or R18 (a peptide that binds to all 14-3-3 isoforms and disables their functionality), elucidate whether or not 14-3-3 proteins are involved in RBC mediated regulation of fibroblast ECM proteins.

4) Utilizing techniques such as High Performance Liquid Chromatography, isolate and purify RBC’s MMP stimulatory factors, and test their therapeutic potential in wound healing (i.e. using the rabbit ear hypertrophic scar model). The overall success of this novel treatment could be evaluated based on variables such as histological scores of the wounds, cellularity and scar thickness.
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