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FIBRONECTIN FOR USE IN HEMOSTATSIS AND WOUND STABILIZATION IN TRAUMA

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FIBRONECTIN FOR USE IN HEMOSTATSIS AND WOUND STABILIZATION IN TRAUMA

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Exploring the phase during wound healing when fibrinogen (FBG) and fibronectin (FN) interact forming a small thin layer for cells to migrate and proliferate at the injury site is necessary for the primary building blocks and initial stages of skin recovery. The aim of this thesis is to illustrate that administrating a reasonable amount of (FN) and FBG, in a semi-organizational technique, to the wound site will encourage cell population and ECM formation, as well as, improve the wound healing process. WE hypothesized that FBG and FN interactions will configure the construction of dermal cells and their final fate to remodel and flourish the wound.

One section of this thesis will compare the functional integrity of the different forms of FNs. Plasma derived fibronectin (Pd-FN), although different in structure and composition could function similarly to the cellular-derived fibronectin (c-FN).

The short peptide sequence Arg-Gly-Asp-Ser (RGDS), expressed on the structure of FN, helps the molecule to interact with a wide variety of crucial dermal cell receptors known as integrins, The α,β combinations of integrins expressed on the cell's surface are triggered by external stimulus. The interactions are the initial steps in skin regeneration and recovery. They are responsible for cell migration, differentiation, and proliferation. The interactions are also responsible for ECM formation and anchoring cells to the ECM.

Acknowledgment:

I respectfully pledge my warm and full appreciations to my advisor, Dr. William Velander. He has been there for all of his students when they needed guidance and direction to a successful and bright future. He has spent much of his valuable time to understand and evaluate my strengths and weaknesses so he can help me to fulfill my dream. He is a humble research professor and will do his best to help students. Again, thank you very much Dr. Velander.

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Chapter 1

The Molecular and Cellular Biology of Wound Repair:

Skin, the largest organ in the body, is important in covering, protecting, and thermally insulating the internal organs and tissues of the body. It consists of two organized stratums, the epidermis outer layer and the dermis inner layer that interact with each other to maintain a steady-state central to body wide "homeostasis". Skin is frequently exposed to injury by the environment and therefore must possess a self repair mechanism for regeneration. The skin's regenerative ability is coupled to the process of stopping hemorrhage and fluid (plasma) loss while initiating the multiple step sequence of the regeneration of new layers of skin to restore normal tissue function.

At the core of re-establishing a tissue after disruption due to injury are processes that require the deposition of bioheteropolymers. These heterogenous insoluble biopolymers are formed from mixtures of monomeric species like fibrinogen, fibronectin, pro-collagen and other proteins. Fibrinogen and Fibronectin are both large proteins with molecular weights >200 kDa. While these proteins are all initially soluble prior to deposition, all become insoluble via the process of activation by proteolysis (ie., pro-collagen to collagen) and covalent chemical cross-linking (ie., fibrinogen to fibrin and then cross-linked fibrin, fibronectin to cross-linked fibronectin with fibrin). The deposition process is less structured and mostly blood borne during the immediacy of stopping blood and

fluid loss and regaining a barrier to the environment. This is essence and urgency of the acute phase response. In contrast to the acute phase response, the deposition process becomes highly ordered, directional and cell intensive during the rebuilding of skin layer by layer. This is the wound healing phase which beings to manifest itself at the wound site within hours after the post acute phase response is initiated.

Wound healing is a complex, dynamic process of re-establishing cellular elements of tissue structure and restoring them as organized layers of tissue. In humans wound healing can be described by three different consecutive phases that begin to happen after the acute phase response: the inflammatory phase, the proliferative phase and the remodeling phase (4). Within these three general phases there are complex and coordinated events including chemotaxis, phagocytosis, collagen degradation, and collagen remodeling, followed by angiogenesis and epithelialization. Figure 1 illustrates the general steps the skin takes to complete regeneration (14).

The Inflammatory Phase:

The first phase of wound healing is called the inflammatory phase. The inflammatory phase begins with the migration of blood neutrophils (white blood cells or leukocytes) and macrophage progenitors to the wound site from surrounding blood vessels to cleanse the wound(4). Following wound cleansing, other chemotactic agents are released, including fibroblast growth factors (FGFs), transforming growth factors (TGF-b and

TGF-a), plasma derived growth factor (PDGF), and plasma-activated complements C3a and C5a (anaphylactic toxins) (1b). Later, monocytes (macrophages) from neighboring vessels engulf any contaminates or pathogens to further remove debris from the injury site (4).

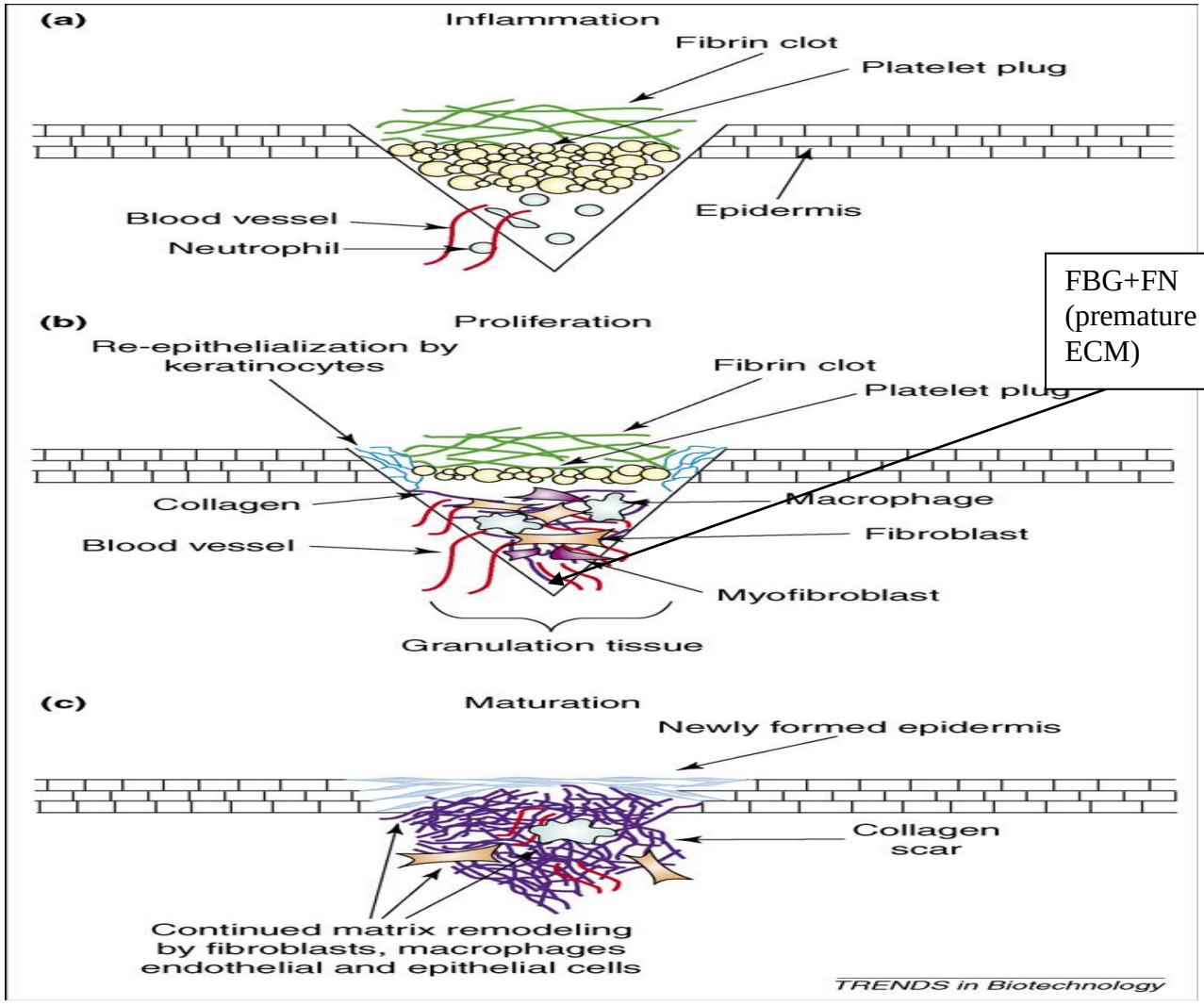


Figure 1. Phases of wound healing that manifest themselves post acute phase. Wound healing process can be divided in consecutive sequence of three phases: inflammation (a), proliferation (b) and collagen remodelling (c) by the constituent cell types of the healing wound.

Cellular Proliferation Phase

The second phase of wound healing is the proliferation phase, which consists of fibroblast migration, matrix formation and re-epithelialization. The first new skin layer to be formed is called granulation tissue. Granulation tissue forms as a result of a precursor layer of extra-cellular matrix (ECM) secreted by fibroblasts. It is later modified by fibroblasts to restore normal skin tissue. The initial inflammatory reaction includes fibroblasts which are modulated from inactive cells to migratory, proliferating and biosynthetically active cells. This process is triggered by different fibroblast growth factors. Fibroblasts also mechanically contract the new synthesized ECM, providing the tensile strength required for supporting the function of skin (14). Fibroblasts secrete numerous growth factors and cytokines that act on other cell types to initiate tissue repair. Signals, including reactive oxygen species, chemokines and cytokines, promote the chemotaxis and collagen deposition of fibroblasts and have the potential to prompt signaling cascades with downstream effects on other cell populations critical to granulation tissue differentiation (14).

The exact origin of all fibroblasts which are found infiltrated into wounds or fibrotic tissue remains fairly unclear. A certain fraction of fibroblasts within wounds and scars originate from resident cell populations or surrounding vessels (14). The balance between growth factor activity and the incorporation of fibroblasts into an environment ensures cell survival and gene expression patterns required for normal tissue remodeling.

Changes in mechanical stiffness and release of growth factors from inflammatory cells can be the initial stimulus for the activation of resident tissue fibroblasts. This followed by the formation of contractile actin stress fibers and firm attachment via fibroblast integrins to the ECM (14). It is thought that a significant percentage of fibroblasts in dermal wounds can be derived from bone marrow.

Fibroblasts can transform into myofibroblasts, the contractile activated cell type, after some external stimuli or from circulating mesenchymal precursor cells (2c). The main functions of myofibroblasts are the maturation and transformation of granulation tissue by producing new ECM which leads to the contraction and the remodeling of the wound. Myofibroblasts differ from fibroblasts by expressing alpha smooth muscle actin (α-SMA), which is the most widely used marker, for myofibroblasts (2c).

ECM Remodeling phase:

The ECM remodeling phase starts with migration of keratinocytes to the injury site as well as changes in granulation tissue. This is followed by the activities of adhesion, proliferation and differentiation that eventually will produce the outer layer of the epidermis. During this phase the granulation tissue continues to mature by its constituent cells. The remodeling of ECM by all of the cells present at the injury site is also defined by collagen deposition and the establishment of connective tissues made by fibroblasts and myofibroblasts. Myofibroblasts promote wound and scar contraction by establishing

cell-cell and cell-ECM contacts and contraction forces. Myofibroblasts form bundles of contractile microfilaments and extensive cell matrix contacts, which attach the microfilaments to the surrounding ECM. In the final stages of normal wound closure, myofibroblasts go through apoptosis, which leads to loss of mechanical tension (14). A failure to truncate this myofibroblast activity results in tension that leads to an excessively contracted and therefore dysfunctional scar tissue.

As the remodeling phase proceeds with a layer by layer restoration of functional skin, the need for blood supply elevates. The creation of blood vessels (angiogensis) starts from cells surrounding the wound, especially endothelial cells(ECs) and fibroblasts.

Angiogenesis:

The initial event in angiogenesis is the directed migration of ECs to the wound site. During this event, vascular endothelial growth factor (VEGF) is expressed by ECs which are central to catalyzing vessel regeneration during wound healing. During the infiltration of ECs into the forming tissue needing angiogenesis, there is a directionally apical to luminal fragmentation of the venous basement membrane, possibly mediated by collagenase and plasminogen activator derived from ECs. ECs develop pseudopodia that protrude through the disrupted basement membrane and the entire cell migrates into the perivascular (luminal) region. Hypoxic conditions appear to stimulate migration of cells and this phenomenon is assisted partially by a macrophage derived angiogenic factor

expressed during times of low oxygen tension (2). Fibronectin (FN) plays an essential role in the migration process where there is an increase in FN surrounding the neo-vasculature of the healing wound. FN adhesive properties act as scaffolding upon which cells can migrate. FN has also been shown to be chemotactic for ECs.

Angiogenesis is triggered through signaling controlled by the αvβ3 receptors and FGFs. It is also directed by the stable interaction between integrin α5β1 and Tie-2 tyrosine kinase receptors that regulates endothelial cell response to angiopoietin-1(2). It has been reported that substrate-bound FGF2 promotes endothelial cell adhesion by directly interacting with integrin αvβ3 and induces endothelial cell proliferation, motility, and the recruitment of fibroblast growth factor receptor (FGFR1) in the cell substrate contact (1).

General cellular signaling associated with migration and proliferation:

During skin recovery cells both receive and transmit paracrine and juxtracrine signals in a coordinated manner that is necessary for the healing to proceed in an ordinary fashion. It is central to ECM formation and maturation. For example, skin cells migrate to their final destinations as a result of different polypeptide growth factors that will also play a regulatory role in proliferation, maturation and subsequent progression to angiogenesis. For example ECs require FGFs to support proliferation and prevent apoptosis. In addition, FGFs promote EC migration and increase synthesis of several proteins that are important in degradation processing during ECM remodeling that happens during cell

migration or angiogenesis, including collagenase, urokinase plasminogen activator, urokinase plasminogen activator receptor, and plasminogen activator inhibitor. Furthermore, FGF regulates EC adhesion by modulating expression of integrin receptors and increases expression of vascular endothelial growth factor (VEGF), another angiogenic protein that regulates and enhances the formation of new blood vessels (45).

Signaling is not only achieved by interactions between growth factors and cell receptors but also between cell receptors and matrix proteins. In the case of growth factor and cell receptor signaling, Wijelath (2002) found that VEGF bound to FN enhanced the capacity to stimulate EC migration due to the association of FLK-1 (VEGF binding region) and α5β1. In an example of complex growth factor, matrix protein and cell receptor interactions, fibrinogen (FBG) binding of FGF-2 plays a role in proliferation. It enhances EC proliferation through the coordinated effects of αvβ3 and FGFR1 (FGF binding receptor) resulting sustained cell activation (45). Tsou and Isik (2001) showed that vitronectin altered the expression of FGF and VEGF receptors and those matrix integrin interactions that regulate EC responsiveness to growth factors. Moro (1998) showed that the binding of fibroblasts to specific matrix proteins activate the epidermal growth factor receptor (EGF). Thus, specific interactions between growth factors and matrix proteins appear to be critical in the regulation of cell properties. In that regard, in subsequent chapters we will discuss in detail the role of fibronectin in cell regulation in the context of its presence as a soluble protein and insoluble protein once it is incorporated into ECM.

Chapter 2: Regaining homeostasis through the sequential actions of hemostasis and then cell colonization and tissue reconstruction.

Introduction:

Homeostasis is the system driving force for the body to regulate its internal environment which strives to maintain a stable, steady state condition. During disruption of normal tissue and its physiology, homeostasis exerts an overarching and systemic effort to restore steady state. In the case of blood vessel disruption, stopping hemorrhage is accomplished by an orchestrated and defined process primarily involving cells and blood borne proteins. The process starts by the formation of a FB clot. This clot not only stops bleeding, but is the initial foundation by which cells associated with healing are recruited and catalyzed to begin a sequentially long recovery process.

A priority process of maintaining homeostasis is the intensive monitoring of the integrity of skin as it protects the internal environment and regulates body temperature. The disruption of structure and function of the skin ignites the acute phase during which clot formation to regain a barrier and stop fluid loss occurs and then also activation of specific dermal cells to gain attachment to the existing clot. This leads to initial colonization and almost simultaneous synthesis of an immature ECM. Central to the ECM installation

process is the secretion of FN and other ECM proteins in a directional installation from these colonizing cells. This contrasts the greatly more random process of the deposition of FB clot which includes cross-linked FN whose origin is from blood plasma. Thus, this initial ECM and also subsequent more mature ECM layers use cellularly directed deposition and further the progressive proliferation of the cells.

Acute phase response at the protein level:

Regaining homeostasis begins by stopping hemorrhage and fluid loss (from semi permeable oozing of plasma i.e. burn wounds with cauterizing injury), which can also be described as the coagulation phase. A hemostatic plug is formed from the reaction of FBG, thrombin and factor XIII. Soluble fibrin (FB) monomers aggregate and noncovalently polymerize after proteolysis of FBG by thrombin. An insoluble FB clot is made from the FB aggregates by factor XIIIa-mediated covalent cross-linking. FBG is also up-regulated as part of the innate immune response to inflammation during the acute phase (1b). With respect to FBG as a signal arising from the FB substratum, along with FN, it chemotactically mediates skin cell migration, especially epithelial cell movement during wound repair (43). During the formation of the FB clot, many adhesive glycoproteins (e.g. Fibronectin) originating from blood plasma are incorporated into the hemostatic plug by Factor XIII and Factor XIIIa activity. This amalgam of proteins creates an initial surface where cell migration and proliferation can be conducted. FB clots are random structure containing many proteins in addition to growth factor‘s storage

(plasma platelets). When platelets are incorporated, this creates a locally concentrated pool of growth factors, proteases, and protease inhibitors, and a substrate for induction and modulation of cell function (1). Thus, the clot layer (now to be identified as the immature ECM) can promote the attachment of other dermal cells on the injury site.

There are several protein and cellular level activities that are characteristic of the acute phase response to tissue disruption. The acute phase response includes the non-adaptive immune system that catalyzes the activation of macrophages and neutrophils. As a result, macrophages and neutrophils engulf foreign bodies and secrete proteases at the site of tissue disruption. This also leads to the induction of acute phase responses that catalyze various cell types to produce cytokines (1b), such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor (TNF-1). Cytokines signal aggressive changes plasma protein compositions that result in the production of various glycoproteins and then further activation of cells in the immune system.

The post-acute phase transition to the inflammatory and early stages of the formation of ECM like substratum formation:

The first cells to infiltrate the wounded tissue are inflammatory cells that proteolyze damaged tissue and also proceed to breakdown FB clots (proteases like neutrophil elastase, trypsin and plasmin from plasma/tissue made by the tissue plasminogen activator). During this “debridement” process, other dermal cells will begin a migration

and adherence to an “immature ECM” formed from deposition of plasma derived fibrin and fibronectin. These include fibroblasts, ECs and keratinocytes which penetrate the clot to begin the process of layer by layer reconstruction of the skin.

After adhesion to the wound site, fibroblasts undergo changes in gene expression governing the synthesis of matrix constituents and cell surface receptors. These include a sequence that creates an intermediate state of cell adhesion, followed by reorganization of their actin-based cytoskeleton and a change in morphology, and improved matrix deposition leading to a planar cell polarity, wound contraction, and ultimately a very strong cell adhesion to bring about resolution of wound injury (2c). Although fibroblasts secrete and use the ECM to populate and proliferate at the injury site they also play an important role in helping the premature ECM assembly. This occurs in part through integrin receptor activation by growth factors. Growth factor signaling is essential to ability of ECs to adhere to proteins presented on the ECM surface. In the absence of ECM engagement; ECs will eventually undergo apoptosis by a process termed “anoikis” terminating their involvement in the wound healing process. This is mediated by the constitutive expression of Akt which halts anoikis in the absence of cell attachment (47).

A second wave of colonizing cells consisting of tissue progenitors and primary cells migrate from the wound perimeter after the acute phase. Some of these cells are epithelial and ECs. These cells also interact with the preexisting ECM through integrin receptors

via ECM proteins. These interactions catalyze an acceleration of the maturation of the ECM.

Maturation of ECM and the organized deposition of fibronectin:

The general maturation of ECM structure happens in an iterative fashion. This is maturation is centered about the directional and sequential installation of FN and FBG by attached cells. As the ECM enlarges, more cells migrate to the wound site to proliferate and mature which in turn further matures the ECM. The process begins with epithelial cell–derived FBG that unlike plasma derived FBG, uniquely assembles into a mature ECM independently of conversion to FB by thrombin. This is an important difference between plasma- and cellularly-derived FB. The cellular installation of FBG into the ECM occurs in a heparin sulfate proteoglycan (HSPG)–dependent manner. In particular, FBG residues Bβ1-42 and A-RGD sequences play a role in matrix-FBG–mediated enhancement of wound closure (42) and its association with FN in the ECM. This is reflected by integration of FBG into matrix fibrils that co-align uniformly with FN fibrils. The functionally important role of FBG Bβ1-42 region is exposed and regularly distributed throughout the matrix (42).

Cell membrane based receptors are important regulators for the maturation of the ECM. However, FN (Chernousov (1985); McDonald (1987) and FBG assembly into the ECM requires metabolically active cells and not the synthesis of new cell surface receptors or

other matrix constituents. In this process of FBG secretion from epithelial cells (Guadiz, 1997) and FN secretion from ECs (Kowalczyk, 1990), both molecules are polarized to the baso-lateral face of the cells, directing these glycoproteins to the ECM for binding.

The interactions between FBG and FN in the ECM require the polymerization of FN into fibrils and the adhesion with FBG through receptors. It starts with the integrin receptor α5β1, which plays a central role in mediating the assembly of FN into the ECM, most likely by regulating a series of sequential self-interactions that result in the polymerization of FN. In addition, some evidence suggests that FBG binds to Mn2+/activated α5β1 on ECs, and fibroblasts bind preferentially to FBG via αvβ3(42). Moreover engagement of αvβ3 also promotes cell migration occurs during metastasis, angiogenesis, and wound repair. Previous studies have shown that the neutralization of αvβ3 ligation reduces assembly of endogenous FN into mature matrix fibrils, where the immature matrix is composed of shorter, thinner fibrils and stitch-like fragments of FN (1). In other studies, the inhibition of the expression of α5β1 integrin on the surface of fibroblast cells did not effect ECM assembly significantly, where anther cell receptor called αvβ3 provides an alternative pathway for the assembly of soluble FN into the ECM by a process independent of α5β1(1). More recent studies have shown that the deposition of FN into the ECM results in an extended conformer that induces expression of latent cell binding domain in the RGDS-containing region in addition to RGDS(42). This conformational alteration enhances the avidity of cell binding to matrix FN over soluble FN. There is sufficient evidence that soluble FN can interact with dermal cells at the mature ECM site. Most importantly, the role of cell receptors and FN's binding sites

interactions serve as a tool for the ECM to be deposited in an organized pattern leading to iterative colonization of dermal cells and the eventual layer by layer maturation to normal skin.

Summary of the phenomena at the interface between acute phase response and wound healing processes:

After tissue disruption, FB and other growth factors associated with clotting rushes to the wound to start the formation of FB clot which have a greatly more random structure relative to ordered ECM made by cells. The goal of the acute phase response is to stop hemorrhage and fluid loss and restore the barrier with the environment, albeit using a temporary barrier consisting of randomly deposited biopolymer. The clot is also engineered to be a surface (immature or preliminary ECM) for initial deposition of inflammatory and dermal progenitor cells. These cells migrate to and start the installation of an organized ECM containing collagen, FN and FBG on top of the immature ECM. Remodeling via proteolytic degradation of the underlying plasma-derived fibrin naturally occurs as cellular ECM is installed. The maturation of ECM alos results in collagen remodeling necessary for normal skin recovery which can occur over months.

Specific Hypothesis and subject of this thesis*:* To speed up ECM maturation and normal layer of skin, we postulate that the application of scaffolds loaded with FN and other skin

recovery factors at the injury site will accelerate the healing process (ECM maturation) to form normal function skin (instead of scarring) while also being hemostatic.

Thus, we hypothesize that applying plasma drive FN (pd-FN) in semi-organized pattern will increase cells migration needed to form a mature ECM. Thus, the installation of this less random, semi organized matrix will result in an amplification of the cellular mediated, directional deposition of FN and FBG at the wound site. The use of Pd-FN incorporated at the nano-scale dimension of the scaffolding (filamentous or particular) to achieve organized pattern of deposited FN.

To test this hypothesis, we must acquire sufficient amounts of pd-FN, for experiments to be used in developing and studying deposition techniques. *Second* we need to develop deposition techniques which are less random and more patterned as is FN installed in ECM. Thirdly, we should prove that pd-FN can function similarly to cellularly installed FN in the ability to mature the ECM and interact with various types of dermal cells. The subject of the experimental work of this thesis is confined to the purification of pd-FN.

Chapter 3- Fundametnals of fibronectin structure and function:

Introduction:

After injury and the cessation of bleeding by the formation of the fibrin clot, there are multiple stages in the healing process. Involving a diversity of inflammatory and progenitor cells and also the proteins that they secrete. Part of the complexity of these sequential phases lies in the diversity of proteins that are part of cellular signaling (both paracrine and juxtacrine) as well as proteases and structural proteins that are part of the ECM. We choose here to focus upon the role of FN and its two sources: from blood plasma or from colonizing cells that make ECM.

Whether deposited from blood plasma or made by colonizing cells that are participating in tissue reconstruction, FN plays an important role in chemotaxis and in ECM physico-mechanical structure. FN interacts with other ECM components including fibrin, collagens, and heparin. FN-rich matrices provide substrates for cell adhesion and migration during development, wound healing, as well as affecting many cellular functions including proliferation, survival, and differentiation. Cells encountering fibronectin (FN) that is installed into extra-cellular matrix will adhere and then change their cell morphology by cytoskeleton re-organization.

We here review the attributes of fibronectin at the level of protein and gene regulation. It is seen that fibronectin protein structure and therefore function arises from the variable formation of different mRNA transcripts. Multiple fibronectin structures and functions result from mRNA splicing events.

Global Fibronectin Structure:

Fibronectin is a 250 kDa glycoprotein that exists as a dimer consisting of two subunits joined by disulfide bonds near their COOH termini. Each individual subunit is composed of a series of homologous repeating units of three types (types I, II, and III) (12). FN can be found in a soluble form in plasma and other body fluids and as an "insoluble form" that is integrated into tissues. The "insoluble form" of FN results from being installed into ECM by a covalent cross-linking process after unidirectional secretion by colonizing cells which reconstruct tissue. Because FN is an integral ECM component, it is moderately abundant in the body. The molecular weight of the dimer subunits approximately 460-kDa in blood, and present at 300 to 400 μg/mL, (0.6 to 0.9 μmols/L), in plasma and 0.5 μg per $3x10^8$ platelets in platelet α-granules (20).

The general structure of FN is presented in Figure 2. Both plasma-derived and insoluble FN have 12 units of type I repeats, two of type II repeats and 15-17 type III repeats. Type I is approximately 40 amino acid residues in length and has two disulfide bonds and type II repeats have a stretch of about 60 amino acids residues that contain two intra chain

disulfide bonds. Type III repeats are about 90 amino acids residue without disulfide bonds (35). The type I repeat has stacked β-sheets within a hydrophobic core that has a high content of aromatic amino acids. The type II segment has two anti-parallel β-sheets that are perpendicular to each other. Type III repeats contain seven β-strands organized in two perpendicular β-sheets attached by flexible loops. Type III strands A, B, and E form one β-sheet, and strands C, C', F and G form the other to engulf the hydrophobic core (36). Many types of dermal cells have avidity for the FN binding sites illustrated in Figure 2. These binding domains are at the core of FN's chemotactic and proliferative activity.

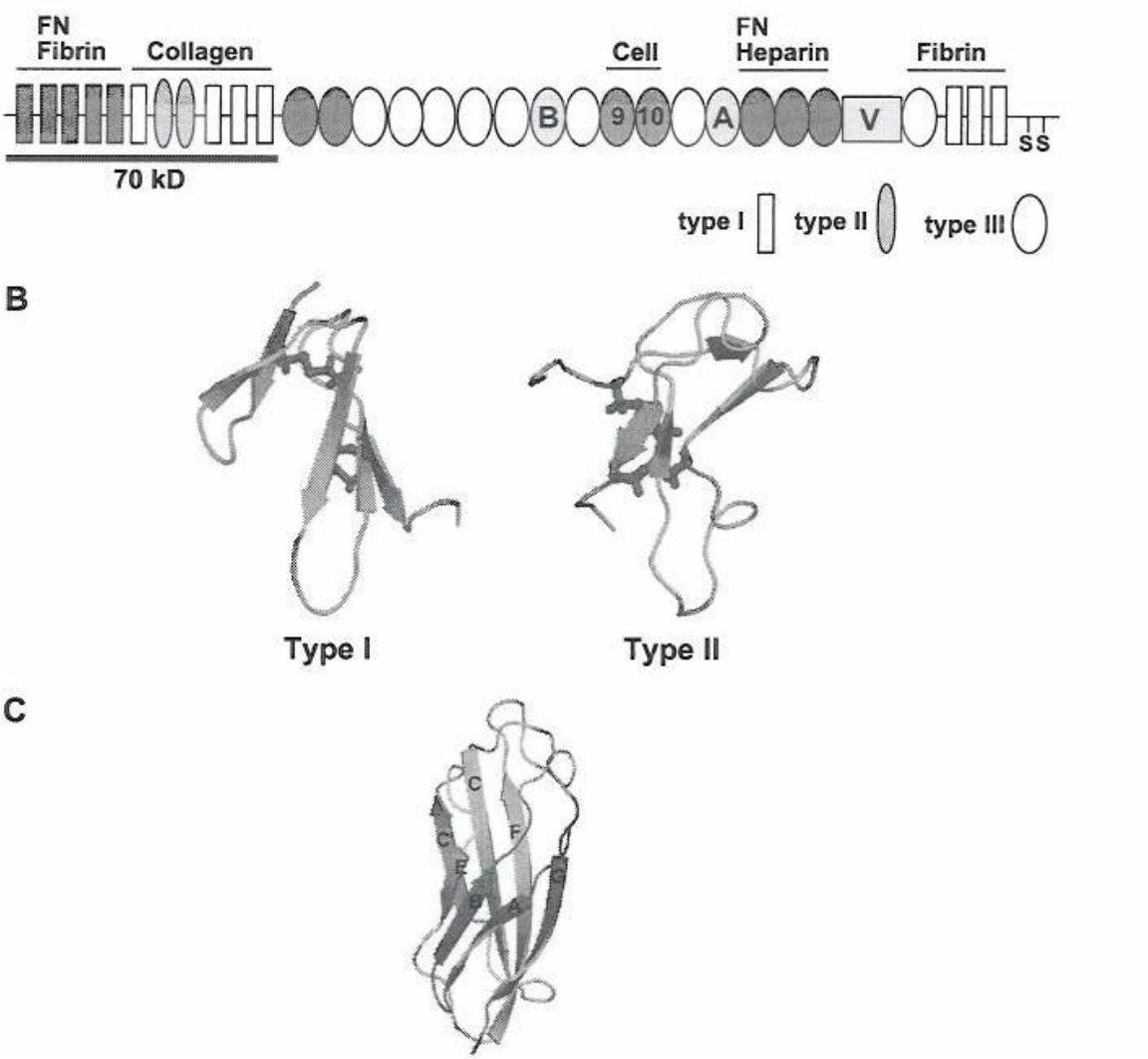


Figure 2. FN structure, as shown multiple binding sites are available for collagen, heparin, and many types of cells. (B,C) represent the directions of β-sheets within the molecule.

Specific variations of FN structure and function occur via variant mRNA splicing events:

Introduction to mRNA splicing phenomena:

Alternative mRNA splicing is an important aspect of gene regulation by which multiple mRNAs (and hence their encoded proteins) can be selectively produced from a single pre-mRNA. This post-transcript processing occurs widely among higher eukaryotic genes and frequently results in functional diversity via production of multiple protein isoforms from a single gene(9).

FN mRNA splicing creates variable structures:

The FN gene has the two exons EIIIA and EIIIB which encode a "type III structural repeat" in addition to others. These regions exhibit "exon skipping" that have species-specific patterns of differential splice site selection resulting in different mRNA transcripts and therefore FN protein structures. In addition there exists a variant FN transcript called the "V" or variable region which pairs multiple 3'-splice sites with a common 5'-splice site. All of these variations of the V region have been shown be produced by a cell-type-specific, splicing event. For example, hepatocytes synthesize pFN exclude EIIIB and EIIIA, whereas fibroblasts and astrocytes synthesize FNs that partially include these two segments (12). In figure 3, regions of EIIIA and EIIIB are included while potential V region sequences are outlined FN structure. Examples of the

species specific diversity that results from V region splicing have been documented: there are 8 different forms of FN in chicken, 12 in rats and 20 in human(23).

It is not yet known how splice event variability is controlled in gene expression. Recent in vitro studies of RNA processing have led to the identification of a number of cellular factors essential for splicing which include several abundant sn-RNPs and a number of protein factors (1). The pattern of exon skipping is also found in many species.

Some researchers have suggested that the intronic TGCATG repeats of fibronectin promote inclusion of the alternative EIIIB exon, probably by formation of a complex that recognizes an evolutionarily conserved pattern of TGCATG repeats (4).

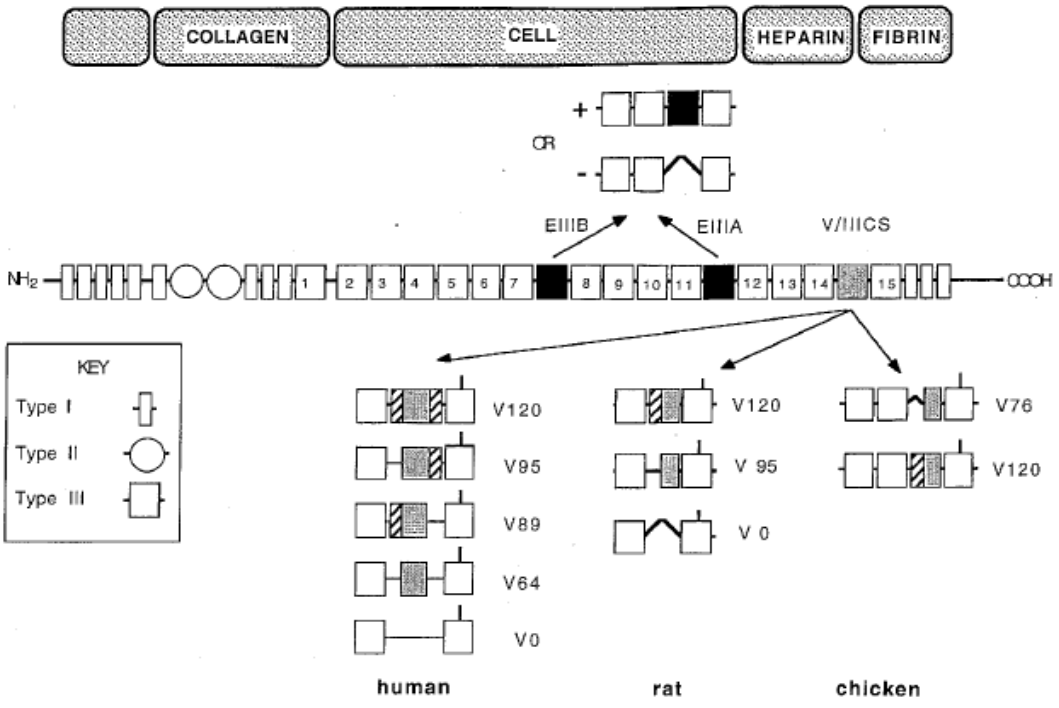


Figure 3: The splicing patterns of the V region for FN by the inclusion and the exclusion of some amino acid in the domain. V represent the variable region, the number associated with V represent the amino acid number in the peptide chain in FN molecule.

Copy right to Constant, C. F. (1995). Alternative splicing of Fibroncetin- Many different proteins but few

Differential functions of the spliced regions of FN:

Several studies by Humphries (1986, 1987) have shown that the V segment (also called IIICS) contains cell-binding activities for certain cell types. To date, the functions of the EIIIA and EIIIB segments have not been delineated. Because multiple transcripts are generated during mRNA processing, multiple FN polypeptide subunits are co-translated. As a result, the assembly of FNs into disulfide bridged dimers creates heterodimers which are mixtures of several different types of polypeptide subunits. This complicates the analysis of FN structure and function as they are difficult to enrich with respect to anyone heterodimer population. The synthesis of recombinant forms of FN has resulted in subpopulations having IIIB or IIIA that exist as monomers. In contrast, about 90% of recombinant FN containing a V region is dimeric. These results suggest that the alternatively included V segment in FN may promote dimerization while the EIIIB and EIIIA segments may prevent dimer formation (12). Interestingly, all these forms of recombinant FNs were found to promote cell adhesion and morphological spreading to a similar extent (12).

Although there was no clear function for the EIIIA and B regions of FN, researchers have used fluorescence staining to measure different rFN incorporation into the ECM, where results have shown all forms of FNs incorporate into the existing matrices. The O (does not contain EIIIA,-B, and V regions) and V forms required higher levels added than did the EIIIA and -B forms to give equivalent staining (staining assay indicates the ability of

FN incorporation within the ECM). The inclusion of EIIIA and -B regions are important to enhance FN incorporation within the ECM (12).

Experimental studies have shown that EIIIA is up-regulated in the sinusoidal ECs, but EIIIB is also up-regulated in different cell populations. Similar studies showed that the EIIIA and EIIIB are expressed in the abnormal palmar fascia, and no FN protein was present in the normal palmar fascia (22). Studies on EIIIA+ forms of FN promoted activation more effectively than EIIIA- in the activation of lipocytes into contractile myoepithelial cells. The activation of these cells can be assessed by the changes in levels of smooth-muscle α-actin. Studies using a recombinant FN with or without EIIIA had no effect on cell spreading morphology, motility, or proliferation, an indication of no RGD sequences present in these forms of FNs (23). These studies are an indication for the functional requirements of each tissue to allow the needed function.

Summary of FN structural heterogeneity arising from mRNA splicing:

Many studies have concluded that FN mRNA splicing is regulated during development, repair, and disease. These observations indicate that each splice region has a distinct function. In addition, each spliced region is controlled and functions independently from other regions. The presence of each of these regions are additive in their contribution to a specific functionality. It is apparent that these different forms of FN are produced in different cells according to time dependant needs for growth and stability.

Fibronectin adhesive and structural functions:

The functions of FN protein are dictated by its many forms and locations in a specific tissue. FN is expressed differently in various body tissues such as muscles, brain, kidney and heart during early embryonic development. For example, experiments on mice lacking FN had a lethal phenotype defect in meso-dermal cell migration and mesoderm formation (George, 1993). FN protein also plays a role in cerebral ischemia disease, which maintains and helps neuronal survival (Sakai, 2001). Other studies indicate an association of fibronectin with the basal keratinocytes of the epidermis (8).

Several functions of fibronectin are evident during wound healing and skin recovery. Initially FN and other proteins can also be deposited from blood plasma as a coating on the wound site surface to lay a temporary foundation for cells to adhere and proliferate. Immediately after injury, FN can also assist in platelet adhesion and the formation of the fibrin clot. FN also helps in the formation of ECM. Cells encountering the ECM protein fibronectin (FN) adhere, spread, form an organized cytoskeleton, and activate biochemical pathways that allow cell cycle progression and prevent apoptosis (5).

FN adhesive function is observable during the attachment of some tissue culture cells to culture vessels. When FN is presented as fixed on cell surfaces it can then serve as an anchorage point for cell attachment. Thus, FN has a role in increasing the attachment

strength of transformed (immortalized) cells to culture vessels and thus changes their morphology (though not their growth characteristics and malignant potential). The mechanism of these two effects is unknown (17). Another example of FN function is when vascular ECs secrete fibronectin into their ECM, which may allow them to remain attached to their basal lamina in the face of high sheer forces of circulating blood.

The other main role of FN is its incorporation into ECM as a structural protein. In the ECM, FN has a binding site that is a short sequence at the N terminus of FN type III repeat, previously identified as the major heparin-binding site. This site is responsible for the formation of stress fibers, focal contact, and filopodia–inducing activities of the ECM (5). In addition, several non- RGD–containing FN type III repeats have been shown to support adhesion, stress fiber and focal adhesion formation when β1 integrins are activated by Mn^{2+} or activating antibodies (5).

FN binding domains and identified functions:

The structural diversity of FN enables its diverse function in many developmental and homeostatically regulated processes. For example, FN has two binding sites in the V region called LDV located in V25 domain and REDV site in V31 domain. Both of these regions are receptors for the α4β1 cell surface ligand. The recognition of V25 by α4β1 is important with respect to cell migration in wound healing(23). The other site included in

the V domain of FN is the LDV region. The LDV region can change cell behavior and provide sites for both α4β1, α5β1(23).

One of the main binding sites in FN is the short peptide sequence Arg-Gly-Asp-Ser (RGDS) in the central region of pd-FN that has been identified as a primary cell binding site (12). The discovery of these sites was done using monoclonal antibody mapping to different segments of FN and responsiveness to the inhibition of FNs adhesive ability to different cell's types. An example of an elucidated FN ligand for α5β1 (a well-known receptor for many dermal cells) is the 80-kD cell-binding domain that contains the RGD sequence. Alternatively, a combination of integrin receptors binds to the sequence EILDV in the alternatively spliced V (or EIIICS) region (10). As an example of other effects caused by FN binding sites, Obara (1988) discovered a region which is amino-terminal to the RGDS sequence that promotes cell spreading (12).

Another main binding region of FN that interacts with α4β1 (cell's receptor) is the 38-kD fragment containing the heparin (Hep) II domain that attaches much more efficiently than an RGD-containing fragment (80 kDa) (4b). The region bonding formation during cell-FN interactions depends on the amino acid combinations. These amino acid combinations are able to form cross-linking with the cell receptor.

The V region present on the A chain of plasma FN contains at least two sites responsible for mediating cell adhesion to FN. As shown in (Fig.8), different binding domains have

been identified for cellular and tissue binding. (4b). For example, a high affinity binding site to FN by T lymphocytes has been located within a 38-kD fragment. The same cells did not adhere to the NH2-terminal 29-kD fragment containing the Heparin I domain of plasma FN (4b).

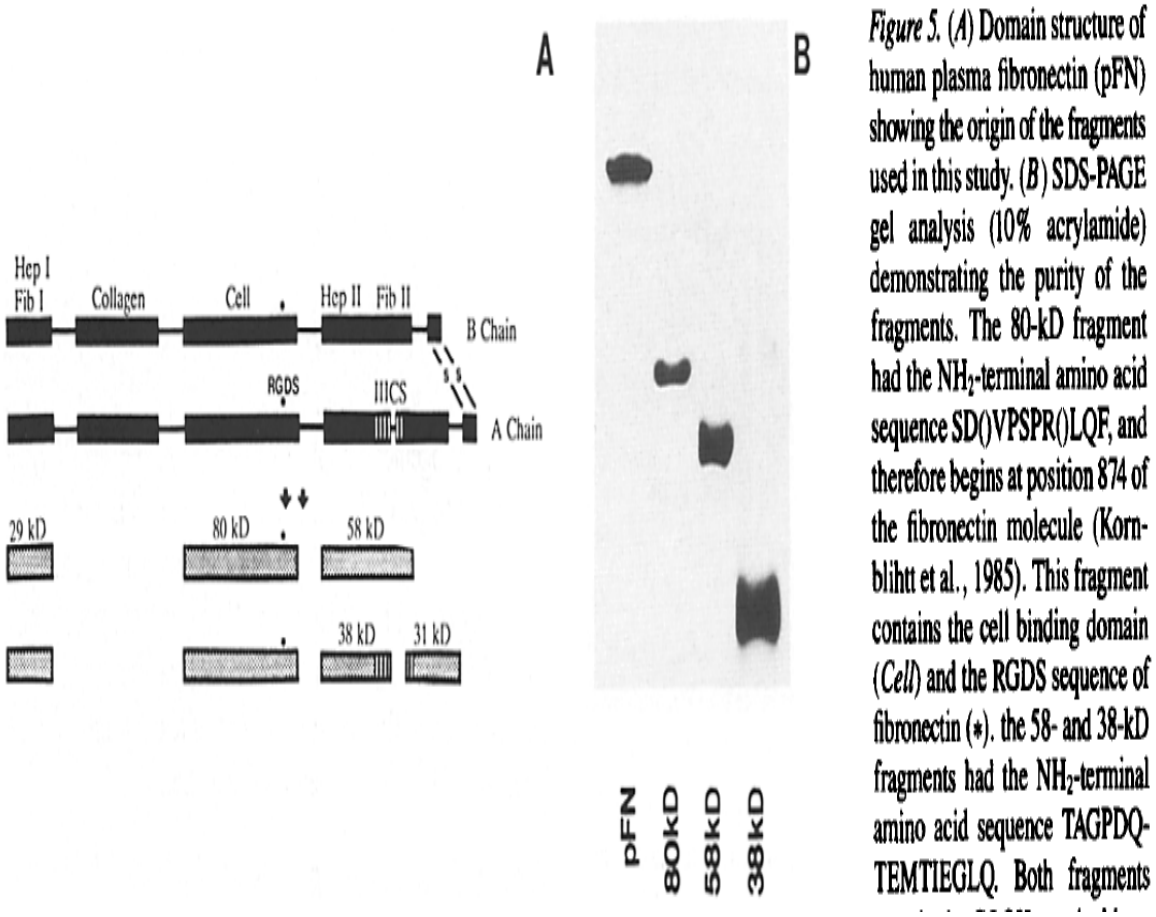


Figure 5. (*A*) Domain structure of human plasma fibronectin (pFN) showing the origin of the fragments used in this study. (*B*) SDS-PAGE gel analysis (10% acrylamide) demonstrating the purity of the fragments. The 80-kD fragment had the NH_2-terminal amino acid sequence SD()VPSPR()LQF, and therefore begins at position 874 of the fibronectin molecule (Kornblihtt et al., 1985). This fragment contains the cell binding domain (*Cell*) and the RGDS sequence of fibronectin (*). the 58- and 38-kD fragments had the NH_2-terminal amino acid sequence TAGPDQ-TEMTIEGLQ. Both fragments

Figure 4: Structrual binding domains of human plasma fibronectin.
This Figure copyright to: Guadiz, G., Sporn, L. A., & Simpson-Haidaris, P. J. Thrombin Cleavage-Independent Deposition of Fibrinogen in Extracellular Matrices. *Blood, 90* , 2644-

Changes intracellular signaling pathways have been correlated with FN-cell binding such as through the reversible phosphorylation of proteins that are involved in regulating metabolic pathways (Edelman, 1987). In addition, protein tyrosine phosphorylation has been implicated in transmitting signals that regulate cell growth and differentiation after cells bind to FN in the ECM (10). In non-transformed fibroblasts, proteins undergoing tyrosine phosphorylation are concentrated in cell-cell or cell-matrix contacts (Maher, 1985). This is an indication that protein tyrosine phosphorylation is possibly involved in transducing signals across membranes when integrins bind to ECM proteins and aggregate into focal contacts (10).

Heparin and Collagen Binding Domains of FN:

Heparin binding domains of FN:

Crucial to the functions of FN are the heparin and collagen binding domains. The heparin binding domain is important during the acute phase responses of clotting and platelet adhesion. This domain is made up of several homologous repeats of about 90 amino acids, known as type III homologies. These 95 or 120 amino acid segments are inserted between the last two type III domains but are not themselves related to these regions. The inserted segments could be involved in binding heparin or hyaluronic acid, in cell adhesion, or in self-association and “fibrilogenesis” (15).

Binding to collagen during collagen remodeling:

Collagen installation and remodeling involves the use transglutaminase (Factor XIIIa) to catalyze the cross-linking of FN from plasma into collagen at mature ECM surfaces (19). FN is one of a limited number of plasma proteins which contain glutamine residues susceptible to the action of Factor XIIIa. The reaction may be important for wound healing and tissue repair. However, types I and III collagen inhibited Factor XIIIa-catalyzed cross-linking between αl(I)-CB7 and FN. Both types of collagen were cross-linked to FN, but only at 37°C in suggestion that this reaction can be achieved at the wound site in human skin (19). Incubation of FN, the αl(I) chain of type I collagen, and Factor XIIIa at 20°C resulted in the formation of high molecular complexes as shown in Fig 5. These large MW complexes are an indication of binding. Large MW complexes could be feasible to enlarge the ECM and increase surface area for epithelial dermal and keratinocytes cells to lay the substratum of the skin.

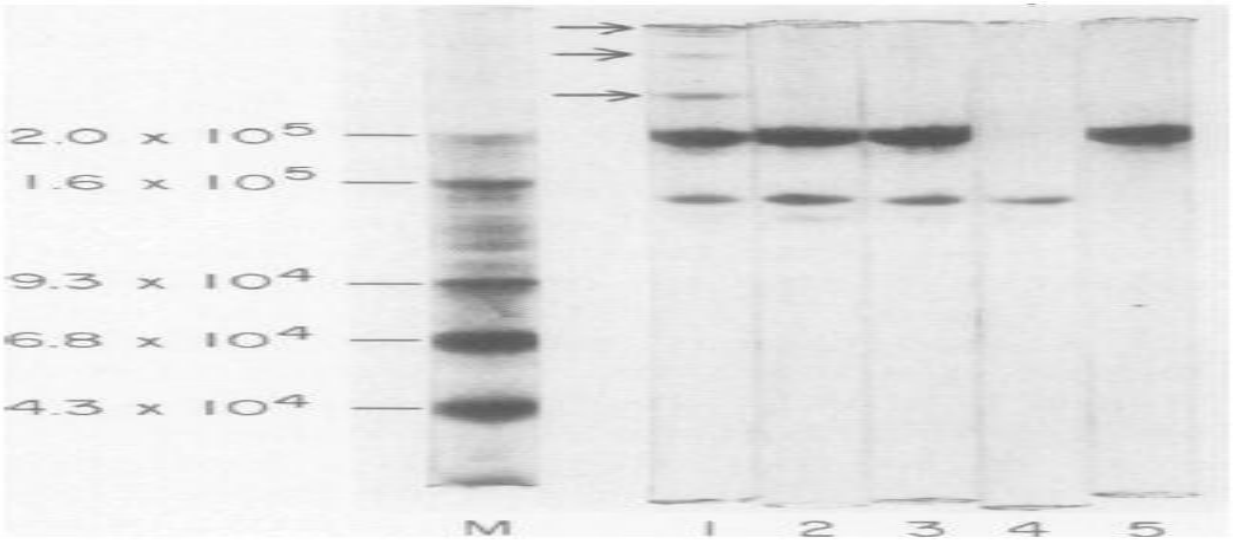


Figure 5: Gel showing the different components that are involved in the experiment. Fibronectin, 2.0 x 10^5; The major component of al(l) collagen chains had an apparent molecular weight of 1.3 x 10^5. Arrows point to the cross-linked complexes of fibronectin and αl(l) chains.
(The copyright to: Kleinman, H. K., Mosher, D. F., & Schad, O. E. (1979). Cross-Linking of Fibronectin to Collagen by Blood Coagulation Factor XIIIa. J. Clin. Invest. *The American Society for Clinical Investigation, 64*, 781 -787.)

A major determinant of FN binding to αl(I)-CB7 is located at the site of digestion of αl(l) chains by vertebrate collagenase and has the sequence Gly-Ile-Ala-Gly-Gln-Arg. Al(I)-CB8 and the penultimate cyanogen bromide fragment of the α-chain of FB also contain a Gly-Ile-Ala sequence, and supports what was mentioned earlier about the interactions between FN and FB(19), suggesting that FB also has a role in collagen remodeling.

The binding to heparin and collagen are useful engineering tools that can be manipulated to purify FN from plasma. This will be discussed in a future chapter. Purified FN can be important component of a synthetic precursor ECM which accelerates the early phase of tissue healing.

Interactions with Platelets:

FN interaction with the platelet is an important step initial step in wound healing. Platelets provide a strengthening of the hemostatic plug to stop the bleeding and by their adhesion to the surrounding skin layers greatly enable its hemostatic barrier action. The adsorption of plasma derived FN to activated platelets increases the efficiency of adherent platelet aggregates that form in response to vascular injury. FN has the potential to interact with platelets via two different 2 sites. The first is centered on module III-10 that contains the RGD sequence recognized by αIIbβ3, αVβ3, and α5β1 integrins. The second comprises the type I modules at the N termini that direct FN to as yet poorly defined sites on surfaces of platelets involved with subsequent fibril formation (20).

FN role in the binding to various cell surface receptors:

Introduction:

FN plays a key role in the recruitment of cells which act in wound healing. It also plays a role in adhesion via surface receptors composed of integrins. Integrins are heterodimeric, transmembrane proteins consisting of an α and β subunit. Integrins act as cell surface receptor proteins for a variety of ligands, including ECM proteins such as FN, laminin, and collagens (10).

Integrins in keratinocytes receptors:

The major integrins expressed by keratinocytes are α2β1, α3β1, and α6β4 (Yamada, 1996). The α2β1 integrin is a type I collagen receptor, whereas the α3β1 and α6β4 integrins are laminin 5 receptors that form critical adhesions to the basement membrane. In contrast, during re-epithelialization the migrating epidermal cells up-regulate integrins that ligate conditional matrix proteins. α5β1 is the main integrin that is expressed on the surface of these cells. Similar studies showed that α5β1 mediates keratinocyte migration on FN (Kim, 1992) while αvβ5 mediates keratinocyte migration on vitronectin (43). Keratinocytes do not produce the β3 and αvβ3 integrin receptors which are recognized by FBG binding regions (43). The migration process is necessary for keratinocyte to reach the top layer of skin and form the hard layer.

Fibroblast and Endothelial Cells Receptors:

Fibroblast and endothelial Cells are among the first to reach the "immature ECM" and start the process of formal ECM installation. These cells contain multiple receptors which interact with ECM proteins that program the progression of migration (chemotaxis), adhesion, morphological changes leading to differentiation, and proliferation. For example, adult human dermal fibroblasts and ECs express clearly detectable levels of the β3 subunit and αvβ3 integrin. This contrasts the acute phase response by platelets and megakaryocytes which express the integrin αIIβ3 that binds FBG (Shattil, 1994). Other

cells including human umbilical vein ECs (Cheresh, 1987), human dermal fibroblasts (Gailit and Clark, 1996) and human micro-vascular ECs (Enenstein, 1992) express αvβ3 and bind FBG.

Fibroblasts are key participates to forming granulation tissue in the early phase of healing. Thus, fibroblasts in culture have been studied to evaluate the dependence on their adhesion and spreading on solid surfaces with and without FN and other ECM proteins. This property, termed anchorage dependence, is reduced or lost as cells become increasingly transformed (10). For example α3β1 functions as a receptor for laminin-5, and a weak receptor for a variety of other ECM proteins, including FN, collagens, laminin-1, entactin/nidogen, and thrombospondin (10).

ECs have been shown to express a variety of integrins, including the following: α1β1, α2β1, and α3β1, which are laminin and collagen receptors; α5β1, αvβ1, and αvβ5, are receptors for FN; α6β1, a laminin receptor; and αvβ3, a receptor for FN, vitronectin, osteopontin, von Willebrand factor, laminin, and collagen (3).

FN binding by other cell types

T lymphocytes use two independent receptors during attachment to FN. α5βl is the receptor for the RGD containing cell adhesion domain, and α4βl is the receptor for a carboxy-terminal cell adhesion region containing the Heparin II and IIICS domains (4b).

Resting peripheral blood and cultured T lymphocytes (Molt 4 or Jurkat) express an affinity for FN without the prototype FN receptor, α5β1. These cells express low or undetectable levels of α5β1 recognized by their functionally defined mAb (monoclonal antibody), P1D6. The inhibition by the same mAb indicates the resting peripheral blood T lymphocytes and cultured T cell leukemia's express multiple independent and functional FN receptors(4b).

Monoclonal antibody technology was further used to identify more receptors on different cells. The mAb inhibition of α4βl did not inhibit T lymphocyte adhesion to an 80-kD tryptic fragment of Pd-FN containing the RGD sequence(4b). This result gives an indication that T lymphocytes have at least two receptors for FN and identify α4βl as the receptor for adhesion site(s) located in the carboxy-terminal region of Pd-FN. It was concluded that T lymphocytes expressed a clear preference for an 38-kD tryptic fragment of Pd-FN. T lymphocytes also attached (with much lower affinity) to a site present in the heparin II domain.

It is important to note that culturing conditions may alter the expression of βl integrins by causing changes in the physiology of some subpopulations of primary cells growing within the culture. For example, expression of α5βl in hematopoietic cells is restricted to subpopulations of thymocytes, peripheral blood lymphocytes, monocytes acute lymphocytic or myelogenous leukemias, activated T cells, migrating hemopoietic

precursor cells, and some cultured T, B, or erythroleukemia cell lines (4). This dependent expression of integrin receptors for many cells could be required whenever different tissues need to heal.

It is clear that many cells adhere to FN using many different types of receptors. For example cultured K562, RD (rhabdomyosarcoma), and HTI080 (fibrosarcoma) cells, and freshly derived PBL adhere to FN -coated surfaces. However, other hematopoietic cell lines, such as K562 cells (Fig.8) exhibited a clear preference for the 80-kD fragment of plasma FN, whereas RD cells expressed promiscuous adhesion to all the fragments of Pd-FN tested, except the NH2-terminal 29-kD fragment. YT cells, which do not express α5β1, adhere poorly to intact Pd-FN or an interior FN 80-kD fragment. These cells require a two to three times longer to adhere to Pd-FN-coated surfaces, than Jurkat or Molt 4 cells (4b). Table I provides a summary of the cell types having α4β1 or α5β1 expression and indicates the level of cell adhesion to plasma FN.

Table II. Distribution of the Fibronectin Receptors α4β1 and α5β1 on Human Cells

Cells	Relative fluorescence intensity α4β1 (P4G9)	α5β1 (P1D6)
Hematopoietic cells*		
PBL	+ + +	+/− *or* −
LGL (CD3−, CD16+)	+ + +	+/− *or* −
Monocytes (CD16+)	+ +	+ +
Granulocytes	−	+
Platelets	−	+
Spleen	+ + +	+
Tonsil	+ + +	+
ALL (T or B)	+ + +	+ +
LGL leukemia (CD3+, CD16+)	+ + +	+/−
AML	+ + +	+ +
BLCL	+ +	+
Molt 4 (CD3+, CD4+)	+ + +	+
Jurkat (CD3+, CD4+)	+ + +	+ +
YT (CD3−)	+ +	−
PHA blasts (CD4+)	+ + + +	+ +
CTL (CD3+, CD8+)	+ + + +	+ + +
LAK (CD3+, CD8+)	+ + + +	+ + +
HL-60	+ +	+
U937	+ +	+
K562-1	−	+ +
Fibroblasts‡		
HFF (p5)	+	+
HT1080	+	+ +
RD	+ +	+
VA13	+	+ +
Epithelial cells		
OC-1§	−	−
OVCAR-4‖	−	−
T47D	−	−
QG56	−	+
HUVEs (p1)¶	−	+ +

Table I : Multiple integrins receptors expressed on different cells types and adhesion to FN.Table copyright to: Wayner, E. A., Angeles Garcia-Pardo, A., Humphries, M. J., McDonald, J. A., Carter. W. G. (1989). Identification and Characterization of the T Lymphocyte Adhesion Receptor for an Alternative Cell Attachment Domain (CS-1) in Plasma Fibronectin. *The Journal of Cell Biology, 109*, 1321-1330. Retrieved From jcb.rupress.org.

ECM Receptors and FN binding:

The ECM consists of a diversity of proteins which have roles in cell signaling and or are structural in nature. Some of these components are termed ECM receptors (ECMRs). They possess unique α subunits bound to an integrin β1 subunit (4b). ECMR-VI is identical to the prototype FN receptor α5β1, platelet glycoprotein (gp) Ic/Iia, and VLA 5; ECMR II is identical to α2β1, platelet gp Ia/Iia and VLA 2; and ECMR-I is identical to α3βl and VLA 3. Monoclonal antibodies to α2β1, α3β1 and α5β1 (P1H5, P1D6, and P1B5) inhibit fibroblast or platelet adhesion to collagen, FN and laminin-coated surfaces as well.

Summary:

FN structure is diverse and its function with respect to binding integrins of different cell types that participate in the acute phase response, the inflammatory and post-inflamatory tissue healing phases is equally diverse. The central theme revolves around cell recruitment, adhesion, morphological differentiation and proliferation. It does not seem to be involved with key aspects of the downregulation of the proliferation of colonized cells once proliferation begins.

Chapter 4:

Introduction:

Cellular and plasma derived FN have many structural and functional similarities. While cellular derived FN is primarily involved with the formation and maturation of the ECM, pd-FN is mostly associated with urgency of the acute phase response and clot formation. The substantial structural similarities between both forms suggest that pd-FN could mimic the function of cellularly installed FN if it were possible to deposit FN with less random deposition process than occurs during the acute phase. We discuss in more detail the functional and physiological attributes of cellular versus pd-FN below.

C-FN vs. Pd-FN in ECM and Clot Formations:

During the first stages of wound healing and after FB clot formation to stop hemorrhage a strong sealant adhesion is formed through FN and FB. The form of FN that is involved in this task is Pd-FN, secreted by the liver and abundant in blood. Cellular Fibronectin (c-FN) can also contribute to clot formation which can bind to both FB and platelets. But in comparison to pd-FN, there is a large difference in the extent of cross-linking to FB. It

has been found that the majority of pd-FN can be converted into the FN- FB α heterodimer while c-FN is only partially cross-linked (22).

It has been shown that pd-FN can be assembled into matrices both in vitro and in vivo, and the reported differences in solubility between cellular and plasma FN have suggested that pd-FN, which is normally a soluble protein in plasma, may be less effective in forming matrices *in vivo* than the forms of FN produced and methodically installed into tissues by cells (12). The difference is thought to be due to the EIIIB or EIIIA binding segments found in c-FN (12). However, pd-FN can be incorporated *in vivo* into the ECM and assist in the formation of ECM layer but at a lower level. Thus, we postulate here that the exogenous application of a high dose of pd-FN *in situ* using a less random process might improve ECM incorporations.

Pd-FN VS C-FN in Structure and Composition:

Both pd-FN and c-FN have very similar amino acid compositions, carbohydrate structures, secondary and tertiary structures. In addition, the types and the organization of many their structural domains are indistinguishable. Nonetheless, plasma and fibroblast FNs have differences in pI (iso-electric point), solubility, numbers of subunits linked together by disulfide bonds, and slight differences in size of certain domains (18). These slight differences could play functionally important roles in protein various tasks. For example, the difference in the rate and pattern of cross-linking of pd-FN and c-FN during

clotting demonstrate that these forms have temporally and structurally specific roles in the tissue repair process (22).

There are several forms of pd-FN which have mRNA encoding differences and there are at least three different types of mRNA that encode different FN subunits spanning stretches of 95 to 120 amino acids. These portions are located 276 residues from the carboxyl terminus of FN, near the cell and heparin binding domains(16). Although these sections are possibly the reason for the difference between the two subunits of Pd-FN, may be through the pattern of splicing in rat liver, which gives rise to pd-FN dimers of characteristic subunit composition: one subunit has the V region, the other lacks this segment (22). The short stretches of amino acids may also be responsible for functional differences between FN species such as in self-association and interactions with other molecules or cells (16). For example, it has been found that pd-FN consists of four types of subunits which are two size classes differing in molecular mass by about 10 kDa (15). Furthermore c-FN subunits have so-called V regions. These c-FN's specific subunits are somewhat larger and more acidic than the large subunits of pd-FN.

There have been several studies of the differences in FN structure found in rodents. For example, differences have been found in rat FN: there are two minor lower molecular weight subunits which have been shown to differ only in their glycosylation (15). In other studies it has been shown that the smallest subunits of rat and hamster FNs are not sulfated in contrast to other domains (15). These tyrosine sulfations are located within a

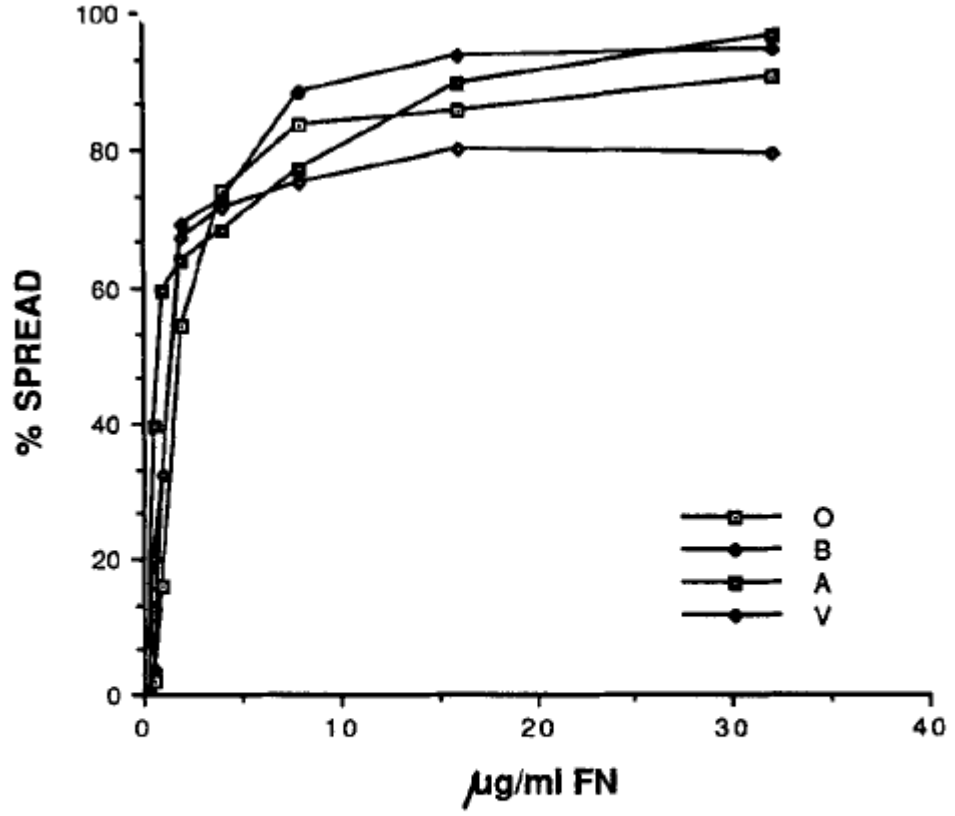
same 40- kDa FN tryptic fragment: there are four tyrosine residues within a 95 amino acid segment (15). The presence of the 95 amino acid segment can be used as a biochemical identifier of both the differences between each pd-FN subunits and of the differences between c-FN and pd-FN.

C-FN and pd-FN origin:

Pd-FN produced by hepatocytes in the liver for supply to blood as a plasma protein. Many cell types produce FN throughout the body as fundamental part of the ECM generation process and is also found in basal lamina, the media of blood vessels, and loose connective tissue. It has been shown that ECs are a major synthesizer of c-FN (17). Cultured human ECs synthesize c-FN with a polypeptide subunit mol wt of 200,000. They will secrete FN into culture media and also incorporate FN into the ECM (17). Cellular FN is also synthesized by fibroblasts and macrophages. Other cells like the astrocytes produce a form of FN that is of slightly higher molecular weight than the cellular form produced by fibroblasts, and the difference is due to preponderance of larger protein subunits rather than to any of the post-translational modifications found in FN (14). Past studies indicate that astrocytes synthesize and secrete FN both dimeric and monomeric forms that are slightly larger than FN from fibroblasts.

Pd-FN ability to function similar to c-FN:

Studies of the Recombinant FN (r-FN) made from different FN subunits suggest that all forms of FN are able to function and perform the critical functions of adhesion, migration and proliferation independently of A and B FN subunits (12). Studies that have generated recombinant FN have characterized the functions of various FN species. It was found that O, B, A and V forms of recombinant FNs (where the O FN lacks A, B, V regions) promote adhesion and spreading of various cell lines (12). In certain cases such as in lymphocytes, none of the A, B, or O FN forms promoted lymphocyte spreading while r-FN having a V domain was effective at inducing morphology changes (12). There are differing results with other cells types such as with B16F10 melanoma cells which is of skin origin as shown in figure 6(12). FN promotes adhesion and morphology changes in the cells listed in table I. In most cases, these cell express the membrane bound receptors $\alpha5\beta1$ and $\alpha4\beta1$ which are recognized by the RGD sequence in the V region of FN.



B16F10 melanoma cells spread on all forms of recombinant FNs. The spreading of B16F10 melanoma cells on all four forms of recombinant FNs were determined and quantitated as described in Materials and Methods. The average scores from three independent experiments are plotted here. The SDs were <10% on all points and are not shown.

Figure 6: Cell migrations.
Figure copyright to: Guan, J. L., Trevithick, J. E., & Hynes, R. O. (1989). Retroviral Expression of AlternativelySpliced Forms of Rat Fibronectin. *The Journal of Cell Biology, 110*, 833-847.

Yamada and Kennedy (1984) reported differences in the ability of FNs to revert the morphology of some oncogenically transformed cells to a normal morphology. FN purified from cell layers was 50-fold more effective in this transformation than was FN

purified from plasma. While this suggests a functional difference between pd-FN and c-FN (12, pd-FN was still able to perform the job at higher dosing.

Anther study was preformed to examine the ability of recombinant FNs to promote cell migration using a modified Boyden chamber assay. Several cell lines including NIH3T3, BHK, B16FIO, and NiI8.HSV were tested in these assays. On a "cells per unit area" basis, all four forms of FNs promoted cell migration to similar extents (12). Table II is a reproduction of that report where µm of cells migration occurring on a surface of FN is given.

Table I. Migration of Nil8. HSV Cells in Response to Fibronectin

	1.25 µg/ml	2.5 µg/ml	5.0 µg/ml	10.0 µg/ml
O	38.7 ± 6.1	138.0 ± 4.0	224.3 ± 17.5	211.7 ± 12.1
B	35.3 ± 4.2	153.3 ± 3.8	211.3 ± 19.5	216.3 ± 16.0
A	43.3 ± 6.4	162.7 ± 21.2	206.0 ± 5.3	215.3 ± 12.5
V	42.7 ± 5.9	159.0 ± 10.8	185.0 ± 4.4	222.7 ± 18.6

Table II: Indicate the incremental migration of Nil8.HSV in response to interactions with FN.

Incorporation of FN into ECM:

Pd-FN circulates in the blood until immobilized by binding and cross-linking to FB during clot formation can also incorporate itself into pre-existing extracellular matrices in vitro and in vivo. For example, differential amounts of exogenously applied FN to ECM *in vitro* of O and V forms relative to A and B forms were needed to achieve equivalent staining in rat ECM (12). These results suggest that inclusion of the EIIIA or EIIIB segments may enhance the ability of FN to incorporate into the existing matrix.

Heteromeric dimerization of FN chains:

Pd-FN has a simple dimer structure in which one subunit contains the V region and the other lacks this segment. C-FN is composed of a more complex mixture of subunits with variations at three sites-EIIIA, EIIIB, and the V region. Because the V region is present in both subunits of c-FN dimmers, but in only one subunit of the pd-FN dimer, it was speculated that this segment might have some effect on the cross-linking of c-FN during clot formation, despite its distance from the amino-terminal factor XIIIa-reactive site (22). Pd-FN is predominantly a heterodimer of V (EIIIC) and O subunits. It was found that 50% of the O form of FN is dimeric, indicating that the V segment is not absolutely required for dimer formation in WEHI231 cells in comparison to A and B forms. Interestingly, inclusion of the EIIIA or EIIIB segment appeared to reduce dimer formation (12). In one experiment using densitometry analysis, about 50% of the O form

of rat FN was found in the dimeric form, whereas; the majority of the B or A forms exist as monomers and ~90% of the V form is dimeric. These results suggest that the alternatively included V segment in FN may promote dimerization while the EIIIB and EIIIA segments may inhibit it (12).

Other structural variations within pd-FN:-

Other than the different segments that are included or excluded in the molecule subunits, or the non-homologies subunits within the same pd-FN, There are small regional areas that could also contribute to different forms of pd-FN. Studies have reported three different FN mRNAs occur by alternative splicing in rat liver which differ in an area (IIICS) located downstream of the ED region(21). The ED region is a 270-nucleotide segment that encodes exactly one repeat of the homology type III. This segment can be used to distinguish FN mRNAs from others(21). The difference sequence does not belong to any of the known internal homologies and is inserted between the last two type III homology repeats, near the COOH terminus. In addition, there are further variations in the equivalent IIICS area of human liver FN mRNA, bringing the total to five alternative motifs for this area. However, the ED and IIICS variable regions are found inserted between the cell-heparin and heparin- FB binding sites(21).

It seems that the function of the ED segment is to increase the spacing between the cell binding tetra-peptide and the heparin binding sites (21). The distance between these

biologically active sites of the molecule may be critical for FN function. For example, pd-FN is about 50% less active than c-FN in restoring morphology and alignment to a transformed fibroblast cell line (21).

Summary:

While there are differences and the similarities between pd-FN and c-FN, pd-FN has been shown to be able to provide the same functions in many aspects of wound healing, especially in platelet adhesion and FB clot formation. It also plays a role in the formation of ECM and cell adhesion.

Testing whether these reported differences in function are dependent on the presence of any of the alternatively spliced segments, researchers tested homogeneous recombinant FN forms containing each or none of the three segments. These experiments showed a only a two to threefold in effectiveness in many wound healing related assays (12). The same studies concluded it appears possible that the reported differences between c-FN and pd-FN in some assay are not due to alternative splicing of FN and may instead arise from differences in preparation or minor contaminants (12). This result is supported by that all the previous data shows all forms of FN are able to perform similarly in cell migration assays, proliferation and adhesion. At the same time the V (EIIIC) region of r-FN was able to be incorporated into the ECM sufficiently but at lower levels than c-FN containing segments (EIIIA) and (EIIIB).

The FB-pd-FN clot is adhesive and serves as a provisional matrix supporting the migration of epidermal cells during wound closure and the influx of inflammatory cells, fibroblasts, and ECs into the wound bed as healing progresses (22). An adhered blood clot contacts interstitial tissue composed of collagens, proteoglycans, and c-FN where fibroblasts migrate from the stromal into the wound (Clark and Henson, 1988). They then begin to synthesize, secrete, and organize c-FN into insoluble fibrils, providing a framework for consequent collagen deposition (22). We hypothesize that augmenting the acute response phase to injury with administration/deposition of less random structures consisting of pd-FN could accelerate this early phase of the regeneration process by increasing the cellular migration, adhesion, and proliferation phases.

Chapter 5

Purifying Fibronectin from Blood Plasma:

Background: Strategies for purifying fibronectin

Plasma fibronectin or "cold-insoluble globulin" was one of the first proteins isolated from plasma, serum, and amniotic fluid (39). It occurs in blood plasma at 300 to 400 μg/mL or 0.6 to 0.9 μmols/L. While plasma-derived FN differs slightly from that secreted from colonizing cells that are making ECM, it includes many of the same multiple cell receptor and ECM binding sites. These include collagen and heparin binding domains. These can be used to extract fibronectin from human plasma. Techniques including gelatin and heparin sepharose can be used to purify FN from human plasma in a one step, high yield affinity purification. FN purification also can be achieved by methods combining precipitation steps, molecular-exclusion chromatography and ion-exchange chromatography. These methods are generally lengthy and the yield of purified protein is relatively low. Fibronectin has also been purified by affinity chromatography on immobilized antibody (41).

Materials and Methods:

The following buffers were used to purify FN from citrated human blood plasma. Equilibrium buffer: TEC stock solution: at PH 7.5 and 24 C composed of 50 mM Tris, 50

mM EACA (Aminocaproic Acid), 20 mM sodium citrate. Washing buffer: composed of equilibrium buffer and 1M NaCl. Elution buffer composed of TEC buffer and 6M Urea. Dialysis Buffer 8L: TBS Solution PH 7.5 composed of 20mM Tris and 150mM NaCl.

Batch mode designed column:

Assembling the column, in a 250 mL funnel flask, the bottom was capped with plastic filter paper, sealed with parafilm, and water was used to check for leaks. Refer to gelatin device in Figure 7 if needed. A 1000 mL side arm flask was placed at the bottom to catch the mobile solutions (eluted solutions). Two bottles of Gelatin Sepharose (GE Health care, USA) were retrieved and swirled gently until the beads were mixed homogenously in the solution. A 5 mL TEC solution was poured through the device, followed by the beads solution. The gelatin bottles were rinsed 2-3 times with TEC solution only. The resin was washed with TEC solution by adding 10 mLs of the solution 2-3 times. The washing solution was dumped from the side arm flask.

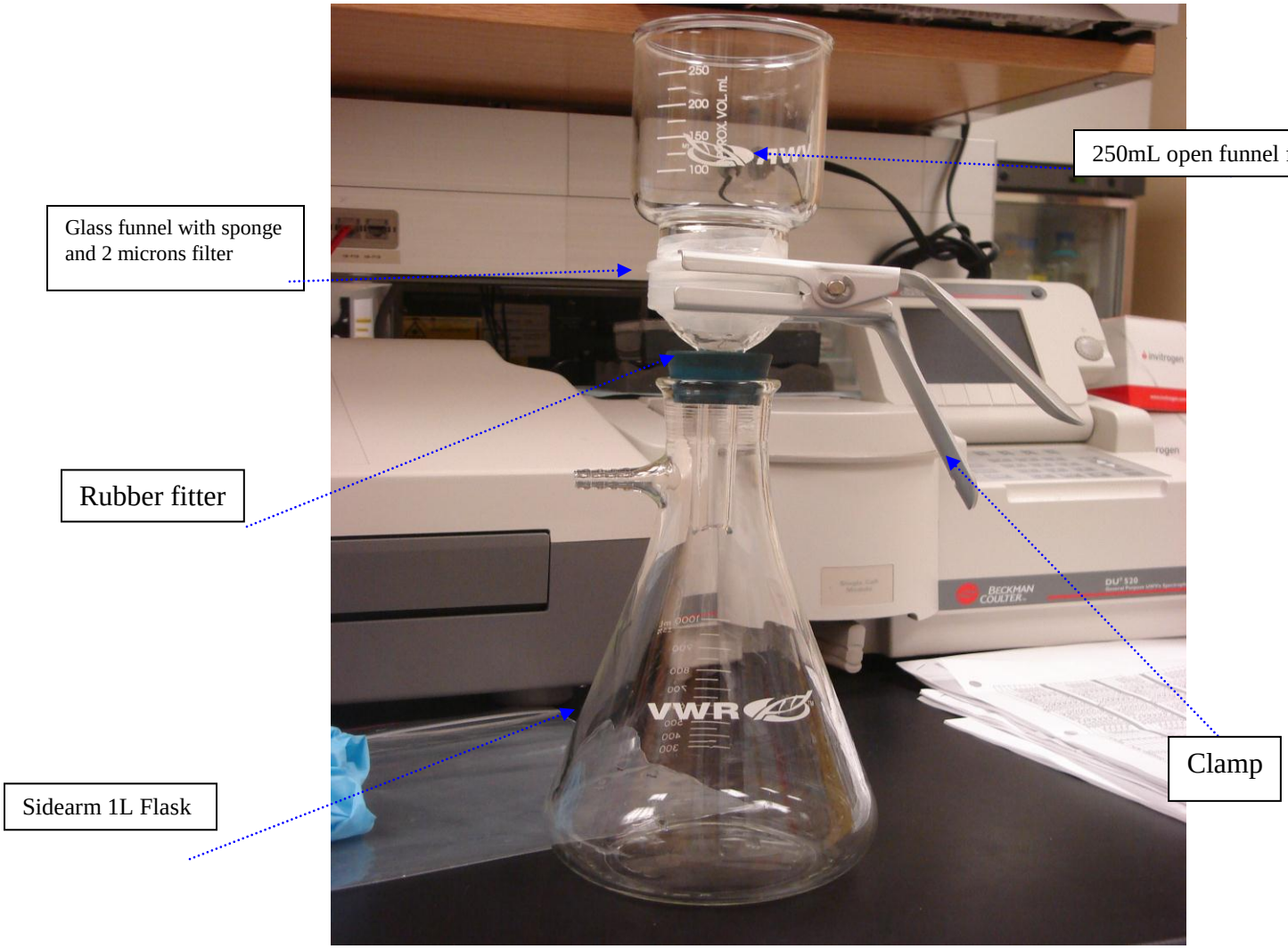


Figure 7: assembled batch mode column with Gelatin Sepharose beads.

Sample preparations:

Three units of plasma were retrieved from the -80°C Ultra Low 5. The plasma thawed at room temperature for at least 1 hour. Placing the samples directly in lukewarm water for faster thawing would shatter the plastic and cause the sample to be lost. The three plasma units were placed in two 4 L beakers that were filled with tap water. The 3 units of plasma were poured into a 4 L beaker and enough Aminocaproic Acid was added to the

plasma for a final concentration of 0.1 M (to inactivate plasminogen). A stir bar was used to mix the solution. The plasma was poured into the 250 mL wide mouth centrifuge bottles and balanced. The bottles were then placed into the swivel rotor in the Eppendorf Centrifuge 5810R-15amp at 2500 rpm for 15 minutes at 15°C. The supernatant was poured into a 4 L beaker without letting the white/yellowish pellet layer (Cryoprecipitate) fall through. After saving the Cryoprecipitate in two 50 mL conical tubes, they were labeled and froze for further use at -80°C.

Loading the sample: A portion of the sample was poured into the column (around the 200 mL mark). The resin and the solution were mixed using a spatula. The loading flow through solution was collected and saved in 1 L bottles.

Eluting the materials: Around 180 mL of the washing buffer (TEC+1 M NaCL) was poured into the column; it dispensed slowly using only gravity. The resin and the solution were mixed using a spatula. The resin was then washed 4-5 times with the elution buffer (TEC + 6 M urea).

Dialyzing the sample: The sample was dialyzed (to remove the high concentration of urea) three to four times of 5 hours each time, or until about 8 L of dialyzing solution was used up.

Running SDS-Page Gel: Aliquots were taken from the elution, loading flow through, washing step, and the dialyzed sample. Aliquots were used to run the gel. Lab procedures followed to run the gel. NU-Page 4-12% Bis-Tris gradient precast gel from Invitrogen (Cat No: NP0321Box) was used. Gels can either be ran as reduced or non-reduced. A reduced gel, using a reducing agent to break disulfide linkages, will reduce the size and the complexity of the protein. The non-reduced SDS-Page will not break the disulfide linkages, but instead leave the protein in its original form. As an identifier for the size of the protein sea-blue plus 2 pre-stained standard (1X) marker from Invitrogen (cat no LC 5925) was used.

Calculating protein Concentrations by BCA:

Follow these procedures to prepare the sample and measure the concentration of the purified protein using bicinchoninic acid assay (BCA).

Reagents:

Reagent A: Bicinchoninic acid solution. Reagent B: 4% Copper Sulfate solution. **Standard**: BSA 1mg/mL.

Setting up the BCA plate:

Dilution standard

#	ug BSA/mL	BSA 1mg/mL [ul]	water [ul]	BSA used [ug]
1	1000	200	0	100
2	800	160	40	80
3	600	120	80	60
4	400	80	120	40
5	200	40	160	20
6	0	0	200	0

sample

#	dilution X	sample ID	sample [ul]	water [ul]
1	2	A	200	0
2	4	B	100 of A	100
3	8	C	100 of B	100
4	16	D	100 of C	100

Table III: Dilutions of BSA standards and sample for BCA.

Procedures:

The BCA working reagent was prepared by mixing 50 parts of reagent A with 1 part of reagent B. Then 25ul of each sample was added into each well (each sample was placed in triplicate). Eight parts of BCA working reagent was used to mix with 1 part of a protein sample (by adding 200ul BCA working reagent to each well + 25ul of the sample). The 96 well plates were covered with plate sealer, and then incubated at 37C for

30 min. Finally, absorbance was measured at 570 nm using a Beckman Coulter AD 340 instrument.

Western Blot Analysis:

The presence of plasma fibronectin was verified using Western Blot Analysis. Lab procedures were followed to run SDS-Page Gel and transfer it to PVDF membrane (Vendor: BioRad, Cat # 162-0177). A positive control plasma derived FN was purchased from Sigma (F2006, Cas # 86088-83-7), and lyophilized from 0.05 M Tris buffered saline, PH 7.5. Rabbit Polyclonal primary antibody (ab2413, AbCam Cambridge MA, 5mg/mL) was added the membrane, followed by polyclonal secondary antibody (sigma, CA9169, Anti Rabbit IgG-HRP, 2mg/mL).

Results:

The binding of FN to both gelatin and heparin is Illustrated in SDS-PAGE Figures 3 and 4, where no protein eluted in the Loading flow trough's line, an indication of a strong FN binding avidity to gelatin and heparin.

SDS-Page Pictures:

In the E lane, fibronectin is the thick band around 200 kDa and about 98% pure. Lane:

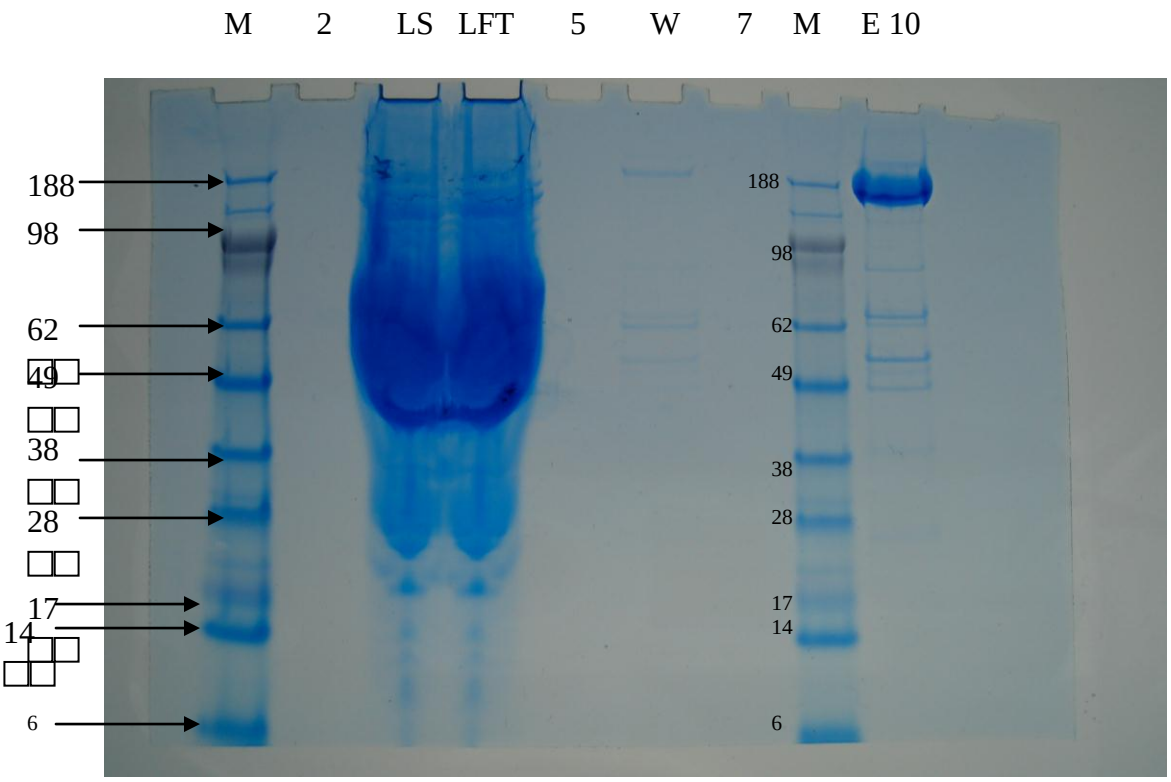


Figure 8: SDS-Page Reduced Gel Picture, representing the molecular weight and the purity of FN after purifying it from plasma with Gelatin affinity chromatography. The letters represent as follow M: Marker LS: Loading sample LFT: Loading Flow Throw W: Wash E: Elution.

Non-Reduced Gel for Gelatin and Heparin:-

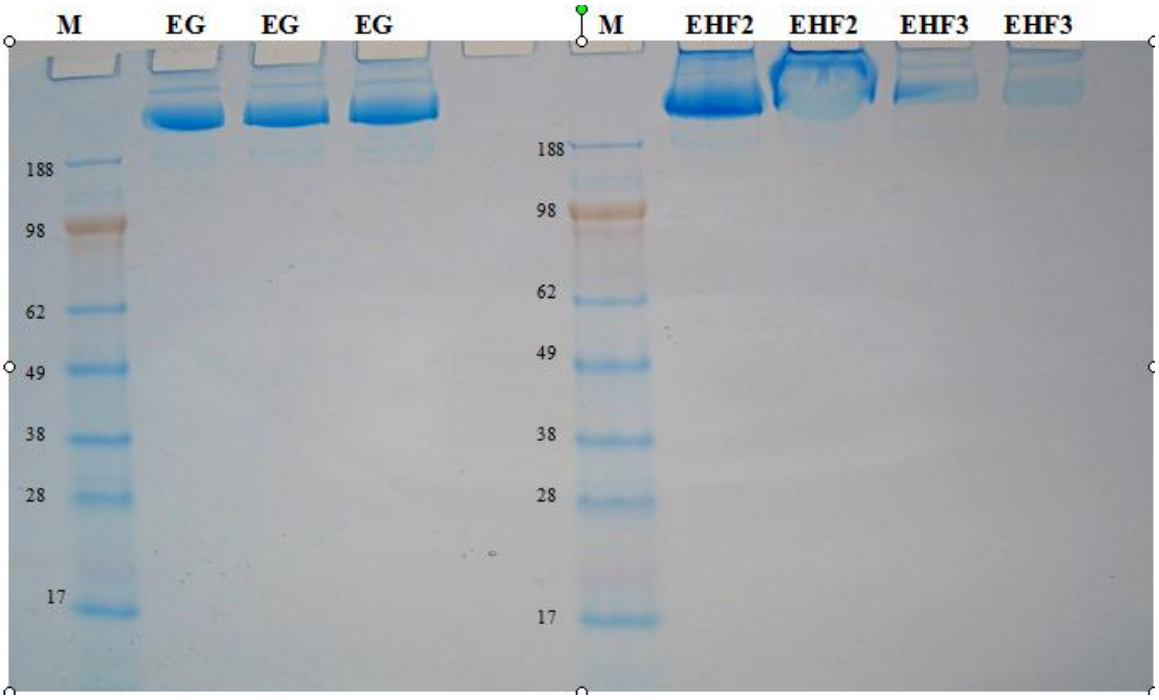


Figure 9: SDS-Page Non-Reduced Gel Picture, representing the molecular weight and the purity of FN after purifying it from plasma with Gelatin and Heparin affinity chromatography. The letters represent as follow M: Marker EG: Elution after Gelatin, EHF2: Elution post heparin fractoin2, EHF3: Elution post heparin fraction 3.

Heparin Run's SDS-Page Gel:

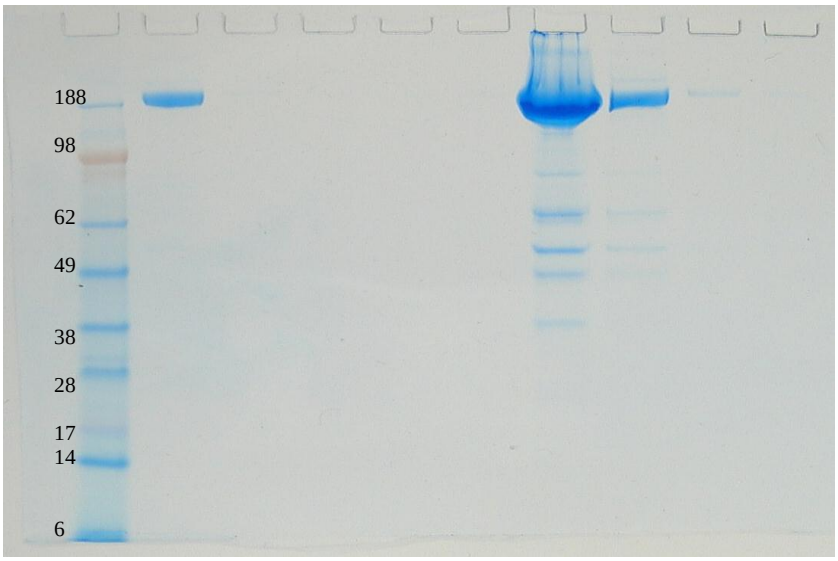


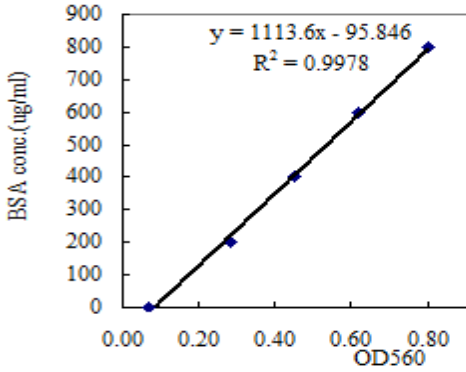
Figure 10: Heparin run SDS-Page Reduced Gel Picture, representing the molecular weight and the purity of FN after purifying it from plasma with Gelatin- Sepharose followed by Heparin affinity chromatography. The letters represent as follow M: Marker, LS: Loading sample, LFT 5: Loading Flow Throw fraction 5, LFT10: Loading Flow Throw fraction 10, W3: Wash fraction 3, E1: Elution fraction 1, E2: Elution fraction 2, E3: Elution fraction 3, E4: Elution fraction 4, E5: Elution fraction 5.

.

Protein concentration: BCA Results: Heparin Run-

Fraction 2 of heparin runs

Results	1	2	3	4	5	6	7
a	0.071	0.07	0.071	0.079	0.079	0.082	1.190
b	0.286	0.285	0.286	0.097	0.074	0.070	0.666
c	0.447	0.452	0.453	0.072	0.066	0.072	0.404
d	0.643	0.602	0.614	0.071	0.071	0.068	0.254
e	0.792	0.792	0.815	0.085	0.083	0.087	0.081
f	0.874	0.868	0.967	0.078	0.079	0.079	0.076
g	0.029	0.030	0.030	0.075	0.074	0.075	0.074
h	0.029	0.030	0.030	0.072	0.075	0.073	0.073



	BSA standard ug/mL	#1	#2	#3	avg	stv	%error
0	0	0.071	0.07	0.071	0.071	0.001	0.817
1	200	0.286	0.285	0.286	0.286	0.001	0.202
2	400	0.447	0.452	0.453	0.451	0.003	0.713
3	600	0.643	0.602	0.614	0.620	0.021	3.402
4	800	0.792	0.792	0.815	0.800	0.013	1.661
5	1000	0.874	0.868	0.967	0.903	0.056	6.147

slope	1113.6
y intercept	-95.84

FN x	#1	#2	#3	ug/mL	ug/mL	ug/mL	1.349 avg	mg/mL stv	diluted 1/1 2.7mg/mL %error
1	1.190	1.268	1.167	1229.344	1316.2048	1203.7312	1249.76	58.950721	4.71696331
2	0.666	0.711	0.682	1291.6352	1391.8592	1327.2704	1336.9216	50.80425	3.800091922
4	0.404	0.414	0.424	1416.2176	1460.7616	1505.3056	1460.7616	44.544	3.04936822
8	0.254	0.264	0.265	1496.115	1585.203	1594.112	1558.477	54.190	3.477

Table VI: BCA Calculations of FN concentrations with initial sample dilution.

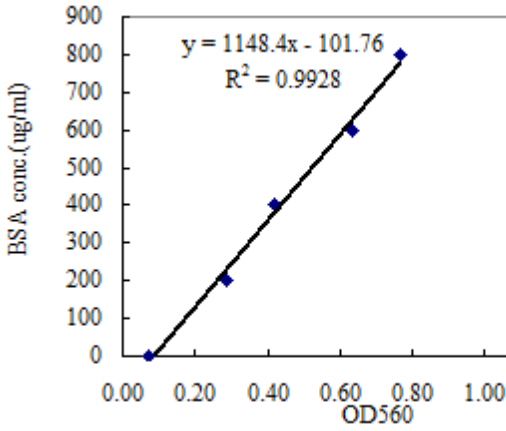
From the BCA Results, the Concentration of Fibronectin after the Heparin Run to be 2.6 mg/mL for fraction 2.**BCA from Gelatin Sepharose:**

Results

	1	2	3	4	5	6	7	8	9	10	11
a	0.084	0.069	0.07	0.569	0.573	0.584	0.567	0.463	0.602	0.031	0.030
b	0.293	0.284	0.289	0.339	0.359	0.357	0.342	0.352	0.358	0.030	0.030
c	0.428	0.418	0.415	0.210	0.223	0.216	0.222	0.216	0.214	0.030	0.032
d	0.643	0.636	0.63	0.147	0.152	0.149	0.154	0.145	0.147	0.030	0.030
e	0.793	0.775	0.727	0.030	0.030	0.030	0.030	0.030	0.032	0.030	0.030
f	0.882	0.911	0.954	0.030	0.030	0.031	0.030	0.030	0.030	0.030	0.030
g	0.933	0.926	0.918	0.030	0.030	0.030	0.031	0.039	0.031	0.031	0.030
h	0.031	0.030	0.030	0.030	0.030	0.030	0.030	0.030	0.030	0.030	0.030

BSA standard

	ug/mL	#1	#2	#3	avg	stv	%error
0	0	0.084	0.069	0.07	0.074	0.008	11.282
1	200	0.293	0.284	0.289	0.289	0.005	1.562
2	400	0.428	0.418	0.415	0.420	0.007	1.619
3	600	0.643	0.636	0.63	0.636	0.007	1.022
4	800	0.793	0.775	0.727	0.765	0.034	4.460
5	1000	0.882	0.911	0.954	0.916	0.036	3.956



slope	1148.4
y intercept	-101.76

FN							0.572	mg/mL	
x	#1	#2	#3	ug/mL	ug/mL	ug/mL	avg	stv	%error
1	0.567	0.463	0.602	549.3828	429.9492	589.5768	522.9696	83.027017	15.876069
2	0.342	0.352	0.358	581.9856	604.9536	618.7344	601.8912	18.564813	3.0844135
4	0.222	0.216	0.214	612.7392	585.1776	575.9904	591.3024	19.124682	3.2343319
8	0.154	0.145	0.147	600.749	518.064	536.438	551.750	43.417	7.869

Table V: BCA Calculations of FN concentrations without initial sample dilution

SDS-Page:

Reduced Gel with FN positive Control:

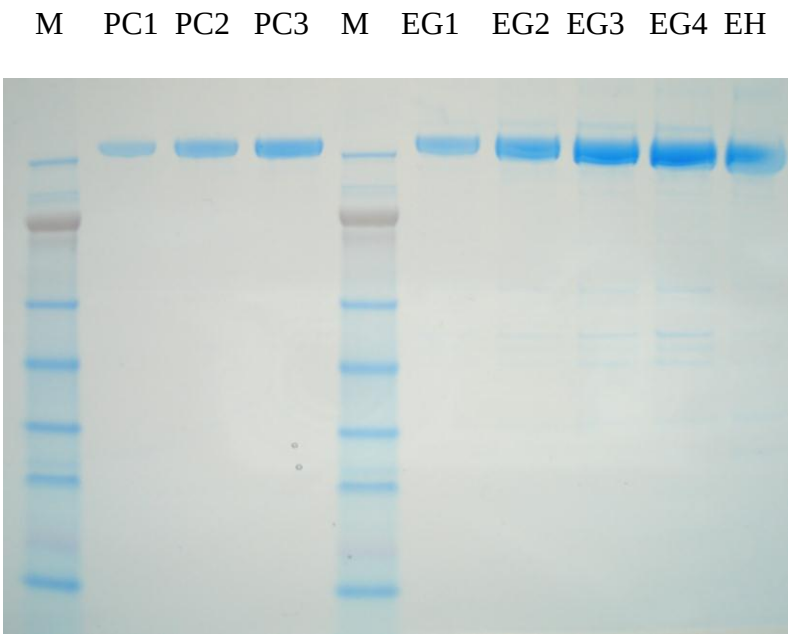


Figure 10a: SDS-Page Reduced Gel Picture with FN positive Control , representing the molecular weight and the purity of FN after purifying it from plasma with Gelatin-Sepharose followed by Heparin affinity chromatography. The letters represent as follow M: Marker, PC 1,2,3: Positive control at at 2.5, 5, 7.5µg, EG 1,2,3,4: Elution from Gelatin at 2.5, 5, 7.5, 10 µg, EH: Elion from Heparin at 10 µg.

Western Blot Results:

M PC1 PC2 PC3 M EG1 EG2 EG3 EG4 EH

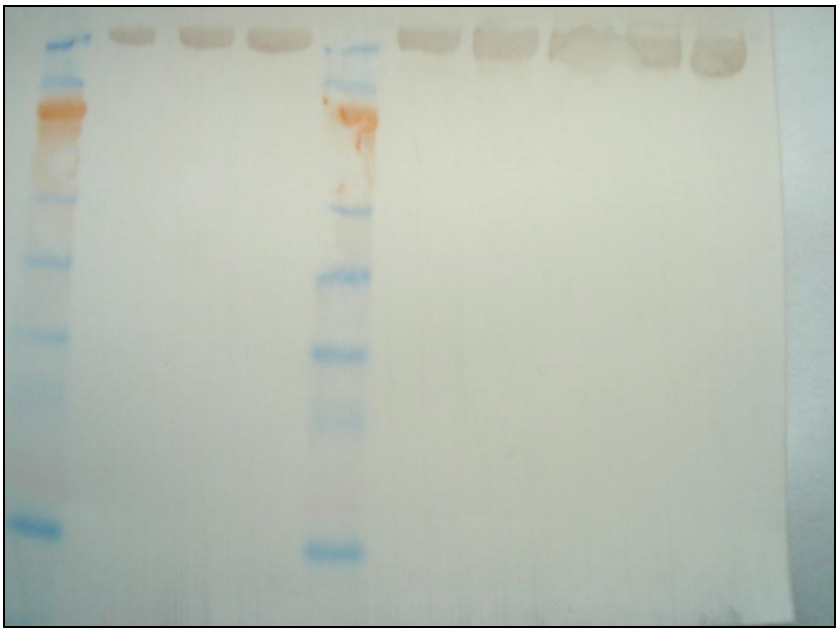


Figure 10b: Reduced western blot analysis picture. The letters represent as follow M: Marker, PC 1,2,3: Positive control at at 2.5, 5, 7.5µg, EG 1,2,3,4: Elution from Gelatin at 2.5, 5, 7.5, 10 µg, EH: Elution from Heparin.

SDS-Page:

Non-Reduced Gel results:

M PC1 PC2 PC3 M EG1 EG2 EG3 EG4 EH

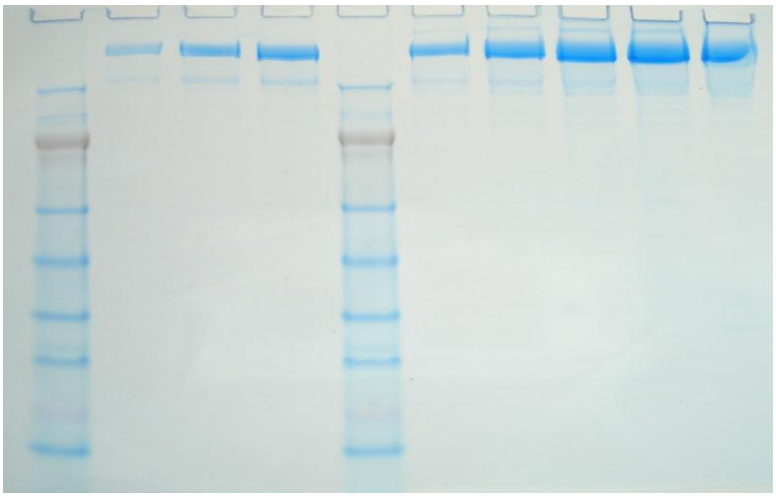


Figure 10c: SDS-Page Non-Reduced Gel Picture with FN positive controls, representing the molecular weight and the purity of FN after purifying it from plasma with Gelatin-Sepharose followed by Heparin affinity chromatography. The letters represent as follow M: Marker, PC 1,2,3: Positive control at at 2.5, 5, 7.5µg, EG 1,2,3,4: Elution from Gelatin at 2.5, 5, 7.5, 10 µg, EH: Elution from Heparin.

Non-Reduced Western Blot Picture:

M PC1 PC2 PC3 M EG1 EG2 EG3 EG4 EH

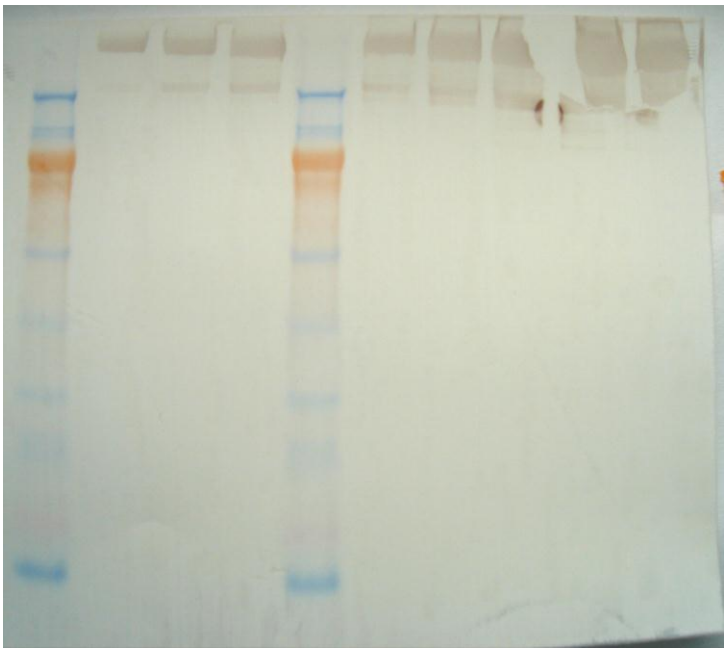


Figure 10d: Non-Reduced western blot analysis results. The letters represent as follow M: Marker, PC 1,2,3: Positive control at at 2.5, 5, 7.5µg, EG 1,2,3,4: Elution from Gelatin at 2.5, 5, 7.5, 10 µg, EH: Elution from Heparin.

Material Yields:

Three units of plasma yields: about 700 mL/ unit = 2100 mL.Volume of Loading Flow is approximately = 2300 mLs. Volume of Elution after Gelatin Sepharose run = 200 mLs. Volume of Elution after dialysis = 350 mLs.

For the Heparin 1 mLs column Run, as mentioned earlier we loaded on 5 mLs of loading sample. Volume of Loading Flow Throw = 10 mLs. Volume of the Wash = 3 mLs. Volume of Elution = 5 mLs. So for the Gelatin Sepharose: we had about 350mL after

dialysis at approximately (350*0.572mg/mL = 200mg) of FN. For Heparin, we had about 1 mL of fraction 2 at approximately 2.6mg/mL = 2.6mg. For fraction 3 it was about 0.3 mg/mL = .3mg. Total FN concentration: 2.9mg out of 5 mLs. Also 5 mLs of post Gelatin loading sample yielded (5 mLs*0.572mg/mLs= 2.86mg). This reflects that there was no significant lose of material during the Heparin procedures.

Discussion:

Some contaminants are still present in the FN after affinity purification. The non-reduced gel picture result indicates that the contaminant's bands on the gel could be fragments of FN. The electrophoretic appearance of the contaminants is changed by reducing agent.

The gel figures in 5a and 5c show that the positive control matches exactly with the FN's purification results. Since the bands are matching in size and purity, it is an indication of purified FN protein. The western blot results in Figures 5b and 5d indicate a successful interaction with FN primary antibody proving plasma derived fibronectin is the primary species in the purified product.

As mentioned earlier, the purity of the material after one purification step with gelatin was about 98% (refer to figure 8 and 10). Heparin affinity chromatography using Heparin Sepharose did not result in an increase in purity.

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