CHARACTERIZATION AND QUANTITATION OF COLLAGEN-I OXIDATION IN TGF-β STIMULATED FIBROBLAST CULTURE

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ABSTRACT

Incisional hernia is one the most common postoperative complications of abdominal surgery. Wound healing studies detailing the events of scar formation have shown that scar collagen composition and structure changes with the maturation of the scar. Cytokines, such as TGF-β, and reactive oxygen species (ROS), such as H_2O_2, present in the environment during the wound healing process can influence the events of scar formation and the properties of scar tissue. Few studies, however, have examined how these factors can influence the structure and stability of collagen fibers. This study uses a MRC-5 fibroblast culture preparation, and 2D gel profile of a CNBr digested cells and media preparations to identify ROS-oxidation changes in collagen structure, characterize the collagen structure of TGF-β-treated cultures, and evaluate the ROS sensitivity of this collagen in TGF-β-treated cultures. Results showed that MRC-5 cultures treated with TGF-β (0.5%) displayed a 2D gel collagen profile distinct from their untreated counterparts. The collagen content in both MRC-5 cells and media was greater and displayed the presence of the collagen fragment, α1CB6. Results showed that oxidation of TGF-β-treated cultures also produced a 2D gel collagen profile distinct from the untreated controls. Oxidation yielded more high molecular weight collagen peptide fragments and generated the appearance of an additional collagen CNBr fragment, α1CB8. The results of these studies suggest that oxidation and TGF-β treatment can alter the collagen fiber structure and organization in the MRC-5 culture.

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WOUND HEALING AND SCAR REPAIR

Hernia repair is one of the major problems faced by surgeons. Approximately 10% of abdominal surgical incisions result in incisional hernias and approximately 40% of these require additional corrective surgery (Burger et. al. 2004). Although surgical techniques are constantly improving, cell-based therapies that augment surgical scar formation have been utilized more and more frequently as an alternative (Franz et. al. 2007). Cell-based therapies have been particularly effective in animal models and have gained increasing application in clinical studies (Hodgson et. al. 2000). Reports have identified a variety of endogenous signaling molecules that direct the events of wound healing (Tsang et. al. 2003).

Wound healing and scar repair occurs in 3 phase: the inflammatory, proliferative, and maturation phases. These phases begin following tissue injury and end with reorganization of collagen fibrils.

I) The Inflammatory Phase

The inflammatory phase is divided into 2 parts - the hemostatic portion, and the cellular portion. The hemostatic portion of the inflammatory phase involves the activation of the coagulation cascade and platelet activation. These play an important role in wound healing by releasing a number of growth factors and cytokines. The synthesis of kinin by plasmin, and prostaglandins by blood vessel wall in the wound region cause vasodilatation and enhance vascular permeability which leads to the formation of edema and pain (Reviewed in Eming et. al. 2007). Within few hours of injury, immune cells called neutrophils appear, which phagocytose bacteria at the wound site. Leukocytes have

a short life span and decline in number at the wound site after three days in the absence of infection (Tziotzios et. al. 2012).

The cellular portion of the inflammatory phase starts with the influx of neutrophils, which are the dominant cell type at 1-2 days and are responsible for the phagocytosis of debris and bacteria (Koh et. al. 2011). Subsequently, an influx of macrophages occurs, which releases a number of active substances to engulf pathogens, and the macrophage becomes the dominant cell type in 2-4 days. Finally, an influx of fibroblasts takes place and these cells become the dominant cell type at 15 days. These fibroblast cells differentiate into myofibroblasts, which secrete collagen and also assist in wound contraction (Gurtner et. al 2008).

II) Proliferative Phase

The proliferative phase is divided into three parts- collagen deposition, neovascularization, and re-epithelialization. Collagen deposition starts by the formation of granulation tissue in the wound, which includes fibroblasts, inflammatory cells, extracellular matrix, fibronectin, and hyaluronic acid. A significant number of fibroblasts first appear in the wound site at day three and reach the greatest quantity on the seventh day. Fibroblasts are the main element which produces most of the structural proteins used during tissue repair. Collagen is one of the major proteins produced by the fibroblasts which also make major components of extracellular matrix such as fibronectin, hyaluronic acid, and glycosaminoglycans (Werner et. al. 2003). At the beginning, collagen deposition appears in a haphazard way in the wound but subsequently becomes aligned by forming cross-links between individual collagen molecules (Eckes et. al. 2010).

Neovascularization of the wound is one of the key events of the proliferative phase. New capillaries arise from the nearby blood vessels and grow into the wound area. Angiogenic stimulation of cytokines (macrophages, lymphocytes) by wound or hypoxia causes endothelial cells to migrate into the wound site. Endothelial cells then grow and proliferate under the influence of cytokines. Re-epithelialization of the wound starts within a few hours of injury and continues throughout the proliferative phase. The duration of re-epithelialization will vary, depending upon the size of the wound margin. The mechanism for stimulating re-epithelialization is not well understood, but investigators have proposed that exposure to fibronectin and cytokines such as myofibroblast in the extracellular matrix mediate this process (Hinz et. al. 2007). Some studies have shown that growth factors, such as epidermal growth factor (EGF), transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF) and insulin-like growth factor-λ (IGF-λ) may also be involved in stimulating epithelialization (Nauta et. al. 2011).

III) Maturation Phase

The maturation phase is characterized by the reorganization of collagen fibrils within the extracellular matrix and begins as soon as the extracellular matrix is laid down. Extracellular matrix consists of fibronectin, forms a fiber network which acts as a substrate for cell migration, cell growth and as a template for collagen deposition by fibroblasts. The unorganized collagen fibrils become cross-linked by hydrogen and di-sulfide bond to form a fibrillar bundle which enhances the tensile strength and stiffness of the healing tissue (Profyris et. al. 2012).

Wound Scar Repair

Scientists have attributed the significant increase in tensile strength of scars to the continuing deposition of collagen, formation of cross-links, and remodeling of collagen bundles (Ponrasu et. al. 2014). These events depend upon both the synthesis and breakdown of collagen during scar formation such that the growth and maturation of wound collagen is a balance of synthesis and catabolism (Bauer et. al. 2005). Collagen synthesis is initially very robust at wound sites but diminishes to normal levels a few months after injury, with remodeling continuing for up to a year.

An *in vivo* study conducted previously by this lab and collaborator and evaluated the effect of bone-marrow derived- mesenchymal stem cells (BM-MSCs) in scar repair. Stress-strain analysis of the scar tissue of BM-MSCs treated rats' tissue indicated that these tissues had greater tensile strength than scar tissue from untreated animals (Heffner et. al. 2012). The 2D gel electrophoresis analysis of scar tissue collagen showed that scar from BM-MSC treated animals displayed more collagen (Figure 2), and a different collagen profile than tissue from untreated animals (Figure 1). These results indicated that BM-MSCs can influence wound healing in a way that yields stronger and more resilient scar tissue.

The aim of this project is to extend the BM-MSC studies to an *in vitro* cell culture experimental system which investigate how collagen content and 2D gel profile can be altered by the conditions and cytokines associated with scar repair.

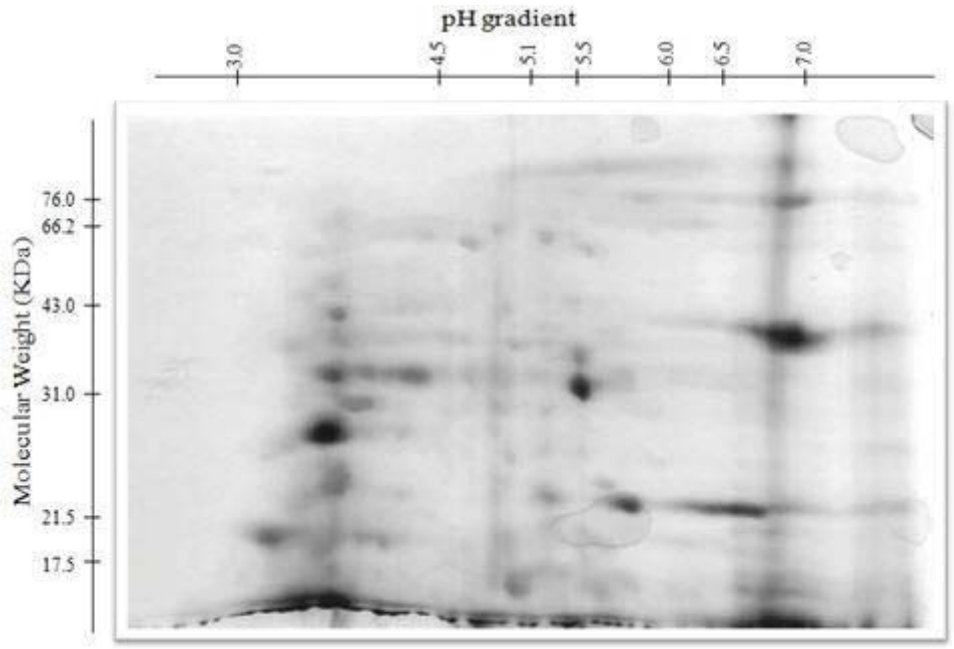

Figure 1: 2D gel electrophoresis profile of CNBr digested collagen rat fascial scar tissue (50µl) (Krontiris-Litowitz et. al. 2012).

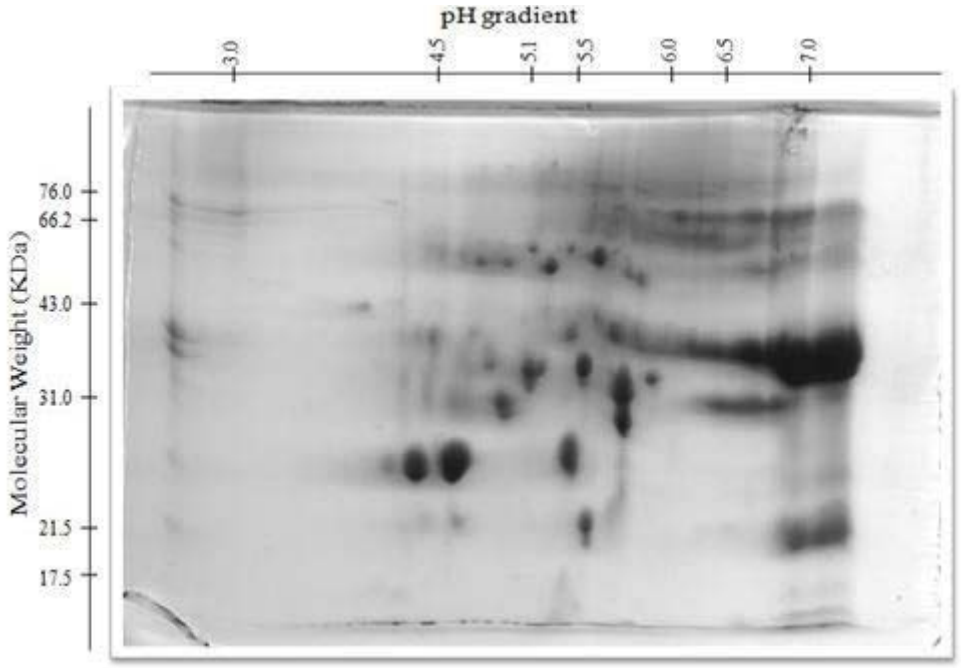

Figure 2: 2D gel electrophoresis profile of CNBr digested collagen rat fascial scar tissue (50µl) from BM-MSC treated rats (Krontiris-Litowitz et. al. 2012).

FIBROBLASTS

In homeostatic, non-injured connective tissue, the fibroblast is responsible for secreting the components of the extracellular matrix such as collagen, glycoproteins glycosaminoglycans, proteoglycans, and laminin (Hinz B, 2007). After tissue injury, fibroblasts become activated and turn into myofibroblasts, which make extracellular matrix (ECM) proteins that assist in wound healing. Fibroblasts and myofibroblasts play an important role in ameliorating the wound healing by producing a contractile force at the wound site to contract the wound (Tomasek et. al. 2002). Tissue injury displays an increased stimulus for fibroblasts and induces them to produce wound healing proteins.

Fibroblasts can have different origins in the body. Fibroblasts are differentiated from mesenchymal cells, but can be produced from epithelial cells as well. The process by which epithelial cells become fibroblasts is called epithelial-mesenchymal transition (EMT). However, fibroblast can also produce epithelia by mesenchymal-epithelial transition (MET) process which is normally observed during tissue repair at the wound site (Zavadil et. al. 2005).

Fibroblasts can exist in different forms, and inactive fibroblasts known as fibrocytes become active in response to tissue injury. Active fibroblasts are rich in endoplasmic reticulum, however, inactive fibroblasts are smaller and spindle-shaped. In wounded tissue, fibroblasts are activated, and differentiate into myofibroblasts, which release ECM proteins and help in reducing the wound size. Myofibroblasts are an intermediate cell type between fibroblasts and smooth muscle cells. Myofibroblasts are identified by the expression of α-smooth muscle actin (α-SMA) which contain contractile actin bundles called stress fibers, and also by appearing to be smooth muscle cells which

do not possess muscle markers such as desmin and myosin (Desmouliere et al., 2003; Eyden, 2005).

Fibronectin, a major component of the extracellular matrix, acts to promote cell adhesion of fibroblasts at the wound site and coordinates cellular interaction with the extracellular matrix. There are two forms of fibronectin: one is soluble, and another one is insoluble. Soluble fibronectin is found in the blood plasma and helps in blood clotting and wound healing, whereas insoluble fibronectin is found in the extracellular matrix and is secreted by the fibroblasts (Alberts et. al., 2002).

Fibroblast Origin and Migration

The presence of TGF-β (TGF-β1, TGF-β2, and TGF-β3) by macrophages and T cells during the inflammatory phase causes both structural and functional differentiation of the wound fibroblasts. This change in function and morphology is necessary so that fibroblasts can establish the scar extracellular matrix (ECM) and promote wound closure (Kim et al. 1999; Hinz et al. 2007). TGF-β1 and TGF-β2 are the major cytokines that stimulate fibroblasts, and are responsible for driving the differentiation of fibroblasts into myofibroblasts (Hinz et al. 2007).

The exact origin of the myofibroblasts seen in the healing wound is not clear while it is known that fibroblasts can differentiate into myofibroblasts. The majority are recruited locally from the dermis and tissues around the wound site (Hinz et al. 2007). Fibroblasts migrate into the wound site via a mechanism called contact guidance where fibroblasts do not invade in a haphazard way but travel in line with the collagen

orientation that is already in place. Myofibroblasts have also been shown to express integrin (αvβ3) which allows them to adhere to and migrate on fibrin (Gailet et al. 1997).

Fibroblasts are also found in uninjured tissue where they maintain the ECM. They move towards wound site via chemoattractants, for example, platelet-derived growth factor (PDGF), interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α) secreted from platelets and macrophages as a part of the inflammatory response (Kim et. al. 1999). Chemoattractants help in directing fibroblasts to the wound site by binding to the specific receptors on the cell surface (Alberts et. al., 1994). This binding allows the fibroblasts to orientate themselves according to the concentration of attractant and set up a guiding edge in the direction of the highest concentration.

Fibroblast interaction with the Extracellular Matrix (ECM)

The extracellular matrix is produced at the wound site is comparatively rich in collagen III, fibronectin and hyaluronic acid (Trebaul et al. 2007). Collagen type-III is formed immediately with the early matrix and acts as a barrier to pathogens. It is later degraded by proteases, remodeled by fibroblasts and replaced by type-I collagen (Witte et al. 1997). The other components of the extracellular matrix (ECM) contribute to stimulating fibroblast migration and fibrin adherence at the clot (Clark et al. 2004). Cells synthesize matrix metalloproteinases (MMPs) which remove denatured proteins and provisional matrix-associated materials which are not required in the healed wound. These proteinases are tightly controlled by tissue inhibitors of metalloproteinases (TIMPS) produced by fibroblasts (Soo et al. 1999).

Myofibroblast activity can affect the ECM composition (Schultz et al. 2011). When myofibroblasts move into the ECM and bind to fibronectin in the ECM, integrins transmit a signal into the cell to activate collagen synthesis (Tomasek et. al. 2002). However, this synthesis can be controlled by the extracellular environment. Studies show that when TGF-β is in the extracellular space collagen synthesis is enhanced (Clark et al. 1995). Conversely, if myofibroblasts are in a collagen rich matrix, they no longer produce collagen, even in the presence of TGF-β.

Fibroblasts and myofibroblasts play a key role in providing the contractile force that brings the wound edges together. Two opposing theories are considered regarding the method of wound contraction (Porter 2007). One theory is that the cell traction forces (CTFs) exerted by fibroblasts on the ECM during migration lead to compaction of the ECM and, therefore cause wound closure (Harris et al. 1981). The another theory hypothesizes that myofibroblasts anchor to the ECM and contract in a similar manner to that of smooth muscle (Gabbiani et al. 1971).

Role of Fibroblasts in Scar Development

Fibroblasts which appear at the site of injury during the end of the inflammatory phase and the beginning of the proliferative phase cause the degradation of the fibrin clot by producing various matrix metalloproteinases, and also synthesize various extracellular matrix components such as collagen I–IV, XVIII, glycoproteins, proteoglycans, laminin, thrombospondin, glycosaminoglycans, and hyaluronic acid (Li et al. 2011). The complex matrix is then prepared for the fibroblast migration and capable of signaling for angiogenesis, tissue generation and epithelialization (Rozario et al. 2010).

STEM CELLS

Stem cells are undifferentiated cells with the ability to develop into many different cells in the body. Stem cells are derived from different sources, and can be categorized into two main classes- embryonic stem cells and adult stem cells. Embryonic stem cells are derived from blastocyst which remains undifferentiated throughout the early phases of embryonic growth. In the early embryonic stages these cells are called totipotent cells, which can develop a full functional organism. During early embryonic development cell divisions make mostly totipotent cells, but within days, totipotent cells turn into pluripotent stem cells. Pluripotent stem cells, like totipotent stem cells, can develop into any tissue, however, they cannot produce a fully functional organism. There is another type of embryonic stem cell known as the multipotent stem cell which has the potential to make a finite range of cells within a tissue type.

Adult stem cells are multipotent cells which replace cells that have lost their functional capacity. These cells remodel and repopulate the injury site by regenerating themselves and differentiating into the cell types demonstrated in the original tissue. Adult stem cells, known as somatic stem cells or tissue-specific stem cells, produce only a limited set of the specialty cells characteristic of the source tissue. A small group of well-characterized and extensively investigated tissue-specific stem cells, such as hematopoietic cells, endothelial cells, mesenchymal cells, and epidermal cells, show promise as clinical therapies (Bajada et. al., 2008).

Mesenchymal stem cells (MSCs), a subclass of adult stem cells normally found in the bone marrow but also isolated from any other tissues, have the ability to form

adipocytes, cartilage producing chondrocytes, calcium producing osteocytes, tendons, muscle, and skin. Like other stem cells, MSCs are highly regenerative, multipotent and have the potential for use in tissue repair (Nakagawa et. al., 2005).

Stem Cell and Wound Healing

The ability to proliferate and differentiate make stem cells very effective in the regeneration and repair of damaged skin. Evidence has shown that in the case of severe injury, the number of circulating stem cells increases dramatically and contributes to the repair and recovery process (Kucia et. al., 2004).

Studies using bone marrow-derived mesenchymal stem cells (BM-MSCs) have demonstrated that they enhance many of the events essential for wound healing. Their healing potential is attributed to synthesizing large amounts of collagen, and releasing growth factors and angiogenic factors (Nakagawa et. al. 2005). Successful wound closure in patients with leg ulcers was observed after treatment with collagen matrices embedded with BM-MSCs (Falanga et. al., 2007). A decrease in wound size and a significant increase in the dermal vascularity was observed in foot ulcers of diabetic patients following implantation of autologous skin fibroblasts with MSCs (Vojtassák et. al., 2006).

Cells and biochemical factors at the wound site can influence mesenchymal stem cells activity and mobility. *In vitro* MSCs produce cytokines such as platelet-derived growth factor, insulin-like growth factor-1 and TNF-α which exhibit chemotactic properties toward wound healing (Mishima et. al., 2008; Hemeda et. al., 2010). MSCs can regulate the cells and the environment at the wound site. MSCs express several

chemokine receptors, which then migrate to regions of inflammation and become responsive to these agents (Ponte et. al., 2007). MSCs also regulate the response of leukocytes that invade tissues following injury (Rasmusson, 2006; Djouad et. al., 2009). Studies show that they regulate the activity, recruitment, and proliferation of T-cells and suppress the proliferation of B-cells and natural killer cells (Corcione et. al., 2006; Sotiropoulou et. al., 2006). This combination ameliorates the acute immune reaction to the wound and reduces pro-fibrotic responses associated with the sustained inflammation during wound healing (Ashcroft et. al., 1999; Red et. al., 2004).

Neutralization of reactive oxygen species in the wound

MSCs play an important role as mediators in promoting tissue regeneration and inhibiting scar formation at the wound (Salem et. al., 2010). During wound repair, neutrophils at the wound site secrete various reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, and alkyl peroxides which contribute to increasing the formation of fibrotic tissues (Muriel et. al.,1998). The ROS are controlled in part by the presence of nitric oxide at the wound site. Nitric oxide at the wound site cleans up the reactive oxygen species to make reactive nitrogen species which are less reactive than ROS and prevent the oxidative damage to DNA and membrane lipids (Wink et. al., 1999).

MSCs play a key role in regulating ROS formation. MSC expression of inducible nitric oxide synthase (iNOS) increases in response to their interaction with T-cells in a pro-inflammatory condition. Studies have shown that this iNOS expression can decrease

the ROS or reactive nitrogen species equilibrium to prevent the synthesis of fibrotic tissues (Ferrini et. al., 2002).

Mesenchymal stem cells enhance dermal fibroblast function

Throughout the proliferation stage, fibroblasts at the wound site produce granulation tissue, a connective tissue composed of extracellular matrix, which helps to make new dermal tissue. Fibrocytes entering the wound site during a prolonged inflammatory reaction or entering a TGF-β1-rich extracellular environment are unable to transform into granulation tissue, and proliferate instead into myofibroblasts (Opalenik et. al., 2005; Abe et. al., 2001). When TGF-β1 produced by injured blood vessels becomes elevated, endothelial cells experience epithelial-mesenchymal transition and become myofibroblasts (McAnulty et. al., 2007). Mesenchymal stem cells at the wound inhibit epithelial-mesenchymal transition and myofibroblastic differentiation by producing hepatocyte growth factor (Yang et. al., 2005), and prostaglandin E2 (Zhang et. al., 2006).

MSCs initiate some paracrine signaling which enhances the wound healing functions of dermal fibroblasts by producing ECM and regulating different cellular responses such as cell survival, proliferation, and migration (Smith et. al., 2010). MSCs also take part in the structural reformation of dermal and epidermal tissues to promote cutaneous wound healing through differentiation and release of proangiogenic factors (Wu et. al., 2007).

COLLAGEN

Collagen is one of the most abundant proteins found in animals, particularly in connective tissues, blood vessels and the skin of mammals. It is an insoluble and fibrous protein that makes up one-third of the protein in the human body. Collagen is an extracellular matrix molecule used to maintain the structural integrity of tissue and to provide firmness to the skin (Hopkinson, 1992).

Collagen has extensive roles to play in the wound healing process and is both, directly and indirectly, involved in each of the phases of wound healing (Schultz et. al., 2009; Guo et. al., 2010). During the inflammatory response, collagen influences cellular mitogenesis, differentiation, and migration (Rangaraj et. al., 2011). During the maturation phase, fibroblasts continue to lay down collagen as part of the framework of the wound (Kumar et. al., 2010). Finally, the covalent cross-linking of collagen molecules provides the tensile strength needed in the construction of scar tissue (Chin et. al., 2005).

Collagen-based matrices are thought to influence wound healing in numerous ways. Collagen is synthesized by fibroblasts, which also make other components of extracellular matrix such as fibronectin which acts as an adhesion of fibroblasts at the wound site, elastin which is responsible for maintaining the elasticity of the tissue, and hyaluronic acid which maintains the moisture content at the wound (Eisenbud et. al., 2004). Studies have shown that collagen also benefits wound healing by inhibiting MMP function (Rangaraj et. al., 2007), stimulating angiogenesis (Hodde et. al., 2001), serving as a chemoattractant for endothelial cells and fibroblasts (Li et. al., 2004; Brett et. al.,

2008), providing support for fibroblast ingrowth and attachment (Voytik-Harbin et. al., 1997) and generating the formation of granulation tissue (Brown-Etris et. al., 2002).

Arrangement of Amino Acids and the Triple Helix Structure of Collagen

Collagen is composed of various amino acids, but glycine (Gly), proline (Pro), hydroxyproline (Hyp) and arginine (Arg) are the four amino acids which are the most abundant in its structure. These amino acids often follow a sequence pattern Gly-Pro-X or Gly-X-Hyp where X can be any other amino acids. Proline or hydroxyproline make up almost one sixth of the total sequence (Koide et. al., 2005), whereas glycine is found in every 3rd position in the polypeptide chain and constitutes one-third of the collagen sequence (Gelsea et. al., 2003). The presence of proline in collagen structure helps in producing the helical conformation of each α chain because its ring structure causes a twist in the peptide chain.

The collagen molecule is composed of three alpha chains which are individual alpha-helical polypeptide strands that are fundamental to the structure and function of the molecule. In collagen-I there are two identical alpha-1 chains and a third alpha-2 chain. The three alpha chains complex forms a triple helix which is a single collagen molecule.

Intermolecular collagen cross-linking occurs outside of the cell, which is essential for maintaining tensile strength in collagen fibrils, and normal connective tissue function, since the tensile strength and biological function of connective tissue are significantly changed when the formation of cross-linking is restrained. The tensile strength of collagen depends upon the formation of covalent intermolecular cross-links between the individual protein subunits (Brodsky et. al., 2005).

Collagen Formation

Collagen molecules are synthesized in fibroblast cells. The initial molecule, pre-pro-collagen, is assembled in the rough endoplasmic reticulum of fibroblast cells after translation of alpha helices. As previously noted, collagen chains are rich in glycine, proline, and lysine. These residues are subject to post-translational processing that is fundamental to the final collagen structure and function. Post-translational modification of collagen includes enzymatic hydroxylation of proline and lysine, glycosylation of hydroxylysine residues, and disulfide bonding between the alpha chains. After processing and assembling pre-pro-collagen, the triple stranded molecules are packaged in the golgi apparatus into secretory vesicles and then exocytosed to become part of the extracellular matrix. These molecules, now known as pro-collagen are further modified in the extracellular environment. The pro-collagen molecule has C-terminal and N-terminal peptide ends which need to be cleaved to form fibrils and require further cross-linking. The C-propeptides and N-propeptides are cleaved by two protease enzymes which are procollagen C- proteinase (pCP) and procollagen N- proteinase (PNP), yielding a molecule of tropocollagen (Orgel et. al., 2006). This reaction is a rate-limiting step in the formation of collagen fibrils and can be facilitated by the glycoprotein, procollagen C-endopeptidase enhancer-1 (PCOLCE1) (Greenspan, 2005). Finally, the tropocollagen molecules associate covalently to each other at lysine or hydroxylysine residues to form collagen fibrils.

Initially, fibril orientations are labile, held together by non-covalent forces which allow fibril molecules to slide across one another. As the molecules mature, cross-links form to confer stability and tensile strength. Once fibrils aggregate, cross-link formation

begins. At this time secreted lysine and hydroxylysine are modified by lysyl oxidase to create aldehydes which spontaneously bond to unmodified lysine and hydroxylysine on adjacent chains to form cross-links (Fratzl et. al., 1997). These cross-links are called reducible and transient cross-links as they are Schiff bases and have varying stability. As the collagen matures, the transient reducible cross-links are replaced by mature non-reducible cross-links, resulting in increased strength of the collagen fibers (McCormick et. al., 1998). The tropocollagens then bind covalently to themselves at the amino acids lysine and hydroxylysine to finally form collagen fibers (Orgel et. al., 2006; Perumal et. al., 2008).

Role of Collagen Degradation in Wound Healing

Collagen degradation allows for remodeling of the ECM during wound healing. Initially Collagen-III is laid down in ECM but this is later replaced with Collagen-I. Collagen undergoes remodeling before the final scar is formed and these events are directed by MMPs, which are responsible for degradation.

Much of the remodeling process focuses on the collagen fibrils which are constantly refashioned through secretion of new collagen, degradation of existing collagen, and reorganization of the collagen fibrils in the ECM. Much of the remodeling of the ECM is dependent upon matrix metalloproteinases, zinc-dependent endopeptidases that are part of the matrixin protease family (Seals et. al., 2003; Sterchi et. al., 2008). MMPs have the capacity to break down a wide range of proteins as they remodel the wound site and have been shown to degrade extracellular matrix proteins, such as collagens, elastins, gelatins, and proteoglycans. They have also been associated with cleaving cell surface receptors, releasing apoptotic ligands, and inactivating chemokines

or cytokines (Van et. al., 2007). The proteolytic action of MMPs is influenced by the presence of extracellular activators and inhibitors as well as by reactive oxygen species (Murphy et. al., 2011; Nagase et.al., 2006). ROS released at the wound site by activated neutrophils and macrophages, initially activate MMPs through oxidation of cysteine. Later in the wound healing process the ROS combine with myeloperoxidase, an enzyme secreted by inflammatory cells, to inactivate MMPs (Fu et. al., 2003).

Matrix metalloproteinases are inhibited by an extracellular protein family known as the tissue inhibitors of metalloproteinases (TIMPs) which are released by the epithelial and stromal cells, and play an important role in regulating the effects of the ECM, cytokines, chemokines and growth factors on cell phenotype (Murphy et. al., 2011). Studies have shown that TIMPs have the potential to influence cell growth, differentiation, migration, apoptosis, and angiogenesis (Moore et. al., 2012).

Matrix metalloproteinases can be classified into six groups based upon the substrate specificity and similarity (Sternlicht et. al., 2001; Visse et. al., 2003). Collagenases (MMP-1, MMP-8, MMP-13) digest triple-helical fibrillar collagens I, II and III, ECM molecules and soluble proteins (Visse et. al., 2003; Woessner et. al., 2000). Gelatinases (MMP-2 and MMP-9) digest gelatin and ECM molecules, laminin and collagen types- IV, V and XI (Patterson et. al., 2001). Stromelysins (MMP-3, MMP-10, and MMP-11) digest collagen II, IV, IX, X, gelatin and fibronectin (Luo et.al., 2002). Matrilysins (MMP-7 and MMP-26) degrade collagen III, IV, V, IX, X, and proteoglycan ECM molecules (Marchenko et. al., 2004). The membrane-associated MMPs, known as Membrane Type-MMPs (MT-MMPs) in mammals, appear as either transmembrane proteins type-I (MMP-14, MMP-15, MMP-16, and MMP-24) or as glycosylphosphatidyl

inositol-anchored proteins (MMP-17 and MMP-25). This class of MMPs degrades gelatin, fibronectin, and laminin proteins (English et. al., 2000). There are seven other MMPs (MMP-12, MMP-19, MMP-20, MMP-21, MMP-23, MMP-27, MMP-28) which are not part of the categories listed above because, while they have similar structures, they are expressed in different locations (Kerkela et.al., 2001; Hou et.al., 2004).

In case of wound healing, MMP-1, MMP-8, MMP-13 play a pivotal role degrading the fibril forming collagen I, II, III. MMP-13 acts on collagen II whereas MMP-1 and MMP-8 preferentially cleave collagens I and III (Klein and Bischoff 2010).

The Role of the ECM in Wound Healing

Wounds that do not heal in a timely manner are classified as chronic wounds (Percival et. al., 2010). The normal healing process is retarded in a chronic wound due to some systemic dysfunction. Chronic wounds exhibit a considerably higher level of protease, particularly MMPs expression. Chronic wounds also exhibit a lack of integrin receptors for fibronectin binding and a lack of keratinocyte migration when compared to acute wounds (Trengove et. al., 1999). In addition to the elevated levels of proteases, chronic wounds exhibit elevated pro-inflammatory cytokines (Chin et. al., 2005) and reactive oxygen species (Tarnuzzer et. al., 1996) which inhibit healing. Tissue inhibitors of metalloproteinases which reduce the function of MMPs are present at low levels in chronic wounds, thus enhancing the degradation of collagens in the wound (Ladwig et. al., 2002). These physiologic differences inhibit cell migration and the formation of the ECM. As a result, the formation of viable granulation tissue is disrupted and limits the re-epithelialization of a wound site.

The expression and deposition of collagen depends upon inflammatory reactions at the site of tissue injury. Studies have demonstrated that the inflammatory cytokines-interleukin-17 and tumor necrosis factor enhance the deposition of type-I collagen (Amara et. al., 2015). Conversely, a low level of inflammation leads to reduced deposition of collagen (Schultz et. al., 2005).

Researchers have demonstrated that collagen-III degradation by matrix metalloproteinases and consequently replacement with stronger collagen-I plays a significant part in the progression of wound healing, and the development of a scar. MMP expression and production are greatly influenced by mechanical stretch at the wound site (Nagase et. al., 2006). Studies have shown that the mechanical stresses at the wound can lead to the release of reactive oxygen species (ROS). The ROS in turn activates MMPs (Asanuma et. al., 2003). When the mechanical equilibrium is restored and the mechanical stretch at the wound has subsided, the level of MMPs decreases.

TRANSFORMING GROWTH FACTOR-β

TGF- β is one of the important growth factors which exhibit pleiotropic effects on wound healing by influencing cell development, fibroblast proliferation, collagen synthesis and transformation of fibroblasts into myofibroblasts (Liu et. al., 2004). Three isoforms of TGF-β family (TGF-β1, TGF-β2 and TGF-β3) have been isolated, purified, and characterized in mammals (Grimaud et.al. 2002). These three factors are secreted by macrophages, fibroblasts, keratinocytes, and platelets (Barrientos et. al, 2008). TGF-β signaling is transmitted by a pair of transmembrane serine/threonine kinase receptors known as the type-I and type-II TGF-β receptors, and Smad proteins are the major intracellular mediators of TGF- β family members. TGF-β superfamily ligands first attach to type-II TGF-β receptors which subsequently phosphorylate a nearby type-I TGF-β receptor, resulting the activation of its kinase. The activated type-I TGF-β receptor transmits the signal by phosphorylating intracellular Smad 2 and Smad 3, which carry the signal into the nucleus and regulate target gene expression.

Biosynthesis of ECM

One of the primary functions of TGF-β is to enhance the synthesis of collagen and fibronectin. TGF- β increases the expression of m-RNA for collagen type I, II, III, V and fibronectin which in turn boosts their biosynthesis (Liu et. al., 2004). Additionally, TGF-β can suppress the activity of proteolytic enzymes produced in the extracellular matrix. Protease suppression is affected by two different mechanisms, which are involved in either the production of protease inhibitors or reduction in protease synthesis. Between

these two mechanisms, protease activity is suppressed and newly produced matrix proteins are protected from proteolytic degradation (Nasatsky et al., 2000).

Roles of TGF-β in wound healing

All three isoforms of TGF-β play a role in the process of wound healing. Studies have shown that the release of TGF-β1 at an early phase of healing stimulates the recruitment of inflammatory cells into the injury site. This isoform also helps wound fibroblasts secrete key elements of the ECM, fibronectin, collagen-I and collagen-III. Additionally, TGF-β1 improves angiogenesis at the injured site by recruiting endothelial progenitor cells and stimulates contraction of the wound (Evrard et. al., 2012). Finally, TGF-β1 is considered to be one of the main factors that enhance collagen deposition at the wound site by inhibiting MMPs that degrade collagen and thus promoting the accumulation of collagen fibers at the site of the wound (White et. al., 2000).

TGF-β2 promotes the recruitment of fibroblasts and immune cells at the wound site and contributes to the formation of granular tissue, angiogenesis, and collagen synthesis (Barrientos et. al., 2008). Studies have demonstrated that TGF-β2 is required for collagen expression and organization, and for the formation of other key ECM components during the healing process (Thompson et. al., 2006). TGF-β3, however, has a different role in wound healing than TGF-β1 and TGF-β2. It has been observed that TGF-β3 has TGF-β1-antagonistic effects in the formation of scars (Waddington et. al., 2010). Studies have shown that in wounds with minimal scar formation, the amount of TGF-β1 decreases as the ratio of TGF-β3 to TGF-β1 increases (Schrementi et. al., 2008). Researchers hypothesize that TGF-β3 directs the decreased scar formation and decreased

collagen-I deposition through its inhibition of myofibroblast differentiation and the stimulation of MMP-9 (Hosokawa et. al., 2003).

The effect of TGF- β type-I on inflammatory cells

TGF-β type-I is well known for its immunosuppressive properties, but it also possesses two opposing characteristics. TGF-β1 can suppress the synthesis of many cytokines, including interferon-γ, tumor necrosis factor-alpha and various interleukins. On the other hand, TGF-β1 can also enhance the secretion of certain cytokines by T cells and promote T cells proliferation (Letterio et. al., 1998).

Studies have demonstrated that the lack of TGF-β type-I signaling in knockout mice results in a substantial decrease in monocyte infiltration at the site of a wound (Ashcroft et.al., 1999). In other studies researchers have shown an increased inflammatory response in the organs of TGF-β type-I knockout mice (Crowe et. al., 2000). These knockout mice displayed an increase in peripheral lymphocytes and neutrophils as well as an increase in proliferating cells in the spleen and lymph nodes. Some studies have shown that TGF-β1 can slow the healing process. Li et. al. have reported that overexpression of TGF-β1 in keratinocytes resulted in chronic inflammation of the wound (Li et.al., 2004). Finally, some research has shown that fibroblast production of TGF-β can vary throughout the wound healing process. These studies showed that TGF-β1 levels were higher with longer periods of fibroblast infiltration (Pakyari et. al., 2013).

OXIDATION OF COLLAGEN AT METHIONINE RESIDUE

The oxidation of many proteins by reactive oxygen species (ROS) can lead to amino acid modification, proteolysis, fragmentation, and loss of biological activity (Chao et.al., 1997). The effects of collagen oxidation include the depolarization of the triple helix, an impaired ability to form fibrils, and increased collagen fragmentation. Methionine, a sulfur-containing amino acid, present in collagen plays a key role in the cell's antioxidant defense through the reversible oxidation and reduction of its sulfhydryl group (Rodney et. al., 2000). Methionine can be readily oxidized by neutrophils oxidants, like hydrogen peroxide, hypochlorous acid, oxygen, nitric oxide, and superoxide.

Regulation via Methionine Oxidation

Sone of the major metabolic byproducts that are formed during injury, wound healing and inflammation are the reactive oxygen species. These are so highly reactive that they rapidly oxidize cellular proteins at susceptible amino acid residues (Chao et.al., 1997). Generally, the oxidation of amino acid residues in a protein can lead to the loss of biological activity or even degradation. For some proteins, the addition of oxygen to the protein structure can cause rearrangement of the original protein folding and increase the surface hydrophobicity of the molecule. As the surface hydrophobicity becomes more extensive, the protein becomes a target for degradation by the proteasome. For some proteins oxidation at amino acid residues enhances the ubiquitin binding, which tags a protein for proteasome processing (Glickman et. al., 2002). Thus oxidation promotes proteasome degradation by through both ubiquitin-dependent and ubiquitin-independent pathways.

Researchers have proposed a variety of defense mechanisms that cells use to scavenge ROS and repurpose or recycle the oxidation energy stored in the ROS. One of the currently proposed mechanisms is based upon a pool of proteins that act as a kind of "oxidation" reservoir. These proteins contain oxidizable amino acid residues that are rapidly available for interaction with ROS (Stadtman et. al., 2002). In this mechanism, these reservoir proteins act to sponge up the ROS and prevent them from attacking and inactivating other functional proteins. In some of these proteins methionine, a sulfur-containing amino acid, serves as the antioxidant.

Methionine is easily oxidized to form methionine sulfoxide [Met (O)] (Vogt et. al., 1995) and can be oxidized by key cellular oxidants such as hydrogen peroxide, hypochlorous acid, oxygen, ozone, and superoxide (Figure 3). In many situations, methionine sulfoxide is recycled and converted back to methionine to be reused as an antioxidant. This process depends on the enzyme methionine sulfoxide reductase (Msr). Methionine sulfoxide reductases can reduce methionine sulfoxide residues through the linked oxidation of NADPH (Ruan et. al., 2002). There are two types of methionine sulfoxide reductases, MsrA, which reduce the S-isomers of methionine sulfoxide (kryukov et. al., 2002), MsrB, which reduce the R-isomer of methionine sulfoxide (Kryukov et. al., 2002).

Proline, another amino acid target of oxidation, is oxidized by hydrogen peroxide to form glutamate (Figure 4). Oxidation of proline eliminates its contribution to the α-helical structure of the collagen molecule, and as a result affects the rigid structure of collagen and causes fragmentation of collagen-I peptide chain (Chunxia et. al. 1991). This ultimately affects the stability and conformation of the collagen-I fibers.

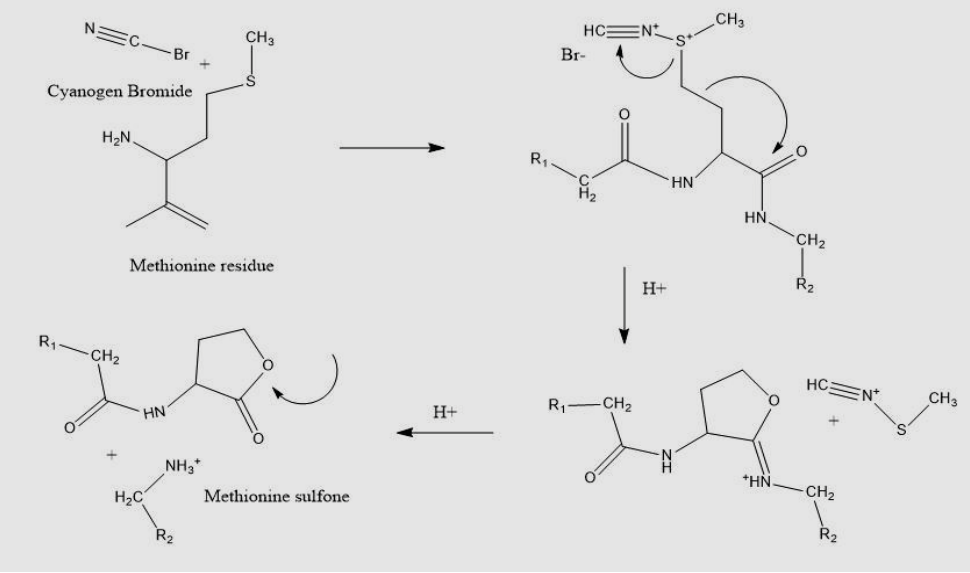

Figure 3: Oxidation of collagen at methionine residue.

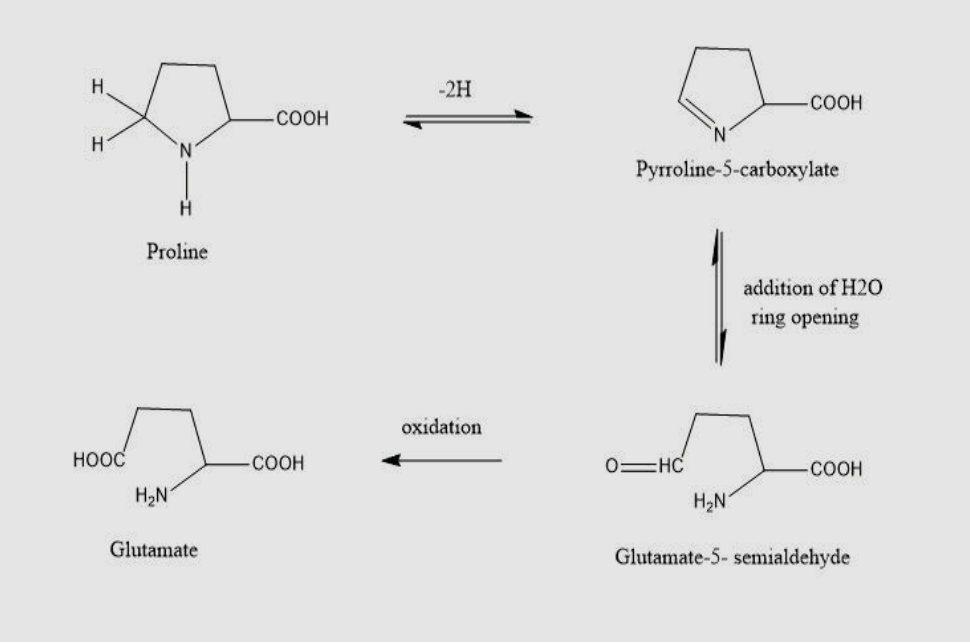

Figure 4: Oxidation of collagen at proline residue.

Some of the strongest evidence supporting a role of methionine in the cells antioxidant defense mechanism comes from studies of aging in animals. Researchers have demonstrated that the amount of MsrA gene expression in rat brain, kidney, and liver decreases with age (Petropoulos et. al., 2001). Studies have shown that MsrA knockout mice have a 40% decrease in their life span, whereas overexpression of the MsrA gene in *Drosophila* (small flies) results in increasing the maximal life span by twofold (Gabbita et.al., 1999). Finally, studies characterizing the surface hydrophobicity of liver proteins and ROS-mediated protein oxidants in rats have shown an age-dependent increase in both protein hydrophobicity and a decrease in methionine sulfoxide capacity (Levine et. al., 2002).

PROJECT OBJECTIVE

Studies have shown that ROS are present in the environment throughout much of the wound healing process and that these agents can influence the composition and properties of the scar tissue. Collagen, one of the major components of scar tissue, is susceptible to oxidation at lysine, proline and methionine residues (Rangaraj et. al. 2011). The biochemical and structural properties of the collagen molecule suggest that oxidation at these residues can impact collagen fibril conformation and collagen fiber stability. Thus, understanding how ROS alters the collagen fiber can provide insight into the stability and strength of the collagen fiber in scar tissue. One of the objectives of this study is to examine the effect of ROS oxidation on the collagen fiber structure and organization by studying the changes in the 2D gel electrophoresis profile of MRC-5 cell culture collagen-I.

Evidence indicates that cytokines are also present in the environment during the wound healing process and play a key role in regulating the progression of wound healing. While there is much evidence documenting that the cytokine, TGF-β, stimulates collagen synthesis, little is known about resultant collagen fiber assembly, structure or stability. A second objective of this study is to examine collagen fiber structure and ROS sensitivity following TGF-β-stimulated synthesis by studying the changes in the 2D gel electrophoresis profile of TGF-β-treated MRC-5 cell culture collagen-I.

In summary, the objectives of this project are to: 1) Identify ROS-oxidation induced changes in collagen structure and composition in the MRC-5 cell culture; 2) Characterize the collagen structure and composition of TGF-β-treated MRC-5 cells and; 3) Evaluate the ROS sensitivity of collagen from TGF-β-treated MRC-5 cells.

Our hypotheses of this project are: 1) Oxidation would cause changes the structure of collagen due to the presence of methionine and proline residues in collagen peptide chain which are more susceptible to become oxidized. 2) TGF-β would enhance the synthesis of collagen and fibril since it is one of the major cytokines present during wound healing. 3) TGF-β would play an important role in wound healing by protecting collagen from being oxidized. An *in vivo* study suggest TGF-β suppress the secretion of nitric oxide (NO) by macrophages and also prevent oxidation.

MATERIALS & METHODS

MATERIALS

Cell Culture

Human lung fibroblast [Medical Research Council Cell strain-5 (MRC-5)] were obtained from ATCC (American Type Culture Collection, Manassas, VA). Sodium pyruvate (1%) and glutamine (1%) by Gibco (Grand Island, NY). Fetal calf serum was purchased from Invitrogen (Frederick, MD). Minimum Essential Medium Eagle's from Sigma-Aldrich Co. LLC (St. Louis, MO). SIGMA*FAST*™ protease inhibitor cocktail (1%) from Calbiochem- EMD Millipore Corporation (Billerica, MA).

2D Gel Electrophoresis

ReadyStrip™ IPG Strip 7 cm, pH 3-10 (# 163-2000-MSDS), Acrylamide/bis-acrylamide (30%) (# 1610158), 1.5 M Tris-HCl, pH 8.8 (#161-0798, 1 L), Sodium dodecyl sulfate (SDS) solution, 10% (w/v) (#161-0416-MSDS, 250 ml), and Tetramethyl ethylene diamine (TEMED) (# 1610801, 50 ml) were obtained from Bio-Red Laboratories Inc. Ammonium persulfate (10%) was prepared with Type-I water. SnakeSkin™ dialysis tube (# 88242, 3.5K MWCO) was purchased from Thermo Fisher Scientific Inc. Coomassie blue (#B0149-25G), Cyanogen bromide (#C91492-25G), Bovine serum Albumin (#A2153-10G), and Catalase (#C9322-1G) were obtained from Sigma-Aldrich Co. LLC (St. Louis, MO).

METHODS

Fibroblast Cell Culture

Human lung fibroblast cells (MRC-5) were grown with MRC-5 media, consisting of Minimum Essential Media-Earles solution, sodium pyruvate (1%), glutamine (1%), and fetal calf serum (10%). Cells were seeded at a concentration of 5 x 10^5 cells per ml, fed with MRC-5 media (10 ml) and incubated at 37°C with 5% CO_2 and 95% room air. The cells were fed every three days until they became fully confluent. Confluent cultures were washed with 1% phosphate buffer saline (PBS) and incubated for 72 hours with either serum-free MRC-5 media (control group) or with serum free MRC-5 media containing 0.5% TGF-β (experimental group) (Illsley et. al. 2000; Shi et. al. 2013). After 72 hours of incubation, the media was collected in a 15 ml conical tube and stored at -80°C. The culture was incubated with 1% SIGMA*FAST*™ protease inhibitor cocktail to avoid the degradation of proteins. Remaining adherent cells were scraped from the surface using S|P® Brand diSPo® Cell Scrape. The adherent and released cells were collected in a 15ml conical tube and stored at -80°C. Both media and cells were then lyophilized separately and processed for collagen analysis.

Tissue Processing

The properties of soluble and insoluble collagen were examined separately in these experiments by analyzing culture media (media lyophilates) rich in soluble collagen, and MRC-5 cells and their associated matrix of insoluble collagen (cell lyophilates). Half of the lyophilates was used for collagen analysis and the other half was for collagen oxidation.

Cell lyophilates were suspended in 4ml PBS with 0.5% protease inhibitor and then homogenized at 1,500 rpm using a Potter-Elvehjem homogenizer attached to a Redeye 5 Speed Drill Process (Global Machinery Company). The resulting homogenate was then centrifuged at 15,000 rpm for 25 min at 4°C. The supernatant was discarded and the cell fragments pellet washed three times with 5ml 2% sodium dodecyl sulphate (SDS) solution. The pellet was then washed with 5ml PBS three times and dried under air for 20 minutes.

Cell pellets and lyophilized culture media were suspended with 5ml formic acid (70%) in a 15 ml conical tube and digested with cyanogen bromide (CNBr) (250 mg) overnight while agitated at 300 rpm on an orbital shaker (Fietzek et. al. 1969; Rauterberg et. al. 1971). The CNBr digestion was then centrifuged at 15,000 rpm for 30 min at 15°C, the supernatant was collected, and dialyzed for 4-5 hours against Type-I water to remove the CNBr from the solution and then lyophilized.

Collagen Oxidation

Half of the lyophilate of the cells and media was dissolved separately in 1 ml 0.02M glacial acetic acid and transferred to 15 ml conical tube. The solution was then incubated with 6µl 1M $CuSO_4$ and 68µl 3% H_2O_2 at room temperature for 30 minutes. The oxidation reaction was terminated by adding 1.5ml 3% catalase solution (Sigma-Aldrich Co. LLC) and incubating at room temperature for 10 minutes (Nowotny et. al. 2014). At this point, 5ml 70% formic acid and 250 mg CNBr were added and the samples were incubated in a shaking rotator overnight at room temperature. The reaction mixture was centrifuged at 15,000 rpm for 30 min at 15°C and the supernatant collected, lyophilized and stored at -20°C until analyzed.

2D Gel Electrophoresis

Cyanogen bromide digested cells and media lyophilates were loaded onto 7-cm immobilized pH gradient (IPG) strips (Bio-Red) by passive rehydration for 18 hours. The strips were focused overnight using a Protean IEF Cell (Bio-Rad). On the day of gel analysis, IPG strips were incubated with 1 ml Equilibration Buffer-I (36% Urea, 25% 1.5M Tris-HCl, 20% Glycerol, 2% SDS, 2% Dithiothreitol (DTT), 15% miliQ water) for 10 minutes and then with 1 ml Equilibration Buffer-II (36% Urea, 25% 1.5M Tris-HCl, 20% Glycerol, 2% SDS, 2.5% iodoacetamide, 14.5% miliQ water) for 10 minutes. The strips were then applied to the surface of vertical 12% SDS–polyacrylamide gels and run at 20mA/gel until the dye front reach the bottom of the gel. Cyanogen bromide digested bovine serum albumin (BSA) was run in a lane to the left of the IEF strip on all 2D gel electrophoresis. This lane was used to mark the molecular weight migration for each gel. The gels were stained in 0.25% Coomassie Blue R, destained in 40% methanol/10% glacial acetic acid/50% miliQ water, and then fixed in 50% methanol/ 10% acetic acid/40% miliQ water. Gels were scanned using HP LaserJet and images were stored as JPEG files that were later analyzed for retention factor (R_f) and isoelectric focusing (IEF) values. Previous studies performed in our lab have shown that the catalase used in oxidation protocol does not appear on the collagen gels. The peptide spots having a molecular weight less than 40 KDa were considered as low molecular weight peptide fragments and those spots having a molecular weight greater than 40 KDa were considered as high molecular weight peptide fragments.

RESULTS

Comparison of Collagen Content and 2D Gel Electrophoresis Profile of MRC-5 Cells and Media

One of the goals of this project was to characterize 2D gel electrophoresis profile for collagen-I of MRC-5 cell cultures, and to identify the changes elicited by ROS-oxidation. The second goal was to characterize the collagen-I profile of TGF-β treated MRC-5 cell cultures, and to identify the changes elicited by ROS oxidation.

Rat-tail collagen type-I (RTC-I) was used as a collagen type-I standard for these experiments. The 2D gel electrophoresis profile of the CNBr-digested rat tail collagen type-I (300 µg) displayed six identifiable spots RTC-1, RTC-2, RTC-3, RTC-4, RTC-5, and RTC-6 (Figure 5). The retention factor (R_f) and isoelectric focusing (IEF) values of these spots were calculated (Table 1) and used to identify 2D gel spots visualized in the experimental preparations. This profile was similar to the rat-skin collagen-I profile published by Tanaka et. al.1981. Six gel spots of the rat-tail collagen type-I in this study had R_f and IEF values (Table 1) similar to the spots α1CB6, α1CB7, α1CB8 observed in the rat-skin collagen-I profile of Tanaka et. al. (1981).

The calculated R_f and IEF values indicate that rat-tail collagen standard spots RTC-1 and RTC-2 correlate with α1CB6; spots RTC-3, RTC-4, and RTC-5 correlate with α1CB7; and spot RTC-6 correlates with α1CB8 (Table 1).

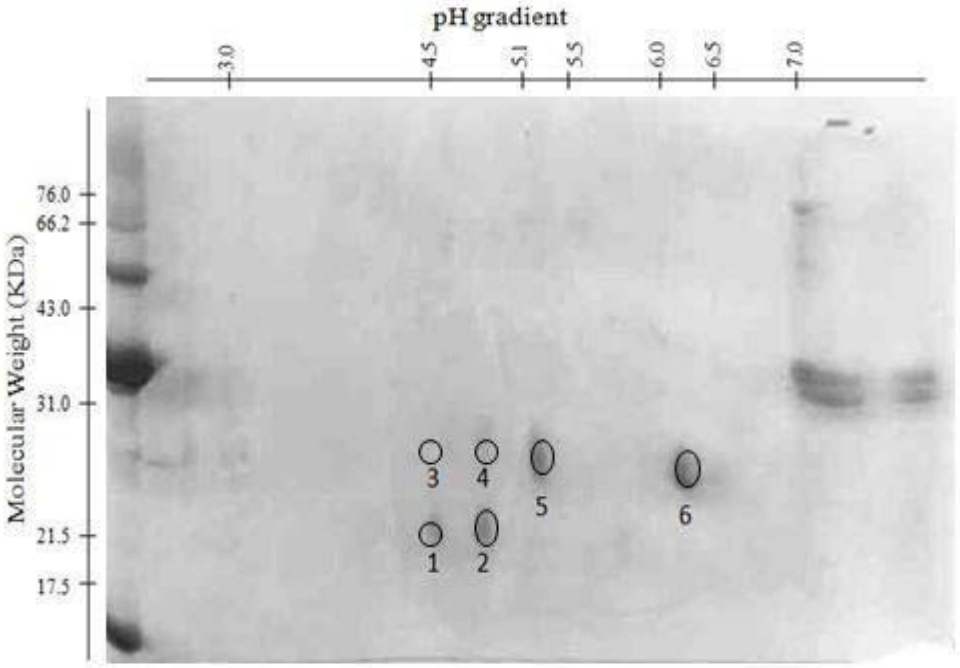

Figure 5: 2D gel electrophoresis profile of CNBr digested rat-tail collagen type- I (RTC-I) (300 µg). Cyanogen bromide digested BSA shown in left lane. The spots 1, 2, 3, 4, 5, 6 represent rat-tail collagen standard type-I standards RTC-1, RTC-2, RTC-3, RTC-4, RTC-5, and RTC-6 respectively.

Table 1: A comparison of R_f and IEF values of rat-tail collagen-I (RTC-I) standard to rat-skin collagen (Tanaka et. al. 1981)

RTC-I Spots	Collagen-I of Tanaka et. al. 1981	R_f	IEF
RTC - 1	α1CB6	0.7393	0.3847
RTC - 2	α1CB6	0.7393	0.4451
RTC - 3	α1CB7	0.6810	0.3959
RTC - 4	α1CB7	0.6854	0.4451
RTC - 5	α1CB7	0.7033	0.5150
RTC - 6	α1CB8	0.7078	0.6931

Collagen content of the MRC-5 cells

The 2D gel electrophoresis profile of MRC-5 cells indicated the protein concentration in this preparation was very low. Eleven peptide spots were identified in the 2D gel profile of MRC-5 cells, and none of the spots could be correlated with RTC-I standard (Figure 3). The spots A, B, C, D, E, F, G were low molecular weight peptide fragments (< 40 KDa) while spots H, I, J, K were high molecular weight peptide fragments (> 40 KDa) typically associated with cross-linked collagen fragments or incompletely digested collagen fibers (Figure 6). The R_f and IEF values of those spots are shown in Table 2.

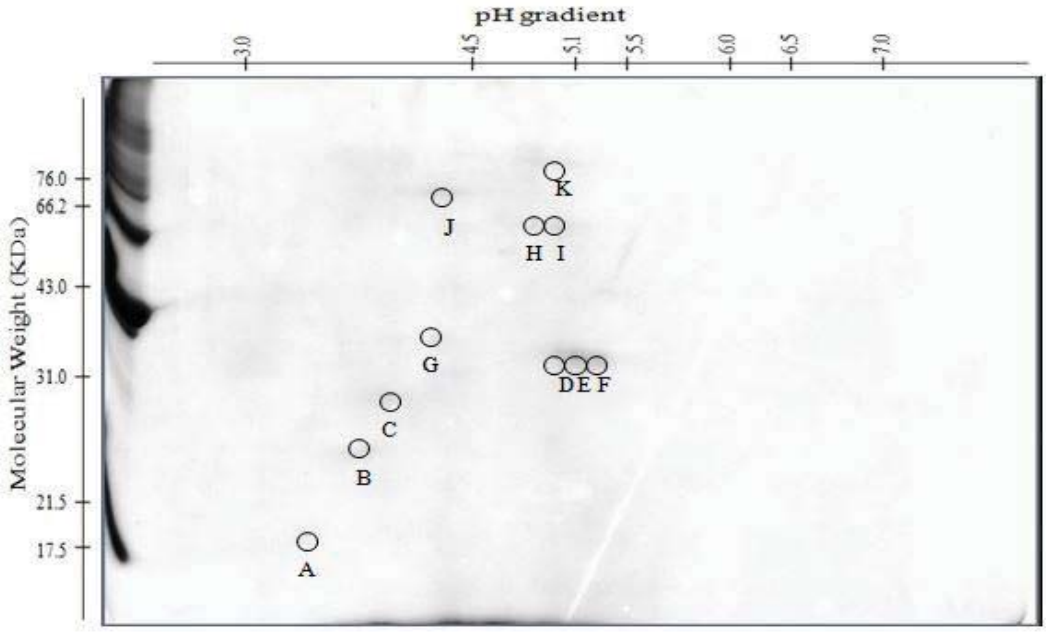

Figure 6: 2D gel electrophoresis profile of MRC-5 cells (75µl) identifying known CNBr digested collagen-I fragments and gel spots. Cyanogen bromide digested BSA shown in left lane.

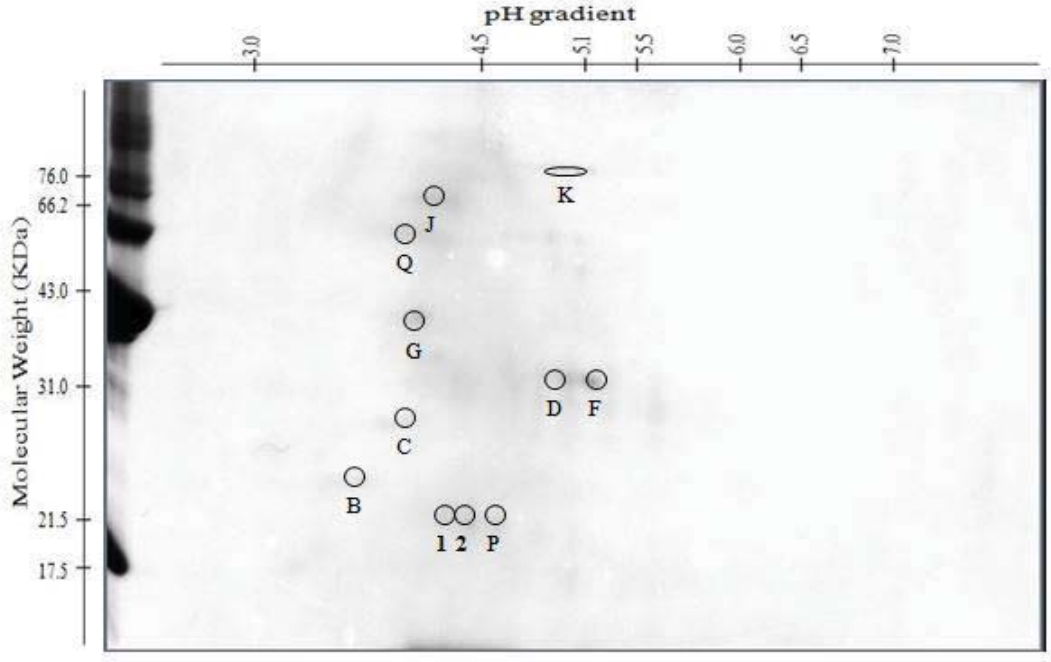

Figure 7: 2D gel electrophoresis profile of MRC-5 media (15µl) identifying known CNBr digested collagen-I fragments and gel spots. Cyanogen bromide digested BSA shown in left lane.

Collagen content of the MRC-5 media

The 2D gel profile of MRC-5 media and MRC-5 cells contained eleven peptide fragments. However, Coomassie Blue staining indicated the collagen fragment content of MRC-5 media was greater than that of the MRC-5 cells (Figure 7). Two of the peptide fragments (1, 2) migrated similarly to the RTC-I standard, and had R_f and IEF values consistent with those of RTC-1 and RTC-2 (Table 3). The remaining nine peptide spots (P, B, C, D, F, G, Q, J, K) represented a range of low and high molecular weight peptide fragments that did not correlate with RTC-I standard CNBr-digested fragments.

A comparison of the 2D gel profile of MRC-5 cell (Figure 6) and MRC-5 media (Figure 7) indicated that both had eleven peptide spots in common, but MRC-5 media had two peptide fragments that could be correlated to those of RTC-I standard (Table 3). In summary, both MRC-5 cells and MRC-5 media contained low collagen content.

Table 2: R_f and IEF values of CNBr digested collagen-I isolated from MRC-5 cells

Spots	R_f	IEF
A	0.8494	0.2244
B	0.6920	0.2871
C	0.5953	0.3148
D	0.5123	0.4926
E	0.5144	0.5102
F	0.5235	0.5350
G	0.4876	0.3585
H	0.2696	0.4634
I	0.2832	0.4854
J	0.2179	0.3760
K	0.1594	0.4869

Table 3: R_f and IEF values of CNBr digested collagen-I isolated from MRC-5 media

Spots	R_f	IEF
1	0.7662	0.3606
2	0.7820	0.3907
P	0.7888	0.4223
B	0.7100	0.2765
C	0.6044	0.3232
D	0.5326	0.5013
F	0.5258	0.5272
G	0.4202	0.3405
Q	0.2741	0.3462
J	0.2179	0.3764
K	0.1662	0.5201

The Effect of TGF-β on Collagen Content and 2D Gel Electrophoresis Profile of MRC-5 Cells

Coomassie Blue staining of the 2D gel indicated that the protein content of TGF-β treated MRC-5 cells was greater than that of the untreated MRC-5 cells (Figure 8). Fifteen peptide fragments were identified in the 2D gel profile, and the R_f and IEF values of these spots were calculated (Table 4). Among the fifteen peptide spots of TGF-β treated MRC-5 cells, two spots (1, 2) could be correlated with the 2D gel profile of RTC-I standard (Figure 8). The R_f and IEF values of spot 1 and 2 in TGF-β treated MRC-5 cells were consistent with those of RTC-1 and RTC-2 standard and characterized as α1CB6 (Table 4). The remaining thirteen peptide spots (L, M, B, C, D, E, F, N, G, H, O, I, J) represented a range of low and high molecular weight peptide fragments that did not correlate with RTC-I standard CNBr-digested fragments.

The collagen profile of TGF-β treated MRC-5 cells was similar to that of the untreated MRC-5 cells. The R_f and IEF values of nine of the untreated MRC-5 cells (Figure 6) peptide fragments (B, C, D, E, F, G, H, I, J) were similar to those in the 2D gel profile of TGF-β treated MRC-5 cells (Figure 8). In summary, MRC-5 cells cultured with TGF-β demonstrated more peptide fragments.

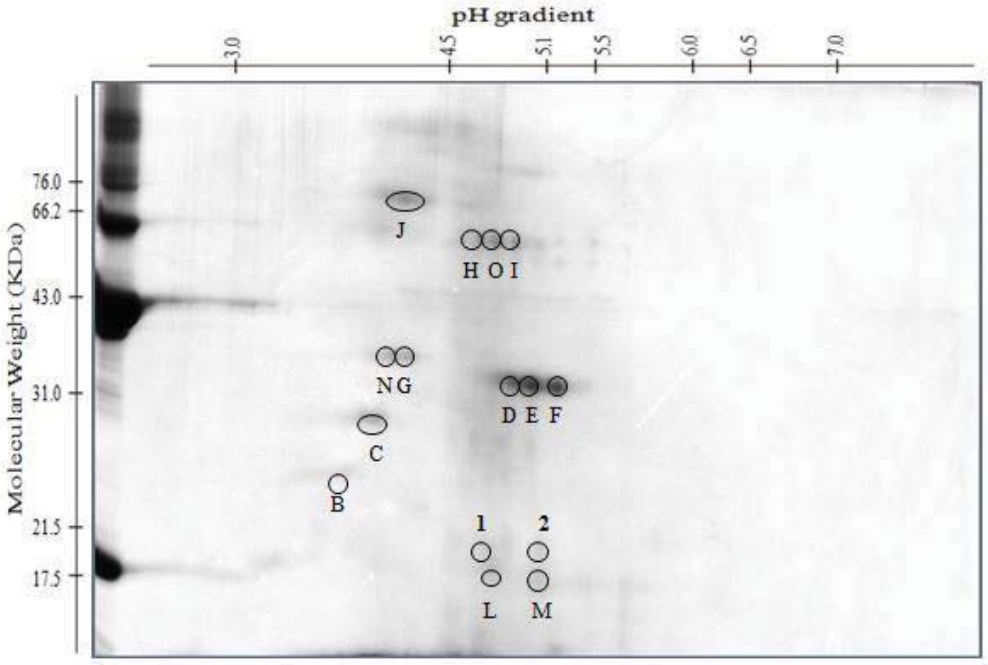

Figure 8: 2D gel electrophoresis profile of TGF-β treated MRC-5 cells (75µl) identifying known CNBr digested collagen-I fragments and gel spots that change as a result of TGF-β treatment. Cyanogen bromide digested BSA shown in left lane.

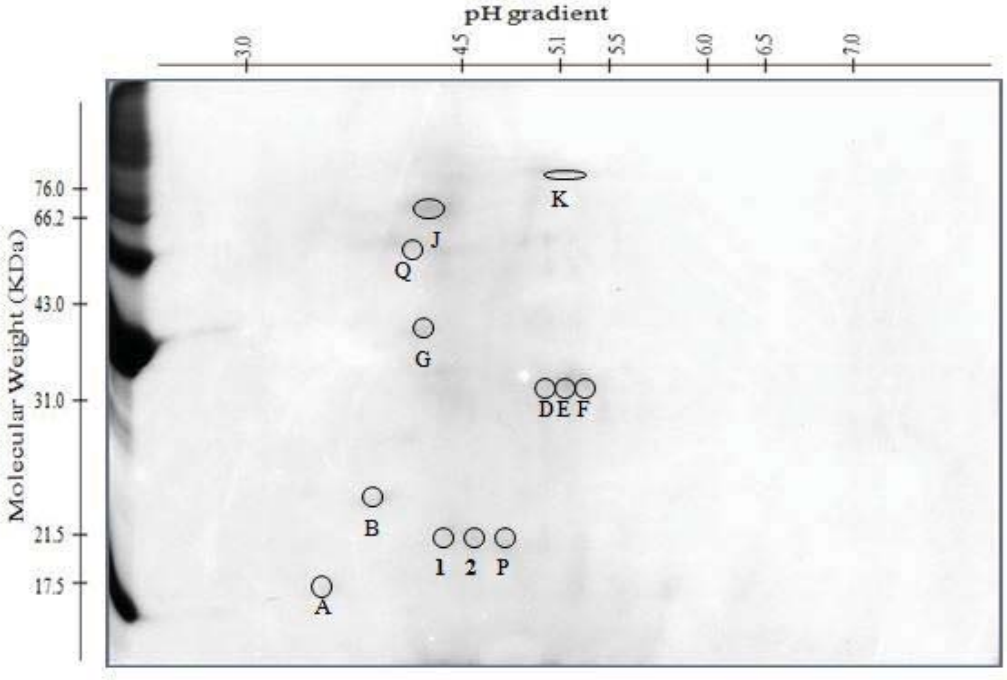

Figure 9: 2D gel electrophoresis profile of TGF-β treated MRC-5 media (100 µl) identifying known CNBr digested collagen-I fragments and gel spots that change as a result of TGF-β treatment. Cyanogen bromide digested BSA shown in left lane.

The Effect of TGF-β on Collagen Content and 2D Gel Electrophoresis Profile of MRC-5 Media

Coomassie blue staining indicted that the collagen content of TGF-β treated MRC-5 media was less than that observed in its MRC-5 cells counterpart (Figure 9). A total of twelve peptide spots were identified in TGF-β treated MRC-5 media. The R_f and IEF values of spot 1 and 2 in TGF-β treated MRC-5 cells were consistent with those of RTC-1 and RTC-2 standard and characterized as α1CB6 (Table 5). The remaining ten peptide spots (A, P, B, D, E, F, G, Q, J, K) represented a range of low and high molecular weight peptide fragments that did not correlate with RTC-I CNBr digested fragments.

A comparison of the TGF-β treated MRC-5 media to the untreated media indicated that the treated media contained more collagen peptide fragments. There were some similarities between the collagen profile of TGF-β treated MRC-5 media and untreated media. First, both preparations displayed collagen fragments RTC-1 and RTC-2. Second, the R_f and IEF values of eight of the collagen fragments in the TGF-β treated MRC-5 media (P, B, D, F, G, Q, J, K) were equivalent to those in the untreated MRC-5 media profile.

Table 4: R_f and IEF values of CNBr digested collagen-I isolated fromTGF-β treated MRC-5 cells

Spots	R_f	IEF
1	0.8162	0.4573
2	0.8230	0.5042
L	0.8706	0.4531
M	0.8978	0.5057
B	0.6983	0.2883
C	0.6009	0.3238
D	0.5329	0.4715
E	0.5442	0.4985
F	0.5418	0.5269
N	0.4875	0.3352
G	0.4851	0.3537
H	0.2771	0.4389
O	0.2879	0.4531
I	0.2856	0.4772
J	0.2155	0.3679

Table 5: R_f and IEF values of CNBr digested collagen-I isolated from TGF-β treated MRC-5 media

Spots	R_f	IEF
1	0.7878	0.3881
2	0.7946	0.4123
A	0.8691	0.2507
P	0.8037	0.4546
B	0.7292	0.3111
D	0.5260	0.4893
E	0.5281	0.5134
F	0.5268	0.5407
G	0.4378	0.3610
Q	0.2822	0.3489
J	0.2348	0.3790
K	0.1624	0.5241

The Effect of Oxidation on Collagen Content and 2D Gel Electrophoresis Profile of MRC-5 Cells

The cells of confluent MRC-5 cultures were split after harvesting; one half was oxidized and the other half served as the non-oxidized control. Overall, thirteen peptide spots were identified in the non-oxidized MRC-5 cells gel preparation (Figure 10). Ten of these were low molecular weight peptide fragments (1, 2, A, R, T, S, P, z, V, F), and three were high molecular weight peptide fragments (H, W, K). Two spots (1, 2) in the non-oxidized MRC-5 cells could be correlated with RTC-1 and RTC-2 standard and were identified as α1CB6 (Table 6). The spot 1, 2 which appear as a common blot in the photograph in Figure 10, were visible as distinct spots on the actual gel.

In contrast, fifteen peptide spots were observed in the oxidized 2D gel preparation of MRC-5 cells (Figure 11). Thirteen low molecular weight peptide fragments (1, 2, 6, X, Y, Z, a, b, c, T, S, P, d, E, F) and no high molecular weight fragments were identified. Two spots (1, 2) were correlated with the collagen standard fragments RTC-1 and RTC-2 and were identified as α1CB6. In addition, a third spot (6) not found in the non-oxidized control was visualized. The R_f and IEF values of this spot correlated with RTC-3 and was identified as α1CB7. The R_f and IEF values of these spots are shown in Table 7.

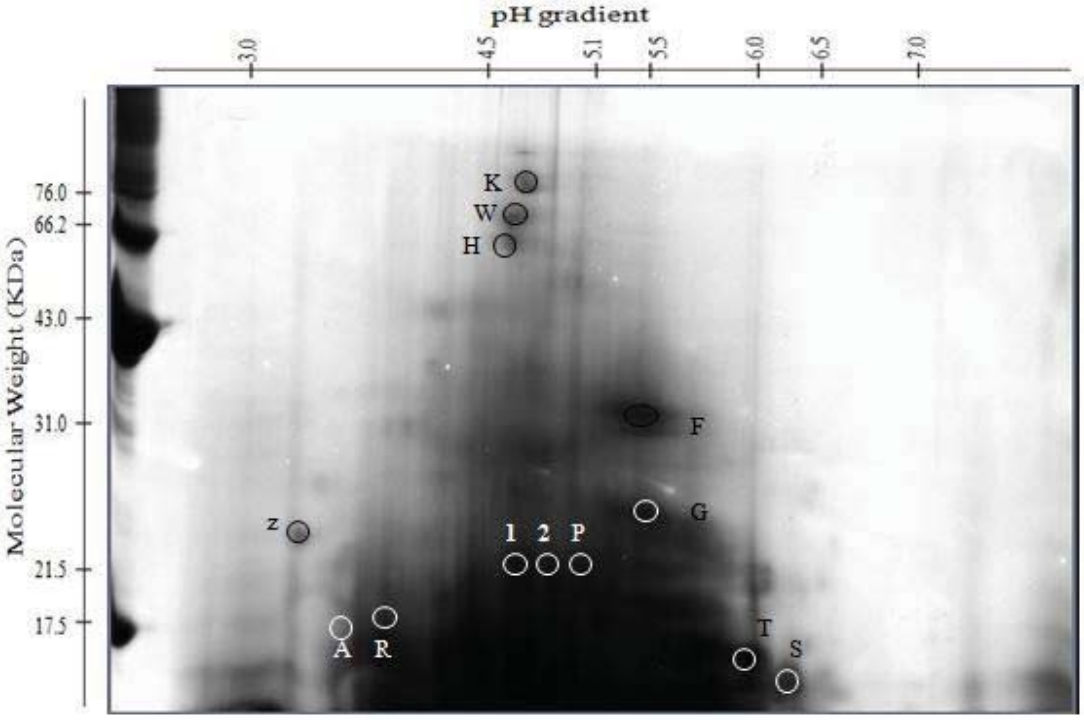

Figure 10: 2D gel electrophoresis profile of non-oxidized MRC-5 cells (75µl) identifying known CNBr digested collagen-I fragments and gel spots that change as a result of non-oxidation. Cyanogen bromide digested BSA shown in left lane.

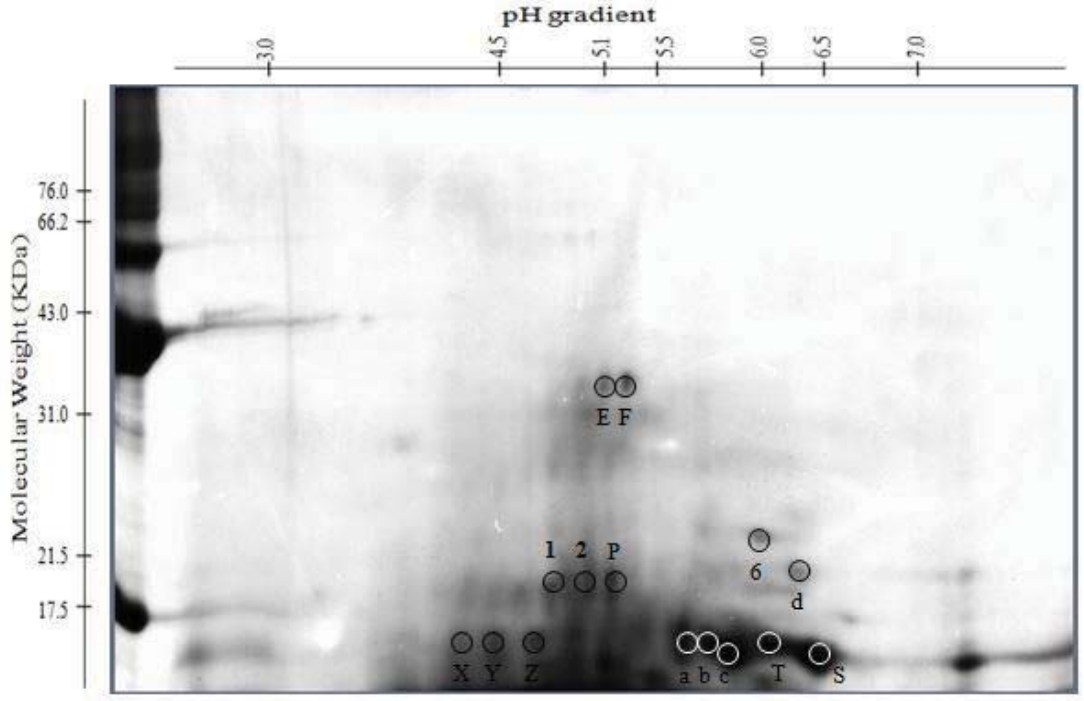

Figure 11: 2D gel electrophoresis profile of oxidized MRC-5 cells (75µl) identifying known CNBr digested collagen-I fragments and gel spots that change as a result of oxidation. Cyanogen bromide digested BSA shown in left lane.

Table 6: R_f and IEF values of CNBr digested collagen-I isolated from non-oxidized MRC-5 cells

Spots	R_f	IEF
1	0.7832	0.4310
2	0.7944	0.4615
A	0.8957	0.2867
R	0.9069	0.3389
S	0.9564	0.7162
T	0.9249	0.6718
P	0.8079	0.4969
z	0.7337	0.1993
V	0.6887	0.5735
F	0.5536	0.5858
H	0.2632	0.4172
W	0.2137	0.4294
K	0.1643	0.4447

Table 7: R_f and IEF values of CNBr digested collagen-I isolated from oxidized MRC-5 cells

Spots	R_f	IEF
1	0.8363	0.4574
2	0.8184	0.4958
6	0.7579	0.6812
P	0.8363	0.5197
X	0.9259	0.3611
Y	0.9261	0.4065
Z	0.9283	0.4490
a	0.9529	0.5991
b	0.9796	0.6320
c	0.9620	0.6585
T	0.9394	0.6997
S	0.9708	0.7478
d	0.8140	0.7181
E	0.5044	0.5127
F	0.5044	0.5438

A comparison of the 2D gel electrophoresis profile of oxidized and non-oxidized MRC-5 cells showed that the oxidation caused cleavage of peptide fragments and thus had more low molecular fragments (Figure 10) than that of non-oxidized MRC-5 cells group (Figure 11). Four peptide fragments (P, T, S, F) were visualized in both the oxidized and non-oxidized MRC-5 cells and had equivalent R_f and IEF values.

The Effect of Oxidation on Collagen Content and 2D Gel Electrophoresis Profile of MRC-5 Media

The media of confluent MRC-5 cultures were split into two groups after harvesting; one half was oxidized and the other half served as the non-oxidized control. The non-oxidized MRC-5 media sample (Figure 12) possessed fourteen low molecular weight peptide fragments (A, R, a, b, c, T, S, d, D, E, l, m, n) and five high molecular weight peptide fragments (o, H, O, I, J) which were not visible in the RTC-I standard. One gel spot (spot 6) was correlated with RTC-1 standard and identified as α1CB8 (Table 8).

In contrast, sixteen low molecular weight peptide fragments (3, 4, X, Y, Z, P, a, b, c, T, S, L, M, q, D, E) and four high molecular weight peptide fragments (o, H, O, J) were identified in the oxidized media 2D gel profile (Figure 13). Spots 3 and 4 were correlated with RTC-3 and RTC-4 standard and identified as α1CB7 (Table 9). The R_f and IEF values of these spots in both non-oxidized (Table 8), and oxidized (Table 9) media group were consistent with those of RTC-I standard.

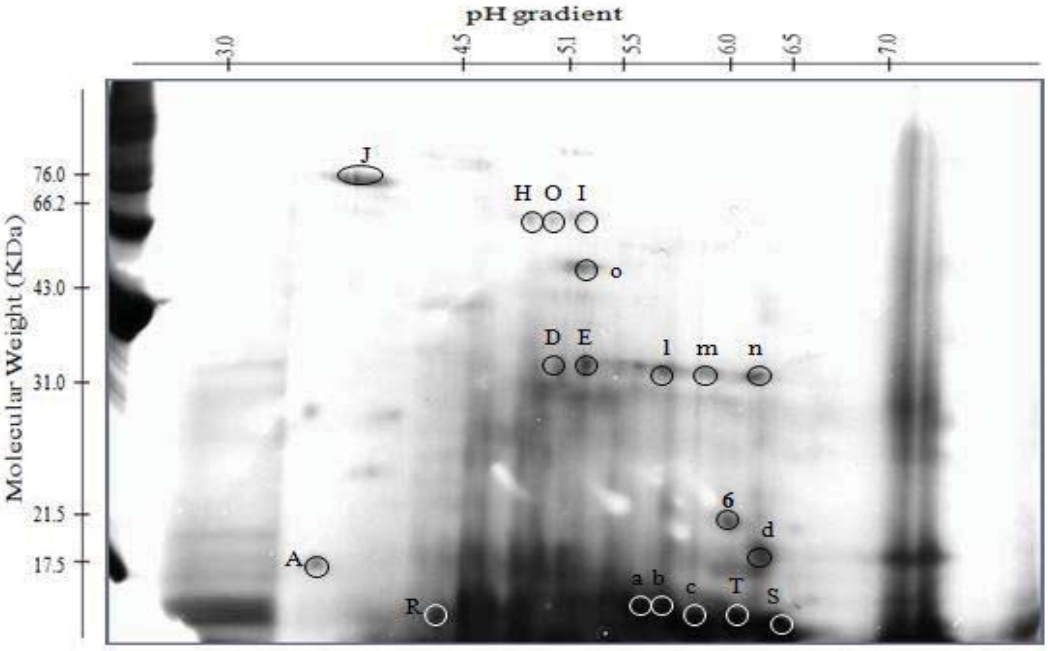

Figure 12: 2D gel electrophoresis profile of non-oxidized MRC-5 media (20µl) identifying known CNBr digested collagen-I fragments and gel spots that change as a result of non-oxidation. Cyanogen bromide digested BSA shown in left lane.

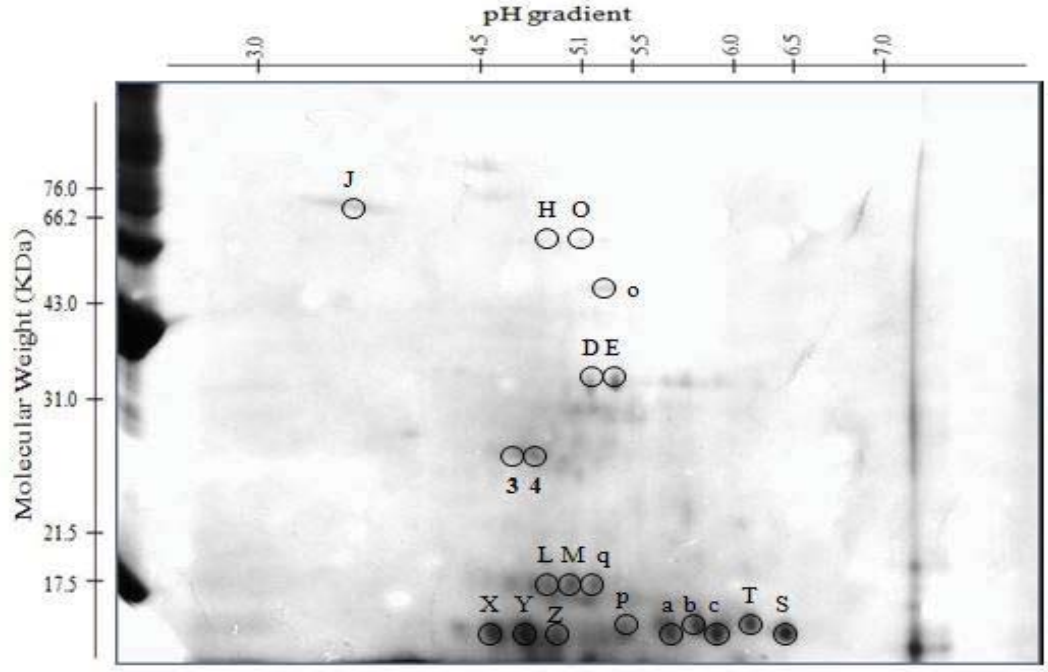

Figure 13: 2D gel electrophoresis profile of oxidized MRC-5 media (20µl) identifying known CNBr digested collagen-I fragments and gel spots that change as a result of oxidation. Cyanogen bromide digested BSA shown in left lane.

Table 8: R_f and IEF values of CNBr digested collagen-I isolated from non-oxidized MRC-5 media

Spots	R_f	IEF
6	0.8005	0.6707
R	0.9729	0.3522
a	0.9637	0.5743
b	0.9701	0.6321
c	0.9593	0.6618
T	0.9842	0.6662
S	0.9842	0.7290
A	0.8753	0.2250
d	0.8617	0.7060
D	0.5305	0.4855
E	0.5237	0.5160
l	0.5237	0.6033
m	0.5305	0.6446
n	0.5373	0.6998
o	0.3400	0.5130
H	0.2584	0.4548
O	0.2608	0.4809
I	0.2516	0.5084
J	0.1928	0.2986

Table 9: R_f and IEF values of CNBr digested collagen-I isolated from oxidized MRC-5 media

Spots	R_f	IEF
3	0.6476	0.4391
4	0.6544	0.4604
X	0.9794	0.4161
Y	0.9818	0.4505
Z	0.9818	0.4835
p	0.9726	0.5608
a	0.9726	0.6052
b	0.9544	0.6349
c	0.9681	0.6612
T	0.9523	0.6924
S	0.9658	0.7318
L	0.881838	0.470388
M	0.881838	0.498376
q	0.884188	0.524664
D	0.536325	0.516468
E	0.529487	0.547704
o	0.363675	0.532859
H	0.284188	0.481831
O	0.286325	0.508273
J	0.22265	0.279573

A comparison of the 2D gel profiles of oxidized and non-oxidized MRC-5 media demonstrated that the oxidized media group (Figure 12) had more low molecular fragments than that of non-oxidized media group (Figure 13). Eleven collagen spots (a, b, c, T, S, D, E, o, H, O, J) were present in both the 2D gel profiles of non-oxidized and oxidized MRC-5 media. All other spots were dissimilar and no other collagen peptide fragments could be identified in both MRC-5 media gel preparation. In summary, oxidation affected the structure of collagen peptide fragments thus less high molecular weight fragments were observed in oxidized MRC-5 media that followed the similar pattern in its oxidized MRC-5 cells counterpart.

The Effect of Oxidation on TGF-β treated MRC-5 Cells: The Collagen Content and 2D Gel Electrophoresis Profile

A series of experiments examined the effect of oxidation on collagen distribution and characterization of TGF-β treated MRC-5 cells. Confluent MRC-5 cultures were treated with TGF-β (0.5%) and split after harvesting; one half was oxidized and the other half served as the non-oxidized control.

There were a total of seventeen protein spots identified in the non-oxidized group of TGF-β treated MRC-5 cells (Figure 14). Fifteen low molecular weight fragments (A, X, Y, a, b, c, T, S, d, 6, C, e, D, E, F) and two high molecular weight fragments (H, I) were visible in the non-oxidized MRC-5 cells 2D gel preparation. The R_f and IEF value of spot 6 was consistent with that of RTC-6 standard and characterized as α1CB8 (Table 10). Four collagen peptide fragments (T, S, F, H) were also visible in the 2D gel preparation of untreated non-oxidized MRC-5 cells (Figure 10).

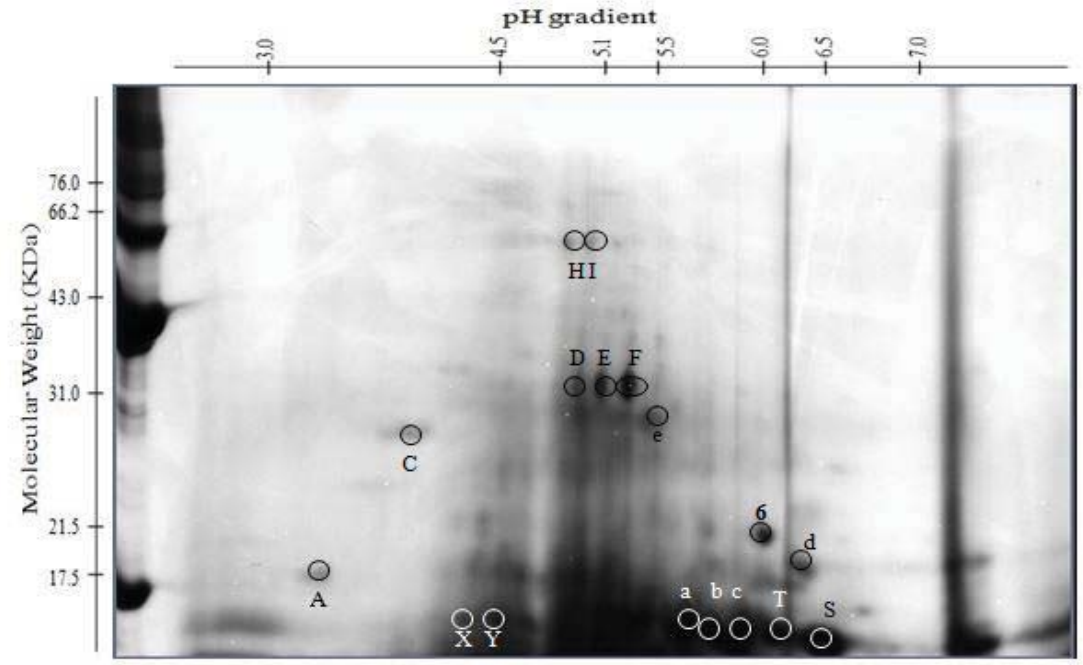

Figure 14: 2D gel profile of non-oxidized TGF-β-treated MRC-5 cells (75µl) identifying known CNBr digested collagen-I fragments and gel spots that changed with non-oxidation. Cyanogen bromide digested BSA shown in left lane.

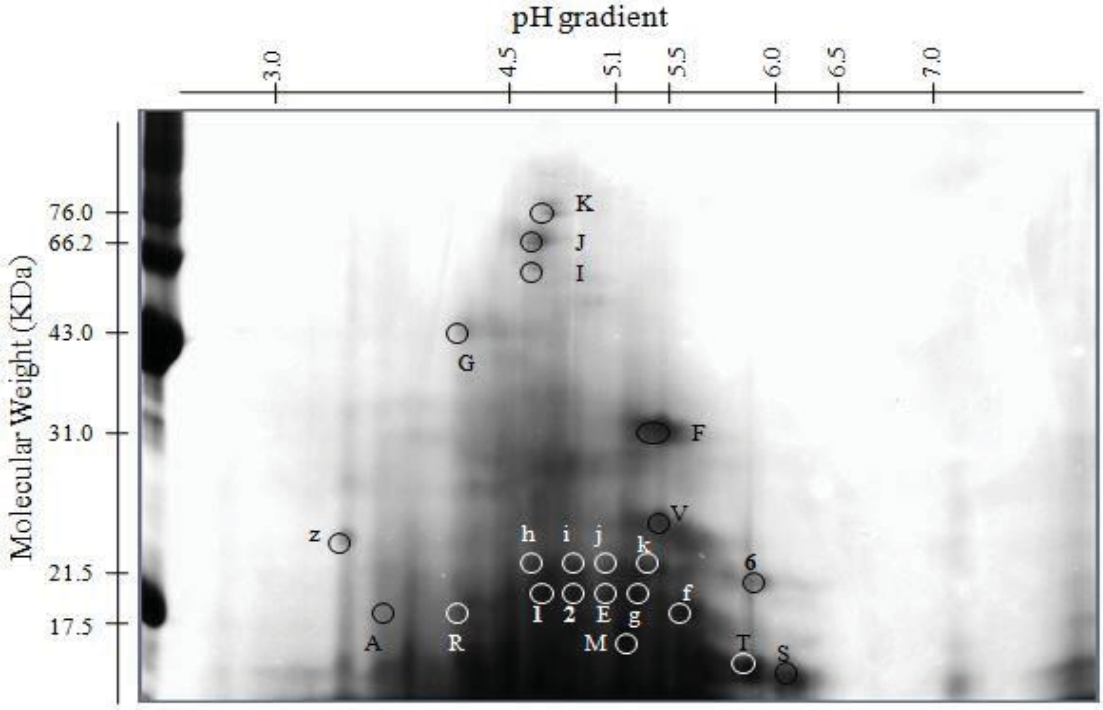

Figure 15: 2D gel electrophoresis profile of oxidized TGF-β-treated MRC-5 cells (75µl) identifying known CNBr digested collagen-I fragments and gel spots that changed with oxidation. Cyanogen bromide digested BSA shown in left lane.

In contrast, the oxidized group of TGF-β treated MRC-5 cells gel preparation contained twenty two protein spots (Figure 15). Eighteen low molecular weight fragments (1, 2, 6, A, R, M, T, S, P, g, f, z, h, I, j, k, V, F) and four high molecular weight fragments (G, I, J, K) were visible in the 2D gel preparation. The R_f and IEF values of spot 1, 2 and 3 were consistent with those of RTC-1, RTC-2 and RTC-6 standard, and characterized as α1CB6 and α1CB8 (Table 11). Seven peptide fragments (1, 2, 6, T, S, F) in the oxidized TGF-β treated MRC-5 cells could be correlated with those visible in the untreated oxidized MRC-5 cells (Figure 11). The spot 1, 2 which appear as a common blot in the photograph in Figure 12, were visible as distinct spots on the actual gel.

Table 10: R_f and IEF values of CNBr digested collagen-I isolated from non-oxidized TGF-β-treated MRC-5 cells

Spots	R_f	IEF
6	0.8077	0.6866
X	0.9457	0.3674
Y	0.9525	0.4045
a	0.9706	0.6054
b	0.9797	0.6310
c	0.9751	0.6666
T	0.9797	0.7036
S	0.9931	0.7634
A	0.8687	0.2180
d	0.8553	0.7265
C	0.6154	0.3247
e	0.6017	0.5711
D	0.5386	0.4885
E	0.5407	0.5157
F	0.5430	0.5456
H	0.2805	0.4743
I	0.2873	0.4971

Table 11: R_f and IEF values of CNBr digested collagen-I isolated from oxidized TGF-β-treated MRC-5 cells

Spots	R_f	IEF
1	0.8223	0.4283
2	0.8291	0.4581
6	0.8111	0.6551
A	0.9459	0.2702
R	0.9617	0.3581
S	0.9774	0.6940
T	0.9503	0.6508
M	0.8941	0.5134
f	0.8561	0.5820
P	0.8268	0.4896
g	0.8268	0.5224
h	0.7729	0.4269
i	0.7774	0.4626
j	0.7818	0.5015
k	0.7885	0.5462
z	0.7279	0.2133
V	0.6965	0.5507
F	0.5573	0.5686
G	0.3729	0.3462
I	0.2629	0.4179
J	0.2202	0.4224
K	0.1729	0.4388

A comparison of the 2D gel profiles of oxidized and non-oxidized TGF-β treated MRC-5 cells demonstrated that oxidized TGF-β treated MRC-5 cells (Figure 15) showed more high molecular weight peptide fragments than non-oxidized TGF-β treated MRC-5 cells. Six collagen fragments (6, A, S, T, F, I) were present in both non-oxidized and

oxidized TGF-β treated MRC-5 cells gel preparation. All other spots were dissimilar and no other fragments could be identified in both preparation. In summary, TGF-β played an important role preventing the MRC-5 cells from being oxidized and caused to form high molecular weight fragments.

The Effect of Oxidation on TGF-β treated MRC-5 Media: The Collagen Content and 2D Gel Electrophoresis Profile

A series of experiments investigated the effect of oxidation on the collagen profile of TGF-β treated MRC-5 media. Media from the confluent MRC-5 culture was split after harvesting; one half was oxidized and the other half served as the non-oxidized control.

Overall twenty four peptide fragments were identified in the 2D gel preparation of non-oxidized TGF-β-treated MRC-5 media (Figure 16). Nineteen of these were low molecular weight fragments (1, 2, A, X, Y, Z, a, b, c, T, S, r, s, t, D, E, l, m, n) and five were high molecular weight peptide fragments (o, H, O, I, J). The R_f and IEF values of spot 1 and 2 were consistent with those of RTC-1 and RTC-2 standard, and characterized as α1CB6 (Table 12).

In contrast, the 2D gel profile of oxidized TGF-β treated MRC-5 media (Figure 17) possessed twenty three peptide fragments. Sixteen low molecular weight protein fragments (u, a, b, c, T, S, v, d, w, 6, s, t, D, E, l, m) and seven high molecular weight fragments (o, H, O, I, J, x, y) were observed. The R_f and IEF values of spot 6 was consistent with that of RTC-6 standard and characterized as α1CB8 (Table 13).

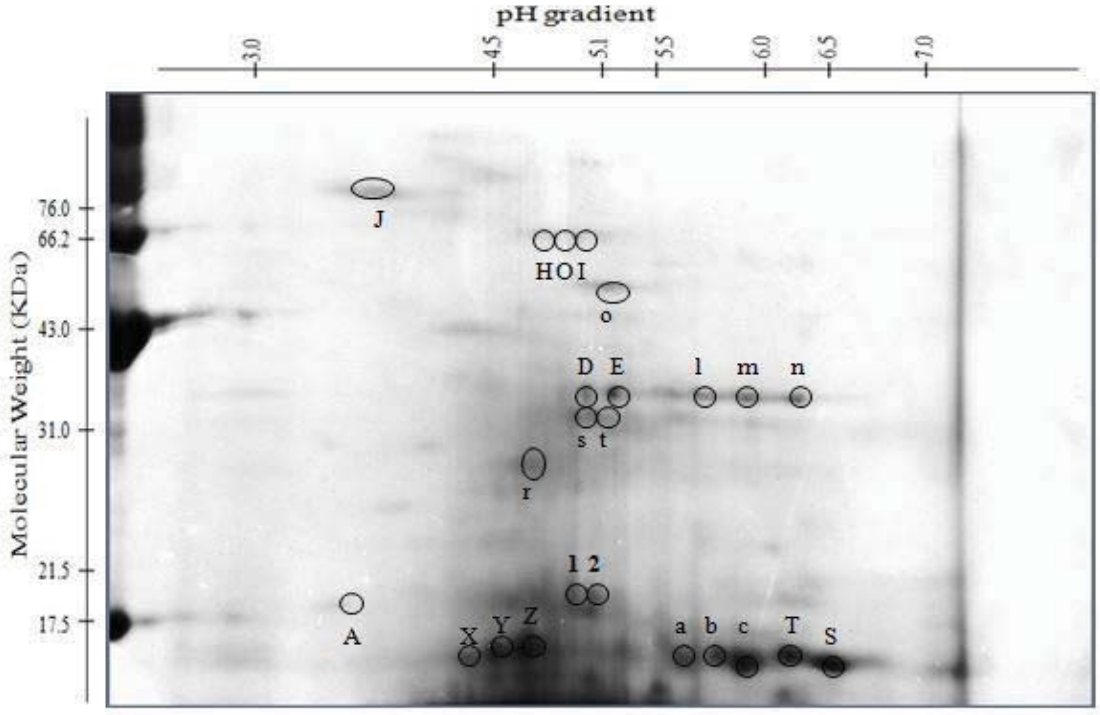

Figure 16: 2D gel profile of non-oxidized TGF-β treated MRC-5 media (25µl) identifying known CNBr digested collagen-I fragments and gel spots that changed with non-oxidation. Cyanogen bromide digested BSA shown in left lane.

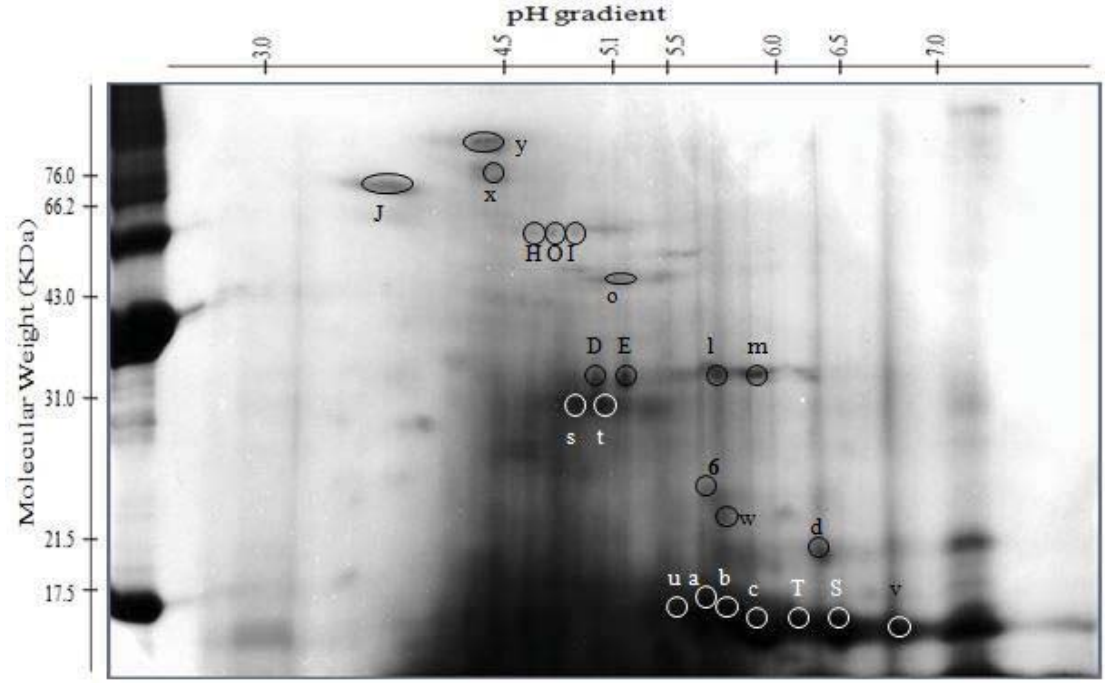

Figure 17: 2D gel profile of oxidized TGF-β treated MRC-5 media (25µl) identifying known CNBr digested collagen-I fragments and gel spots that changed with oxidation. Cyanogen bromide digested BSA shown in left lane.

Table 12: R_f and IEF values of CNBr digested collagen-I isolated from non-oxidized TGF-β treated MRC-5 media

Spots	R_f	IEF
1	0.8465	0.4761
2	0.8465	0.4984
X	0.9323	0.3671
Y	0.9278	0.3970
Z	0.9164	0.4343
a	0.9391	0.5881
b	0.9299	0.6240
c	0.9458	0.6656
T	0.9278	0.6986
S	0.9412	0.7539
A	0.8442	0.2373
r	0.6161	0.4417
s	0.5351	0.4732
t	0.5349	0.4705
D	0.5011	0.4910
E	0.4988	0.5148
l	0.4943	0.6015
m	0.5056	0.6522
n	0.5011	0.7074
o	0.3182	0.5269
H	0.2098	0.4417
O	0.2346	0.4626
I	0.2461	0.5000
J	0.1737	0.2836

Table 13: R_f and IEF values of CNBr digested collagen-I isolated from oxidized TGF-β treated MRC-5 media

Spots	R_f	IEF
6	0.7007	0.6164
u	0.9049	0.5848
a	0.9047	0.6108
b	0.9138	0.6479
c	0.9435	0.6822
T	0.9319	0.7055
S	0.9479	0.7534
v	0.9550	0.8055
d	0.8166	0.7274
w	0.7574	0.6232
s	0.5534	0.4781
t	0.5510	0.5054
D	0.5148	0.4972
E	0.5126	0.5233
l	0.4943	0.6205
m	0.4988	0.6643
o	0.3447	0.5163
H	0.2607	0.4314
O	0.2538	0.4506
I	0.2517	0.4766
J	0.1882	0.3082
x	0.1677	0.4041
y	0.1157	0.3986

A comparison of the 2D gel profiles of oxidized and non-oxidized TGF-β treated MRC-5 media showed that oxidized TGF-β treated MRC-5 media contained more high molecular weight peptide fragments and less low molecular weight peptide fragments (Figure 17) than that of non-oxidized TGF-β treated MRC-5 media (Figure 16). The non-oxidized media group had two spots that could be correlated with those of RTC-1 and RTC-2 standard (α1CB6) whereas the oxidized media group had one (α1CB8). Sixteen peptide fragments (a, b, c, T, S, s, t, D, E, l, m, o, H, O, I, J) were present in both the oxidized and non-oxidized TGF-β treated MRC-5 media. All other spots were dissimilar and no other collagen fragments could be identified in both preparation. In summary, TGF-β prevented the MRC-5 media from being oxidized, and the result followed the similar pattern in its oxidized MRC-5 cells counterpart where addition of TGF-β caused to form more high molecular weight fragments.

DISCUSSION

These studies report that MRC-5 cells and MRC-5 media contain collagen that can be identified by the presence of the well-characterized cyanogen bromide (CNBr) fragment, α1CB6. The collagen-I profile also displayed a variety of low and high molecular weight peptide fragments attributable to complex forms of partially digested collagens. The cells fraction of MRC-5 cultures did not display any identifiable collagen fragments, but some uncharacterized low and high molecular weight peptide fragments were visible. However, the concentration of these fragments was so low that it was difficult to evaluate them.

The addition of a cytokine growth factor, TGF-β, to confluent MRC-5 cultures increased the amount of collagen type-I peptide fragments in both MRC-5 cells and media. We report that TGF-β treated MRC-5 cells and media contained collagen that can be identified by the presence of CNBr digested fragments, α1CB6. This result is consistent with other reports in the literature that show that TGF-β plays an important role in the synthesis of collagen (White et. al. 2000, Liu et. al. 2004).

Some of the collagen fragments previously reported in CNBr digested preparations were not visible in either the TGF-β treated or the untreated preparations. The low molecular weight fragment, α1CB3, and the two high molecular weight fragments, α2CB4 and α2CB3.5, were not present in our preparations. This discrepancy might be due to the variation between culture samples and rat tail collagen. The collagen fiber structure and maturity presented in the fibroblast cultures may not represent the complex structure found *in vivo* tissues such as rat tail.

TGF-β -treatment of MRC-5 cultures resulted in an increase in extracellular collagen modification. We hypothesize that the increase in extracellular collagen in the 2D gel electrophoresis profile of MRC-5 media collagen was a result of changes in the handling and processing of extracellular collagen. For example, an increase in secreted collagen could overwhelm the conversion of procollagen to collagen by procollagen proteinase, leaving a surplus pool of procollagen molecules in the extracellular space. Alternatively, the increase in extracellular collagen fibrils could overwhelm the crosslink formation process necessary to generate stable collagen fibers. Increased extracellular collagen levels might ultimately exceed the capacity of lysyl oxidase, leaving a pool of collagen fibrils waiting to be processed (Fratzl et. al. 1997). As a result, a greater fraction of the extracellular collagen fibrils and fibers would be transiently linked by non-covalent bonds or reducible Schiff bases. These alternate forms of extracellular collagen would explain the difference in the 2D gel profile of TGF-β -treated and untreated cultures.

The oxidized MRC-5 cells and MRC-5 media gel preparation showed that oxidation changed the structure and composition of collagen-I peptide fragments. In both MRC-5 cells and MRC-5 media, oxidation increased the number of low molecular weight peptide fragments and reduced the amount of high molecular weight peptide fragments. These results were consistent with our hypothesis which predicted that oxidation would interfere with collagen cross-linking by reacting with methionine and proline residues.

Physicochemical studies of proline, one of the most abundant amino acids in collagen, indicate that this residue provides tensile strength and rigidity to the secondary and tertiary structure of proteins. Oxidative studies have shown that this amino acid is an easy target for oxidation by endogenous ROS (Hawkins et. al. 1997). Studies show that

treatment of collagen with Cu^{2+}/H_2O_2 causes cleavage of proline ring and generate glutamate (Hawkins et. al. 1997; Chunxia et. al. 1991). This alteration decreases proline content and which in turn promotes the fragmentation of collagen. Thus, oxidation of collagen fiber at this residue could contribute to the formation of a more flexible, weaker collagen and ultimately scar tissue that is more likely to deform and break with stress. Conversely protecting collagen fibers from oxidation could yield stronger, more resilient fibers and scar tissue.

The results of oxidation on TGF-β treated MRC-5 cells and media also support the study hypothesis that oxidation would interfere with collagen stability. TGF-β treated oxidized MRC-5 cells displayed more high molecular weight peptide fragments than the TGF-β treated non-oxidized MRC-5 cells. These results suggested that TGF-β treatment could have caused the methionine and proline residues of collagen-I peptide fragments to become less susceptible to oxidation and CNBr degradation. These outcomes are consistent with a report by Vodovotz et. et. al. (1993) which demonstrated that TGF-β released by activated T cells and macrophages suppressed the production of the oxidizing agent, NO, by inhibiting mRNA expression of iNOS and enhancing the degradation of iNOS protein.

Much of the literature interprets collagen-I study data as though the collagen fiber in all tissues, preparations, and conditions are the same and yield the same CNBr peptide digestion. These interpretations do not consider the potential differences that might exist in collagen fiber structure or organization particularly those differences may accommodate the *in vivo* function of the collagen molecule. In this study, we show that the 2D gel electrophoresis profile of collagen-I can be altered by ROS oxidation and

signal molecules known to be present in the ECM. This suggests that there is potential to vary the extracellular structure and organization of collagen-I fiber, and in a way that can vary the flexibility, tensile strength and other functional properties of ECM.

Previous studies in this laboratory showed that the addition of bone marrow-derived mesenchymal stem cells (BM-MSC) to an abdominal wound could alter the tensile properties of scar tissue (Heffner et. al. 2012). The mechanism by which BM-MSCs increase tensile strength is not understood, but the same studies demonstrated that 2D gel electrophoresis profile of scar collagen was also altered with MSCs therapy. This study suggests that changes in collagen fiber properties might contribute to the observed changes in tensile strength. Data from this thesis project shows that TGF-β, a cytokine present during wound healing, can alter the collagen 2D gel profile in a way that implies a change in fiber organization or structure. Additionally, these experiments show that ROS, known to be present during wound healing, can also influence the collagen 2D gel profile suggesting that oxidation may be another wound healing event that elicits a change in collagen fiber organization or structure. Together these results suggest that the MSCs- induced change in scar tissue tensile properties could be due to the changes in collagen fiber organization similar to those observed with TGF-β treatment or oxidation.

Future studies will focus on the processing of extracellular collagen to see if these extracellular modifications can explain the TGF-β-induced changes in the collagen profile. A first step in this investigations would be to characterize the expression of procollagen C-proteinase enhancer 1 (PCOLCE1) in TGF-β-treated cultures. Work by Steiglitz et. al. 2006 suggests that the PCOLCE1 protein stabilizes the activity of procollagen C-proteinase, thus promoting the formation collagen-I fibrils. Understanding

the effect of TGF-β on the expression of this protein would provide valuable insight into the processing, formation, and structure of the extracellular collagen fibril and fiber.

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