Enzymatic Incorporation of Bidomain Peptides into Fibrin Matrices for Directed Enhancement of Three-Dimensional *in vitro* Neurite Outgrowth and *in vivo* Nerve Regeneration

Thesis by

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In Partial Fulfillment of the Requirements

for the Degree of

Doctor Of Philosophy

California Institute of Technology

Pasadena, California

1999

(Submitted May 3, 1999)

Enzymatic Incorporation of Bidomain Peptides into Fibrin Matrices for Directed Enhancement of Three-Dimensional *in vitro* Neurite Outgrowth and *in vivo* Nerve Regeneration

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Dedication

This work is dedicated to my family for their constant and unconditional support.

Acknowledgments

I would like to thank numerous people for both intellectual and emotional support throughout my doctoral research.

Curtis Herbert, Thomas Uhlich and W. John Kao for teaching me the necessary technical techniques in chemistry and cell culture required for my project as well as how to successfully organize a research project.

Lori Giver for providing enormous amounts of technical and emotional support.

Jocelyne Bloch, Michael Papaloizos, Olivier Jordan and Patrick Aebischer for providing me with the resources to complete the *in vivo* study and performing the delicate microneurosurgery.

Jeff Hubbell for providing me with the opportunity to pursue research in his laboratory and the freedom to develop as a scientist.

All my colleagues from the Hubbell lab who provided a conducive environment for me to conduct my research, especially Sven, Simone, Natalie, Jamie, and Alison.

And finally a large group of people who provided emotional support during the difficult times; including my family, Dave, Tim, Nikki, Dolores, Gargi, Guru, Vidya, and especially Lila for being there for me in my new, foreign home of Zürich.

Abstract

In this research, fibrin has been enzymatically modified using the transglutaminase, factor XIIIa, to incorporate bioactive domains from extracellular matrix and cell surface proteins. Fibrin was chosen as a base matrix as it is sensitive to cell-derived and cell-regulated protease activity. In order to improve the bioactive character of the fibrin, bi-domain peptides were designed where one domain contained a bioactive sequence and the other contained a factor XIIIa substrate sequence. These factor XIIIa substrates were derived from fibrinogen, α_2-plasmin inhibitor and a nonbiological substrate. Each of these peptides were then covalently incorporated into the fibrin during coagulation through the action of the enzyme, factor XIIIa, with the sequence from α_2-plasmin inhibitor incorporating at levels up to 8.2 mol peptide/mol fibrinogen.

Initially, these enzymatically modified fibrin gels were utilized in an academic study to probe the mechanisms involved in RGD-mediated three-dimensional cell migration. Two separate RGD sequences, one linear and one cyclic, were individually incorporated into fibrin matrices and the effect on neurite migration was measured, and it was shown that both RGD peptides have an adhesive-like, biphasic effect on cell migration. The density of peptide corresponding to maximal neurite outgrowth was lower for the cyclic than the linear peptide. However, since the cyclic RGD is a stronger binding sequence, it is likely that these two peptide densities represent a similar adhesive quality.

Development of enzymatically-modified fibrin matrices was also specifically directed towards enhancement of peripheral nerve regeneration. Peptides were individually incorporated in a concentration series and it was shown that these peptides, including RGD, HAV, IKVAV, YIGSR or RNIAEIIKDI, could improve neurite

outgrowth by approximately 20%. A series of formulations were then tested, whereby multiple bioactive peptides were co-cross-linked into fibrin gels. One formulation, which contains the four laminin-derived sequences, RGD, IKVAV, YIGSR and RNIAEIIKDI, incorporated in equimolar densities of 1.7 mol/mol proved to enhance neurite outgrowth by 75% over unmodified fibrin. This formulation was then tested as a filler material for growth guides in peripheral nerve repair. Through these experiments, it was demonstrated that enzymatically modified fibrin is both nontoxic and capable of enhancing nerve regeneration.

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1. Introduction

1.1 OBJECTIVE OF THIS RESEARCH

The objective of this research was to develop three-dimensional matrices that would enhance the regeneration of peripheral nerves across a gap. In order to accomplish this goal, fibrin matrices covalently modified with short neuroactive peptides were employed. Fibrin was chosen as the base matrix for neurite outgrowth because it had been previously shown to support *in vitro* neuronal regeneration (Herbert, Nagaswami et al. 1998). Furthermore, this matrix is completely biocompatibile, with applications as a surgical adhesive, and it is widely available from commercial sources as either a fibrin glue, purified precursor fibrinogen or made from the blood of patients for whom it is to be used. These fibrin matrices were modified by covalently incorporating neuroactive peptides through the action of factor XIIIa. It has been clearly shown within a two-dimensional system that short immobilized peptides derived from extracellular matrix proteins can enhance neurite outgrowth, and these peptides were incorporated into three-dimensional fibrin matrices.

Initially, a method was developed whereby bioactive peptides could be covalently incorporated into fibrin matrices and this level of incorporation was quantified. Then, using a previously developed *in vitro* neuronal outgrowth assay, chick dorsal root ganglia (DRGs) were embedded within the matrices and the subsequent outgrowth of neurites was quantified. Those materials that provided the greatest level of enhancement were then used in pilot studies as filler material for nerve growth guides on severed dorsal root and sciatic nerves in the rat.

This introduction will first describe the current clinical standards for peripheral nerve repair and their limitations. It will then go on to explain the research using nerve growth guides and how this led to the use of fibrin as a base matrix. The related biology of fibrin formation and neurite outgrowth will be elucidated and the effect that individual peptides in neuronal outgrowth and their mechanism are discussed. Finally, the biochemistry of factor XIIIa mediated peptide incorporation will be discussed.

1.2 PERIPHERAL NERVE INJURY

After a nerve bundle is severed, the peripheral nervous system has a modest ability to regenerate and reinnervate the original targets, the two steps necessary for functional recovery. Much is known about the mechanisms of peripheral nerve regeneration, including the identification of many of the cells and molecules involved. The process involves the interaction between both neuronal and non-neuronal cells as well as cellular interaction with residual extracellular matrix proteins derived from the remnants of the distal nerve stump (Kandel, Schwartz et al. 1991). Clinically, when a nerve is severed and the resulting gap is small, the proximal and distal ends are sutured together. The distal stump subsequently degenerates, and it has been proposed that the degenerated stump provides a track of protein signals to which the growing neurites respond (Weiss, May et al. 1994). The tip of the proximal ends (connected to the cell bodies) then form neuronal growth cones that migrate along the original tracks left behind by the degenerated distal nerve stump (Politis 1985; Evans, Bain et al. 1991). When the gap between the two stumps becomes large enough, suturing them together becomes impractical due to the increased tension on the axons. In this case, the nerve bundle is repaired by suturing a nerve autograft into the interpositional region (Mackinnon 1989). The autograft then degenerates in a manner similar to the distal

stump and provides a track to direct the regenerating nerve bundle. However, this requires the sacrifice of a healthy nerve, which is an obvious disadvantage.

The deficiencies of nerve autografting have led to the use of nerve growth guides (NGGs) to enhance nerve regeneration. Instead of placing a grafted nerve in the gap, a polymer-based tube is inserted (Aebischer, Guenard et al. 1988; Uzman, Snyder et al. 1989) (Figure 1.1). These NGGs have been explored to protect the interstump region from ingrowth by scar tissue, to entrap bioactive matrices and to deliver polypeptide growth factors.

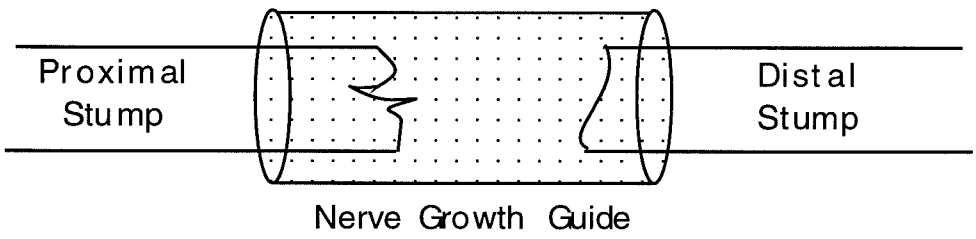

Figure 1.1 Diagram of Nerve Growth Guide
Instead of inserting a resected nerve, the stumps of the severed nerve is placed inside a growth cone. The growth guide then can be filled with a neuro-inductive material.

1.3 NERVE GROWTH GUIDES

While the use of NGGs has not yet become clinically common, they have been used experimentally for bridging large gaps in severed neurons in animal models, with research focusing on the component material of the guide, the physical structure of the guide and the development of materials used to fill the guide.

1.3.1 Physical Characteristics

In the development of NGGs, it was discovered that some physical characteristics, such as porosity and roughness, were crucial while others were less important. Initially, empty silicone guides were used as NGGs, with each end sutured to the epinurium of the nerve bundle. While silicone has traditionally been the polymer used in constructing NGGs (Evans, Bain et al. 1991), success has also been achieved using polyvinyl chloride/polyacrylonitrile copolymer tubes (Aebischer, Guenard et al. 1989). This implies that the specific chemical structure of the polymer used in the guide is not critical.

In contrast, the structure of these growth guides, including the porosity and roughness, has been shown to be important and has subsequently been optimized. One step in studying the effect of NGG structure was to use porous nerve guides. The addition of pores would allow factors from the surrounding tissue to enter the guide and interact with the regenerating nerves. A significant increase in regeneration was noticed when the pores in the guides were small enough to prevent cellular infiltration but large enough to allow proteins to enter (Jenq and Coggeshall 1987; Jenq, Jenq et al. 1987). Furthermore, regeneration in tubes with pores small enough to inhibit proteins larger than 100 kD from entering was much better than in tubes with larger pores (Aebischer, Guenard et al. 1989). This lead to the conclusion that some small, beneficial protein is present in the extracellular matrix and can be made available to the regenerating neuron merely by using a porous growth guide. Another important characteristic was the roughness of the lumen of the guide. If the guide used had a smooth lumen, a bundle of nerves was observed to form in the middle of the tube (Aebischer, Guenard et al. 1990). However, if the interior of the tube was rough, the tube would be filled with connective tissue instead of nerve axons. Clearly then, the physical structure plays a key role and can be optimized to enhance nerve regeneration.

1.3.2 Filling the Nerve Growth Guide

Some success has been obtained in peripheral nerve regeneration by filling the NGGs with bioactive materials, including proteins, three-dimensional matrices or autologous cells. The addition of such bioactive materials has proven to further enhance the level of regeneration that can be obtained with NGGs.

By using empty NGGs, the basic mechanism through which regeneration occurs inside a growth guide was elucidated. Within the first seven days postseverance, a fibrin coagulum forms within the hollow tube (Williams and Varon 1985; Aebischer, Guenard et al. 1990). This fibrin forms a bridge between the two severed ends and retracts from the surface of the guide as it self cross-links. This bridge is then used by an advancing front of fibroblasts, which is followed by Schwann cells and axons (Weiss, May et al. 1994). The growing axons begin to become myelinated after several weeks and the gap is usually completely bridged in 4-5 weeks (Le Beau, Ellisman et al. 1988). This led to research where NGGs were prefilled with materials designed to induce and increase fibrin formation inside the NGG. The first trial involved filling a 10 mm gap in a rat sciatic nerve NGG with saline. In comparing these trials with the unfilled tubes, the saline filled tubes contained more fibrin and a larger number of axons at the midpoint of the tube (Williams 1987). Additional studies were undertaken whereby the NGG was prefilled with fibrinogen-containing blood plasma. This provided results which were even stronger than those for guides filled with saline, with additional increases in the number of axons at the midpoint (Williams, Danielson et al. 1987).

Another significant advance in the development of NGGs was to fill them with a neurotrophic material. Filling the guide with an active protein, such as collagen,

laminin or fibronectin, could potentially allow the synthetic tube to mimic the role of the degenerated distal nerve stump by providing active signals, as well as being able to provide the necessary mechanical support for regeneration. In the past, collagen (Fields, Le Beau et al. 1989) and laminin (Klavjin and Madison 1991) have been used as the active matrix. This research has met with mixed results. When these matrices were incorporated into impermeable growth guides, the addition of bioactive proteins was shown to enhance the number of axons within the guide (Rizvi, Wasserman et al. 1991). However, this result was only noted after long regeneration times, when most of the matrix had been degraded (Le Beau, Ellisman et al. 1988; Rende, Granato et al. 1991). In comparing the effects of the matrix molecules, the addition of laminin increased the number of axons to a greater extent than did the collagen (Klavjin and Madison 1991). In contrast, when these materials were used to fill porous NGGs, the results were significantly worse than those achieved with the use of guides filled with autologous fibrin matrices (Valentini, Aebischer et al. 1987). These contradictory results probably point to the complicated balance between proteolysis and cell signaling in the outgrowth of neurites. While fibrin does not contain many specific active sequences for neurite outgrowth, it is readily and controllably degraded by cell surface proteases, allowing the neurites to migrate through unimpeded. In contrast, molecules such as laminin or collagen contain these active sequences but are not readily degraded by the cells and this may serve to impede neurite migration through the matrix.

Finally, other procedures have been developed that involve NGGs that have been used that were prefilled with autologous cells. This technique involves harvesting cells from the patient, expanding them in culture, and seeding them within the guide. Schwann cells have been shown to invade the NGGs before the axons, so preseeding the cells may stimulate the initial migration of neurites into the guide. This technique has been successfully used in regenerating axons in models where saline has little effect at both long and short times (Guenard, Kleitman et al. 1992; Ansselin, Fink et al.

1997). Unfortunately, this technique has clinical restrictions; as autologous glial cells are limited, it requires a second procedure and the addition of autografts is not possible.

1.3.3 Nerve Growth Guide Models

The goal of employing nerve growth guides is to replace the use of a sacrificial donor nerve for regeneration across a gap. There are several models used to test the efficacy of various matrices in inducing nerve regeneration, with the rat sciatic nerve providing the gold standard. In these experiments, the sciatic nerve is exposed and resected at lengths between ten and fifteen mm. As a standard level of comparison, when a fourteen mm gap is transversed with an autograft, normal levels of axons are observed at nine months along with some functional recovery (Keeley, Atagi et al. 1993). In contrast, use of collagen or saline filled matrices provided similar levels of myelinated axons but a lack of functional recovery. The regenerative capacity of the sciatic nerve in both autograft and entubulation approaches is relativity high (Terris, Cheng et al. 1999), preventing the use of this model to test matrix efficacy at long time points. However, due to the lack of functional recovery with entubulation approaches, most research has focused on measuring numbers of myelinated axons at shorter times or with greater gap lengths. Using this as the standard, it has been shown that the introduction of matrix into a silicone tube can lead to significant regeneration at shorter time points. One example is the use of a collagen matrix, which has reduced the time necessary for regeneration of myelinated axons. With these matrices, a ten mm gap has been shown to be transversed in only six weeks (Chamberlain, Yannas et al. 1998) . Other matrices that have been used with success are agarose and hyaluronic acid. With these matrices, the moderate gaps of ten mm have been transversed in four weeks. (Wang, Nemeth et al. 1998) Finally, matrix precursors that induce formation of a fibrin matrix in the nerve growth guide have had much success. Using these materials

gaps of ten mm have been transversed within two weeks (Williams and Varon 1985) and fifteen mm gaps have been transversed in only eight weeks (Williams 1987).

Due to physical limitations in resecting the rat sciatic nerve at lengths greater than fifteen mm, it became necessary to find a less permissive environment to test highly functional matrices. This has led to use of the dorsal root nerve in the rat. When the dorsal root nerve is severed, the level of regeneration through an empty silicone tube with a gap of four mm is very low. The native dorsal root nerve has approximately 2000 axons and when the bundle is severed and regenerated through an empty silicone tube, the resulting axon count is ten fold lower. Preliminary work on filling these silicone tubes with an active matrix have demonstrated that growth guides filled with YIGSR-derivitized agarose do not enhance the level of regeneration significantly above that seen with saline filled tubes. (Aebischer, unpublished results) While exogenous matrix treatments have had very little success in inducing dorsal root regeneration, the application of more powerful growth factor delivery devices has provided significant results. Porous polymer rods were soaked in a protein solution containing nerve growth factor and placed instead empty silicone growth guides that were used to transect a four mm gap in resected dorsal root nerves. This polymer rod then acted as an uncontrolled drug delivery device allowing the active growth factor to diffuse into the silicone guide. When nerve growth factor was delivered with this method, the number of myelinated axons reached 1600, or 80% of the axons present before resection. (Bloch and Aebischer, unpublished results) This indicates that it is possible to induce regeneration with this model if a highly permissive environment is created, allowing its use in testing highly inductive matrices.

1.4 BIOLOGY OF FIBRIN

In the natural response during peripheral nerve regeneration, severed nerves synthesize a new fibrin-based environment for regeneration. In this section, the biology of fibrin will be elucidated, including the process by which a fibrin matrix is synthesized from the precursor monomers, as well as the structural and biochemical characteristics of fibrin that make it a preferred material for nerve regeneration.

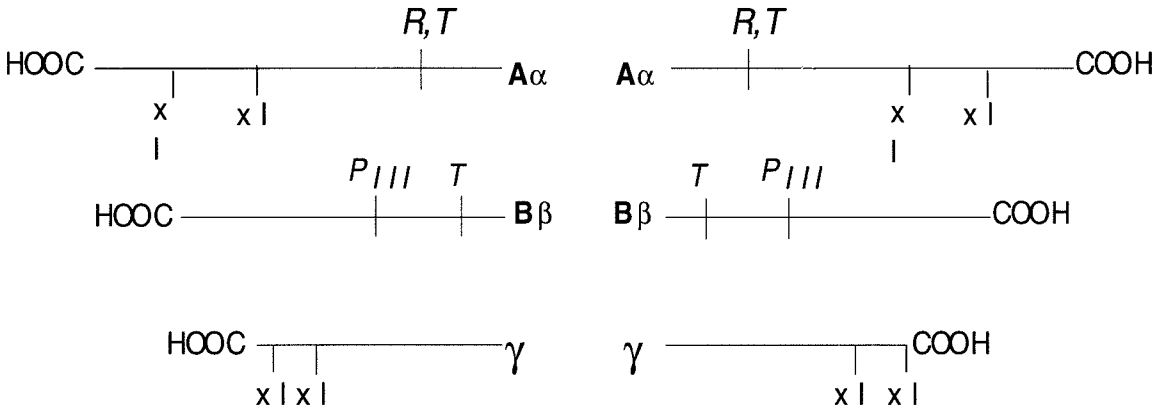

Figure 1.2 Structure of Fibrinogen
Here, the structure of fibrinogen and cleavage sites of the major proteases have been marked (*R* is for reptilase, *T* is for thrombin and *P III* is for protease III). Additionally the sites where cross linking can occur have been marked as xl.

1.4.1 Fibrinogen

Fibrinogen is the precursor monomer that forms the bulk of fibrin gels and much is known about the characteristics of this important molecule. Fibrinogen is a 330 kDa protein that consists of three different chains, an Aα, a Bβ and a γ chain. Each of these chains are aligned together, and the full fibrinogen molecule is a dimer, consisting of pairs of these units (Figure 1.2) Fibrinogen circulates in the blood at a

concentration of 2.5 mg/mL. Fibrinogen circulating in the blood stream is synthesized by the liver and can be produced at a rate of up to 5.0 g/day (Takeda 1966).

1.4.2 Fibrin Formation

The process by which fibrinogen is polymerized into fibrin has been well characterized. Initially, a protease cleaves the dimeric fibrinogen precursor molecule at the two symmetric sites. There are several possible proteases that can cleave fibrinogen, including thrombin, reptilase, and protease III, and each one severs the protein at a different site (Francis, Bunce et al. 1993). Each of these cleavage sites have been located and they can be found on either the Aα or Bβ chain. However, while there are several enzymes that cleave fibrinogen molecules leading to the formation of a fibrin gel, the only endogenous enzyme is thrombin. Once the fibrinogen is cleaved, a self-polymerization step occurs in which the fibrinogen monomers come together and form a non-covalently crosslinked polymer gel (Sierra 1993). A schematic representation of the fibrin polymer is shown in Figure 1.2. After the amino termini of the fibrinogen are cleaved, these new termini adopt a substantially different conformation compared to the uncleaved monomer. This new conformation serves as a non-covalent binding sequence for the carboxy termini of the same chain (i.e., Aα with Aα or Bβ with Bβ) on a separate molecule. Once these new sites are exposed, the binding is quick and self-catalyzed (Stryer 1975). In this manner, a

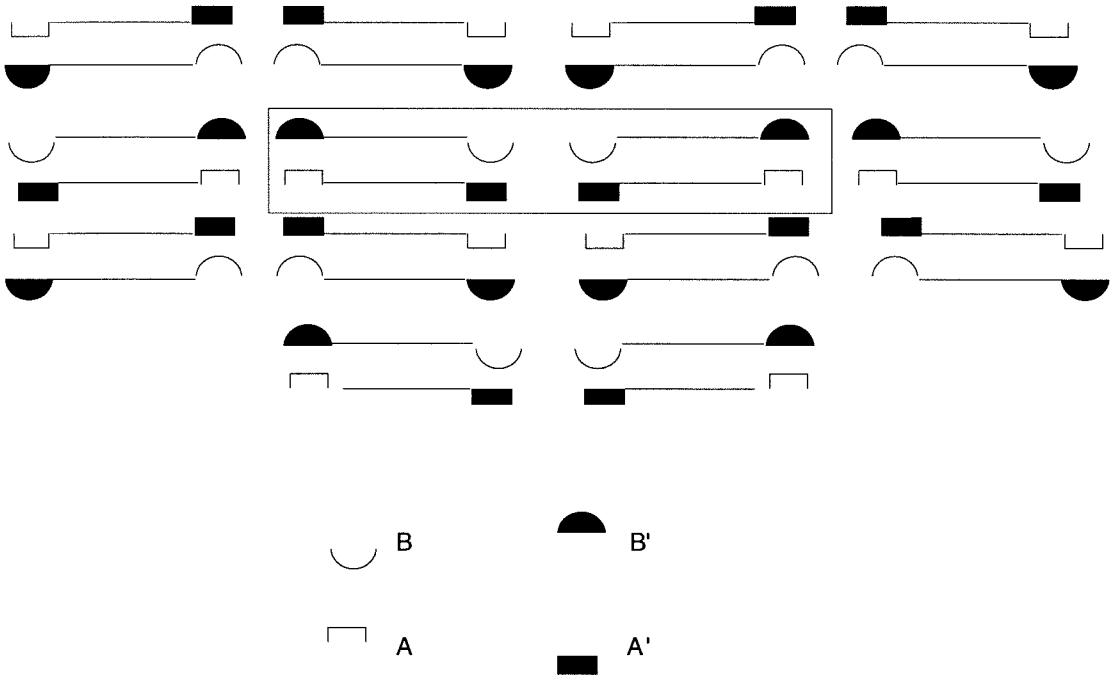

Figure 1.3 Structure of Fibrin Gel
The structure of a polymerized fibrin gel is shown. The gel is held together by the noncovalent binding of sites B to B' and A to A'. A' and B only become available for binding after cleavage by a protease. The polymerization reaction is self-activated and followed by covalent cross-linking to other molecules through the action of factor XIIIa. A single monomer unit is boxed in the center.

polymer chain is formed, as shown in Figure 1.3. Factor XIIIa, activated from factor XIII by thrombin proteolysis, then covalently cross-links the polymer network.

1.4.3 Plasmin

One key aspect to fibrin matrices is their degradability by plasmin. Other biopolymer matrices that have been explored in NGGs, such as agarose, tend to inhibit cell migration, as they physically impede the migration of cells within the matrix. In contrast, the degradability of fibrin allows cells to selectively remove local fibrin matrix. The primary enzyme responsible for fibrin degradation is plasmin. Many different cells produce and regulate plasmin activity near their surface and this provides

infiltrating cells with a method to locally remodel a three-dimensional fibrin matrix. The complex interaction between protease inhibitors, protease activators, proteases and fibrin has been well studied and is well understood.

Fibrin is cleaved in a specific fashion by plasmin, leading to a soluble series (Kane 1984) of degradation products. The degradation products have been named FDP-A, B, C, D, E, X, and Y and have been shown to be bioactive (Doolittle 1984). The presence of these products can lead to subsequent increases in protease, protease activator and protease inhibitor secretion (Lucas, Straight et al. 1983). This provides a possible feedback mechanism that enhances the rate of cell-mediated fibrin degradation. The active enzyme plasmin, a serine protease that attacks arginine-lysine bonds, is derived from the zymogen, plasminogen. Plasminogen circulates in the blood and is cross-linked to blood-accessible fibrin clots through the action of the transglutaminase, factor XIIIa. Additionally, plasminogen binds to the surface of cells, including neurons (McGuire and Seeds 1990). The zymogen probably binds through a variety of sites on the surface including disialogangliosides (Plow, Felez et al. 1991). By binding to the surface of cells, such as neurons, a high local concentration of plasminogen can be obtained and the subsequent formation of the active plasmin can be locally regulated.

The zymogen, plasminogen, is activated through the action of both tPA (Jansen and Reinders 1992) and uPA. TPA is a 70 kDa glycoprotein derived from single-chain tPA. These molecules are present in many cells, including Schwann cells (Baron Van Evercooren, Leprince et al. 1987), venous endothelial cells, kidney cells, muscle cells and many others (Kane 1984; Dano, Andreasen et al. 1985). TPA is also present in blood and is secreted at even higher concentrations in response to vascular injury, exercise and various hormones such as epinephrine. TPA activates plasminogen by binding noncovalently to fibrin or cell surfaces via lysine binding sites. It then forms a ternary complex with plasminogen to then generate plasmin (Kane 1984; Mayer 1990). The amount of active tPA is further upregulated by plasmin itself. The newly formed

active enzyme, plasmin, has an autoregulatory function, as it then serves to activate the formation of additional tPA from single-chain tPA. UPA is a somewhat smaller glycoprotein derived from the precursor single chain uPA. These molecules are also widely distributed in the connective tissues of the body and is found in the blood at concentrations similar to tPA. However, the mechanism for plasminogen cleavage differs from that employed by tPA. UPA does not bind to fibrinogen, but instead interacts with specific cell-surface receptors with a very high affinity (Ellis and Dano 1991). When uPA is bound to a receptor on the surface of a cell, it retains a high level of activity. As an example, it has been shown that the rate at which membrane bound uPA activates membrane bound plasminogen (as described above) is sixty-fold faster than the same reaction between two soluble molecules (Angles-Cano 1994).

The mechanism involved in the inhibition of plasmin activity is equally complex, with plasminogen activator inhibitor-1 (PAI-1), α2-macroglobulin, and α_2-plasmin inhibitor all involved in the regulatory process. PAI-1 is secreted by vascular endothelial cells, monocytes, activated platelets and liver cells (Angles-Cano 1994). It circulates in the blood at nanomolar concentrations in a complex with tPA and acts as the major soluble inhibitor of plasminogen activators. It is able to block the activity of soluble tPA and uPA by forming these 1:1 complexes (Mayer 1990). However, the inhibitory activity of PAI-1 becomes negligible once the plasminogen activators become associated with either fibrin or cell surface molecules. The primary inhibitor of soluble plasmin is the molecule α_2-plasmin inhibitor. α_2-plasmin inhibitor circulates in the blood and binds directly to circulating plasmin, inhibiting its function (Angles-Cano 1994). In contrast to the action of PAI-1, the activity of α_2-plasmin inhibitor is retained with cell surface-bound plasmin, although inhibition occurs at a much slower rate. Additionally, α_2-plasmin inhibitor has the ability to prevent plasmin-mediated fibrinolysis. Through the action of factor XIIIa, α_2-plasmin inhibitor is covalently cross-linked into the fibrin matrix and prevents the activation of plasminogen within the

gel, lowering the rate of fibrinolysis (Sakata and Aoki 1982). A secondary inhibitor of plasmin is the protein α2-macroglobulin. α2-macroglobulin binds to plasmin and inhibits its function in a similar manner as α_2-plasmin inhibitor does. However, while it is present at a significantly higher concentration, it reacts much more slowly, leading to less total inhibitory activity. It probably functions as a reserve inhibitor, duplicating the crucial function of α_2-plasmin inhibitor.

The complexity of the degradation process associated with plasmin allows different cells to have precise control over the local rate of matrix degradation. The secretion of these protease inhibitors, protease activators and proteases is a well regulated and controlled process allowing cells to locally degrade matrix permitting subsequent migration but prevent global degradation. This is true in the process of neurite extension both *in vitro* and *in vivo* and is detailed later in this introduction.

1.4.4 Active Signals in Fibrin

In addition to providing structural support for infiltrating cells, fibrin also contains several bioactive adhesion signals that bind directly to cell-surface receptors on migrating cells. Fibrin contains four separate adhesion peptides that are known to interact with cell-surface molecules. The first sequence is RGD, a common, well-studied motif known to bind integrin receptors on various cell types (described in Chapter 3). Fibrin has two RGD sequences, one on the α chain, and the other on the γ chain. Since fibrinogen is a dimer, there are four RGD sequences on each molecule. It has been previously shown that these two RGD sequences have different bioactivity. In some examples, the Aα chain RGD sequence is active, as in the adhesion of platelets to the fibrin matrix (Phillips, Charo et al. 1991). However, this sequence is not

recognized by endothelial cells, which in turn recognize the RGD sequence on the γ chain (Francis, Bunce et al. 1993). Therefore, while the fibrinogen molecule does contain four separate RGD sequences, individual cells often recognize only one of the two sequences.

Another active sequence in fibrin that has been identified is the Bβ sequence 15-42, which has been shown to be necessary for complete adhesion and spreading of endothelial cells and fibroblasts on fibrin. This sequence is near the amino terminus of the Bβ chain, and after cleavage by thrombin, this becomes the new terminus of the chain. In its initial conformation, this sequence does not have any discernible activity; however, upon the action of thrombin, the exposed sequence becomes active (Francis, Bunce et al. 1993). It is known that the cleavage of the Bβ sequence 1-14 is necessary for 15-42 to become active. This is easily studied, as the polymerization of fibrin with the enzyme reptilase cleaves the Aα chain similar to thrombin, but does not cleave the Bβ chain. The resulting matrices do not support the spreading of either endothelial cells or fibroblasts or the subsequent cell-derived extracellular matrix (ECM) synthesis (Bunce, Sporn et al. 1992). Furthermore, if the fibrinopeptide Bβ 15-42 is removed through plasmic degradation, cell adhesion is similarly reduced to the levels seen in reptilase derived matrices (Hamaguchi, Bunce et al. 1994). The exact mechanism of interaction between cells and this peptide has been investigated as well. It has been shown that a non-integrin 130-kDa cell-surface receptor binds to this sequence and, furthermore, that it is present on endothelial cells (Erban and Wagner 1992). It is also hypothesized that the action of this sequence induces cells to produce greater amounts of ECM proteins, which are subsequently deposited onto the fibrin matrix (Dejana, Lampugnani et al. 1990). The addition of highly active molecules, such as fibronectin, laminin or collage, to the matrix would then serve to further enhance cell attachment and spreading.

A third sequence that has been identified is the peptide KQAGDV on the γ chain. This peptide has been shown to bind to the integrin $\alpha_{IIb}\beta_3$ and is involved in platelet adhesion (Phillips, Charo et al. 1991). Another peptide, KYGWTVGQKRLDSV, also from the γ chain, has been shown to bind the $\alpha_M\beta_2$ integrin and mediate adhesion of leukocytes (Altier, Plescia et al. 1993). While it is known that fibrin mediates neurite and Schwann cell growth and migration, the exact peptides that might interact with these cells are not identified. It is known that neurites respond to matrix-incorporated RGD, so it is likely that they respond to the RGD molecules present in the fibrin matrix. However, it may be that the fibrin matrix serves as a reservoir, retaining ECM proteins secreted by Schwann cells or neurites (Davis, Klier et al. 1987), allowing them to enhance neurite outgrowth.

1.5. BIOLOGY OF REGENERATING NERVE

Through the study of regenerating nerves, much has been learned about the molecular mechanisms involved. Immediately after a bundle of axons is severed, peripheral cells migrate out from the proximal stump. These are then followed by the axons, which migrate toward their target by forming growth cones at the end of each severed axon. This following section describes in detail the biology of these growth cones, the associated glial cells, the complicated role that plasmin plays in this process and some of the extracellular matrix proteins and soluble growth factors that are known to be involved as well.

1.5.1 Growth Cone

Through the study of nerve regeneration, much about the morphological changes that occur to the neurite is known. The general process is as follows. The tip of the severed axon expands to form a growth cone (Jessell 1991). This growth cone consists of two morphologically distinct regions, the lamellipodium and many

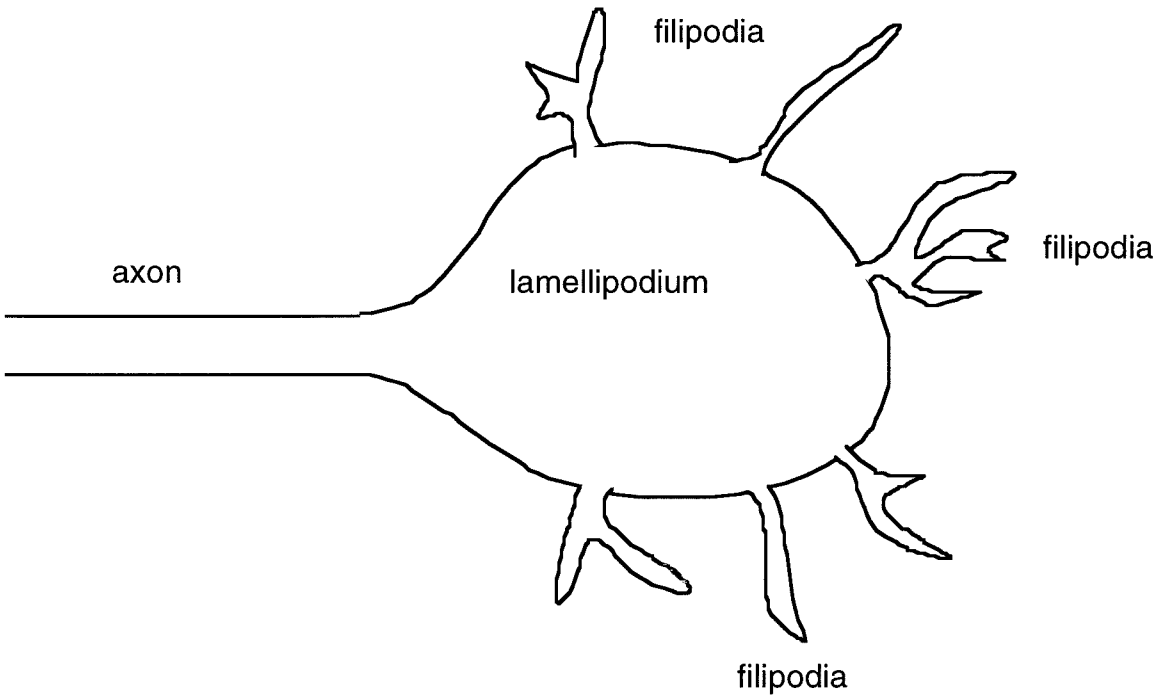

Figure 1.4 Schematic of Neuronal Growth Cone
At the end of a severed neurite, a growth cone is formed. The filipodia constantly extend out from the lamellipodia as the neurite searches for signals. The nerve will only grow in the direction where the filipodia find the correct signals.

filipodia. The lamellipodium is a broad sheet of membrane at the end of the axon stump (Jessell 1991). On the outer sections of the lamellipodium are the filipodia (Figure 1.4). These are spike-like projections that extend from the outer edge of the growth cone and sense the surrounding microenvironment. These filipodia constantly extend

and then fold back into the lamellipodia (Nicholls, Martin et al. 1992). Filipodia contain membrane-bound adhesion receptors that are used to sense the chemical factors in the extracellular matrix (Nicholls, Martin et al. 1992). If these receptors bind to ligands in the surrounding matrix, and the adhesive bond is sufficiently strong, a cascade within the cell leading to additional axon extension in that particular direction is initiated (Bray 1992). This extension is the result of actin polymerization, which can drive filipodial outgrowth at a rate that can increase the surface area of the cell by as much as 25% per hour (Bray 1992). The mechanism is thought to be triggered by both activation induced through receptor binding as well as the increase in tension that occurs when the filipodium attempts to retract. The receptor-mediated activation of actin polymerization occurs through protein kinases, calcium influx or other secondary messenger systems (Bray 1992). The precise mechanism through which the tension induces actin polymerization is unknown at this point.

1.5.2 Glial Cells

One of the first cell types that are known to invade a nerve gap or NGG spanning severed nerve are glial cells such as Schwann cells. Schwann cells enhance neuronal outgrowth through several mechanisms, including secretion of proteins, direct cell/cell interaction through cell-surface receptors and assisting in axon remyelination. Glial cells are found in both the central and peripheral nervous systems, with the primary cell types being oligodendrocyte and astrocytes in the central and Schwann cells in the peripheral nervous system. Previous work has demonstrated that Schwann cells either precede or co-migrate with growing axons and they may be necessary for axon extension in three-dimensional gels (Williams, Longo et al. 1983; Ide, Osawa et al. 1990). This involvement in nerve regeneration was shown in an experiment done

using a nonpermeable growth guide that contained collagen or laminin gel (Klavjin and Madison 1991). Using a severed rat sciatic nerve for the study, a correlation between axonal ingrowth into the tube and the distance that the non-neuronal cells migrated into the tube was observed, in that the regenerating nerve would not grow beyond the point that the Schwann cells had migrated (Williams, Longo et al. 1983). Another experiment was done involving a larger separation between the nerve stumps. Using the same techniques as before, it was observed that Schwann cells precede axons into spontaneously-formed fibrin matrices (Williams, Longo et al. 1983). Additionally, inhibiting Schwann cell mitosis reduced axon ingress into the inter-stump gap (Hall 1986), and Schwann cell mitosis has been reported to reduce motility *in vitro* (Dubois-Dalq, Rentier et al. 1981).

The neural activity of Schwann cells is both diverse and potent. It has been shown that Schwann cells perform a variety of functions in the process of nerve regeneration, including presenting a favorable surface for neurite growth, secretion of soluble active factors and finally assisting in the remyelination of the new neurites. Cells present many membrane bound receptors on their surface, some that interact with other proteins and some that interact directly with other cells. One class of receptors that binds to other cells are the cadherins. It is known that Schwann cells present the cell adhesion molecule, N-cadherin on their surface (Letourneau, Shattuck et al. 1990). Since axons also present N-cadherin, and N-cadherin is homophilic protein (Jessell 1991), a chain of Schwann cells could provide an active path for the axon to follow. Furthermore, N-cadherin is a substrate for neuronal outgrowth, and it has been shown that neurites grow twice as fast upon Schwann cells, which express membrane bound N-cadherin, as upon laminin, another favorable substrate for neuronal outgrowth (Bixby, Lilien et al. 1988). Therefore, when the Schwann cells precede the axons into the NGG, they are able provide a neuro-inductive surface of N-cadherin molecules to enhance neuronal outgrowth.

In addition to presenting surface molecules, Schwann cells also secrete many proteins that enhance neuronal outgrowth. The most potent factor that the Schwann cells secrete is nerve growth factor (Assouline, Bosch et al. 1987). While nerve growth factor is selective for cells of the nervous system, it does activate many neuronal and associated nonneuronal cell types including the Schwann cells themselves, creating a feedback mechanism for the further self-induced activation of Schwann cells. Another series of molecules that Schwann cells secrete are proteins involved in the plasmin cascade, including plasminogen activators and plasminogen. One of the key steps in the migration of the neurites through their environment is the proteolytic degradation of the surrounding matrix. By secreting these molecules, the Schwann cells are able to assist the neurites in this process and prevent the matrix from physically inhibiting neurite migration. Finally, the Schwann cells secrete a series of other proteins that directly enhance neurite outgrowth, including a laminin-heparin sulfate glycoprotein complex (Davis, Klier et al. 1987). Many of these molecules have been well studied and their exact mechanisms are explained in the following sections.

The final step in the process of nerve regeneration is the remyelination of the newly formed axons. Normal, mature axons have a myelin sheath surrounding the axons, and this myelin serves to protect the axons and increase the conduction velocity of action potentials. This remyelination process is sequentially a second function for the Schwann cells. Once the axon has finished growing, the Schwann cells stop secreting other active proteins and begin the process of remyelinating the newly formed axon (Nicholls, Martin et al. 1992).

1.5.3 Plasmin Regulation

One of the key mechanisms in the process of nerve regeneration is the protease-mediated degradation of the surrounding matrix. This is a complex, highly regulated

process that involves the synthesis of proteases, protease activators, protease inhibitors and relevant receptors. In the following sections, the proteins and cells involved will be elucidated.

In order for the neurite to grow, a careful balance of proteases must be achieved at the tip of the growth cone. This balance is obtained through the regulation of protease activators, receptors for these activators and protease inhibitors, and the mechanism employed has been partially elucidated (Figure 1.5). It has been demonstrated that neurons secrete a plasminogen activator that in turn cleaves plasminogen into plasmin, the active enzyme (Krystosek and Seeds 1981; Krystosek and Seeds 1984; Pittman 1985). As the axon grows, this protease is produced at the tip of the growth cone and it clears out a path for the axon to travel through the matrix (Pittman and Buettner 1989). Furthermore, if an excess of plasmin inhibitor, such as aprotinin or EACA, is added to the media, it has been shown that the three-dimensional growth of neurites through a fibrin matrix is diminished (Herbert, Bittner et al. 1996). However, much more about the complex regulation of this protease has been discovered. While it is necessary for the protease to be present in order to remove the matrix that is blocking the path of the neurite, if the concentration of active plasmin is too high, then global degradation of the fibrin would occur, removing adhesion proteins and signal peptides before the filipodia can find them and bind (Monard 1985). Therefore, it is crucial that the level of protease activity be tightly controlled. Finally, the protease also acts at the trailing edge of the neuronal growth cone. At this point, it is necessary for the growth cone to detach from the surrounding matrix, and it is the responsibility of plasmin to detach any cell-fibrin connections that occurred earlier in the neurite extension (Monard 1985).

Based on the sensitivity that neuronal outgrowth shows toward protease activity, it is clear that the amount of active protease surrounding the axonal growth cone must be closely regulated. The primary mechanism employed by the regenerating

nerve to achieve this control involves surface receptors for plasminogen activators. First, the growth cone secretes plasminogen activator, primarily urokinase plasminogen activator (uPA) (Seeds, Haffke et al. 1992). On the surface of the cell are receptors for both uPA and tissue plasminogen activator (tPA). There are two receptors for uPA, and they serve to regulate the protease concentration near the underlying substratum (Seeds, Haffke et al. 1992). One of the receptors binds to the active region of uPA and prevents it from leading to the degradation of the adhesion contacts with the matrix

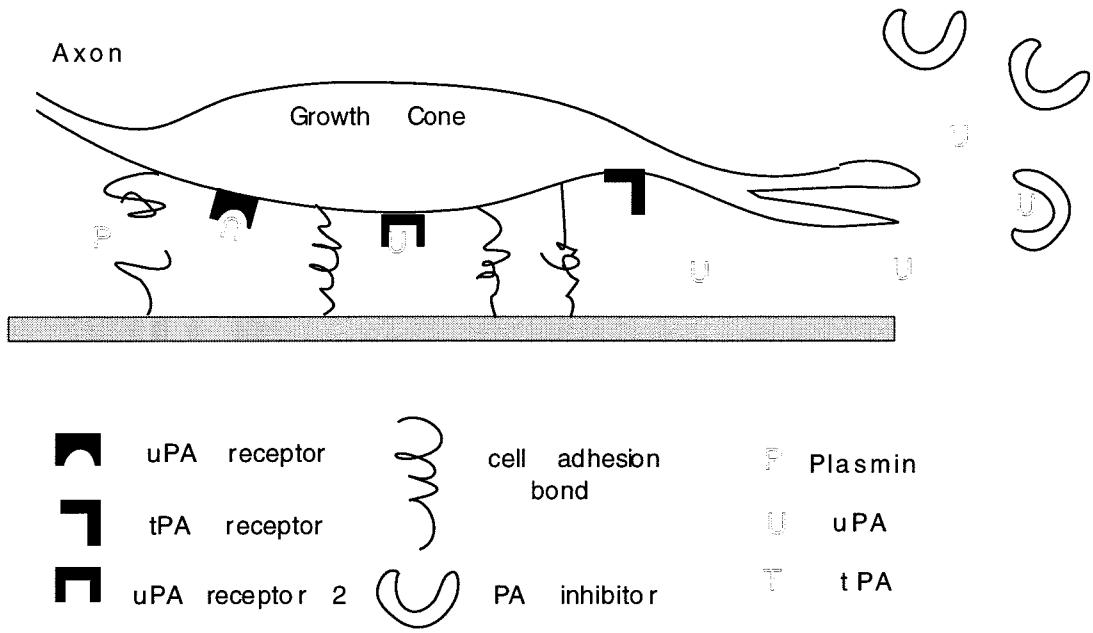

Figure 1.5 Balance of Proteases at Growth Cone
Schematic of the neuronal growth cone. uPA is secreted by the cone and then is either bound to a surface receptor or consumed by protease inhibitors in the matrix. If the protease is bound to a receptor, it can either be in an active state or an inactive state. That is determined by which receptor binds to it. The active protease activator will make protease that will either degrade the matrix ahead of the growth cone or break adhesion bonds at the tail of the growth cone.

(Pittman 1985). The other receptor binds to a non-active site on the protease (Pittman and Buettner 1989). This receptor binds to the uPA in a manner that it retains its activity, allowing the uPA to cleave plasminogen. Therefore, the plasminogen activator that is secreted by the cell often binds to the surface of the growing axon (Pollanen, Hedman et al. 1988), serving to localize the plasmin activity near the surface of the cell. This is highly beneficial for several reasons. First, it helps to keep the plasminogen activator, and therefore the generated plasmin, localized. This limits the amount of global matrix degradation that occurs. Second, it prevents the plasminogen activator from being destroyed by many of the numerous protease inhibitors present in the extracellular matrix, which minimizes the amount of activator that must be synthesized. And finally, it allows the cell to control the plasmin activity at its surface. This control is obtained by the distribution of the two uPA receptors. The distribution of these two receptors is such that the inhibitory urokinase plasminogen activator receptor would be found near the leading edge of the growth cone where adhesion contact is critical and the activating urokinase plasminogen activator receptor would be found farther down the axon, where removal of the binding is just as important (Pittman and Buettner 1989). Finally, the neuronal cells contain a tPA receptor on their surface as well (Tiberio, Farina et al. 1997). This is important for protease regulation as well, since the associated glial cells synthesize tPA instead of uPA. A schematic of the distribution of receptors and proteases at the growth cone is shown in Figure 1.5. The role that tPA plays in matrix degradation will be explained below.

In addition to regulation of protease activity directly at the growth cone, the concentration of plasmin in front of the advancing growth cone must be carefully regulated as well. This is so that the active protease synthesized does not remove active signals before they are recognized but can still prevent the matrix from impeding migration (Monard 1985). This is accomplished by the presence of a high concentration of plasminogen inhibitors ahead of the advancing axon. One such

inhibitor is α_2-plasmin inhibitor. Through the action of factor XIIIa, a transglutaminase, α_2-plasmin inhibitor is covalently bound within the fibrin matrix. This protein then serves to remove plasminogen activator and plasmin from the fibrin matrix. Therefore, at large distances from the advancing neurite, very little active plasmin is present because of these inhibitors. However, at small distances, much of the inhibitor has been consumed and some active plasmin is present. This is beneficial not only because it removes the matrix, but some research has suggested that partially degraded fibronectin may serve as a good signal transducing agent (Werb and Tremble 1989). Clearly then, a balance of protease at the leading edge of the neurite is vital.

While neurites play a major role in producing and regulating the local and global plasmin activity, the associated glial cells play a supporting role in this process. As mentioned earlier, the Schwann cells migrate into the fibrin matrix before the neurites enter. Therefore, in order to enter the damaged region and participate in nerve regeneration, the Schwann cells must be able to degrade the matrix. The method used by Schwann cells is similar to that employed by growing axons (Krystosek and Seeds 1984), in that plasminogen is converted into an active protease, plasmin. One difference is that Schwann cells secrete tissue plasminogen activator instead of urokinase plasminogen activator (Baron Van Evercooren, Leprince et al. 1987). The tPA then binds to the Schwann cells and local degrades the surrounding matrix, allowing these cells to migrate into the fibrin. An additional benefit is gained by employing a separate plasminogen activator for the Schwann cells. Tissue plasminogen activator is not directly mitotic for Schwann cells, preventing them from continual self-upregulation. However, tPA is able to activate urokinase through the action of plasmin (Collen, Zamarron et al. 1986). The subsequently formed urokinase plasminogen activator, a product of migrating axons, has been shown to be mitotic for Schwann cells (Baron Van Evercooren, Leprince et al. 1987). In this way, Schwann cells have developed an auto regulatory mechanism which is activated in the presence of

regenerating nerves, so they can rapidly proliferate when they are needed for nerve regeneration.

1.5.4 Growth Factors and ECM

The growth, maintenance and regeneration of neurons involves the action of many different molecules including soluble growth factors and active extracellular matrix molecules. The presence of some of these molecules during development or regeneration in the peripheral nervous system has led to the study of their respective roles and potential for improving nerve regeneration.

The range of soluble growth factors that affect neuronal activity include specific neurotrophins, such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3), and nonspecific growth factors, including basic fibroblast growth factor (bFGF) and insulin like growth factors (IGF). Many of the individual neurotrophins have been identified, although their specific activities are not as clearly defined. Neurotrophins have a major role in the development of the nervous system (Chalazonitix 1996), the subsequent regeneration after injury and in regulating cell death (Barde 1994). NGF, the best known neurotrophin, has been shown to be synthesized by both neurons and glial cells (Assouline, Bosch et al. 1987; Thoenen, Zafra et al. 1991). NGF acts on neurons, binding through specific NGF receptors on the axon (Chao, Casaccia-Bonnefil et al. 1998; Frade and Barde 1998) and undergoing retrograde transport (DiStefano and Curtis 1994; Altar and DiStefano 1998). Furthermore, it has been shown both *in vitro* and *in vivo* that exogenously added NGF enhances neurite outgrowth in a dose dependent fashion (Sakiyama and Hubbell; Bloch and Aebischer, unpublished results). Other neurotrophins have similar activity, with retrograde transport often involved in the process of activation (Altar and DiStefano

1998). The specificity for the neurotrophins is derived from their targets. While each neurotrophin seems to have similar activity, they act upon different cell types, resulting in a certain level of specificity. The role of other growth factors has been studied as well and had shown to have an effect both endogenously and exogenously. During peripheral nerve injury, it is known that the concentrations of FGF, both acidic and basic, are changed (Eckenstein, Shipley et al. 1991). This is true for other growth factors, including IGF (Ishii, Glazner et al. 1994). Many cells are involved in the process of nerve repair, including neurites, fibroblasts and glial cells present at the site, as well as monocytes that migrate to the site of injury. It may be that these growth factors elicit responses from the associated nonneuronal cells present in addition to neuronal cells. However, it has been shown that bFGF can increase neurite outgrowth *in vitro*, proving that the activity is not totally dependent on blood borne cells (Ray, Peterson et al. 1993; Iwasaki, Shiojima et al. 1995). Other proteins active for nerve regeneration are present but have not been characterized yet. One such protein is involved in migration of Schwann cells to the site of injury. It is known that macrophage produce a mitogenic growth factor which recruits Schwann cells (Kandel, Schwartz et al. 1991), but the corresponding cell surface receptor is not known.

Many common extracellular matrix molecules have been found to be neuroactive both *in vitro* and *in vivo*, including laminin, collagen and fibronectin (Lander 1989). This dense network of proteins is required to support the cells and many proteins in the matrix have been shown to control cell adhesion, spreading, migration and differentiation (Carey 1991). Perhaps the most active extracellular matrix protein in neurite outgrowth is laminin, a large glycoprotein made of three covalently linked chains (Martin 1987). Many studies of laminin have already been conducted, and it has been shown that laminin plays a vital role in the development and regeneration of nerves *in vivo* and in the extension of neurite outgrowth from nerve cells *in vitro* (Williams 1987; Williams, Danielson et al. 1987). Laminin has specific regions,

described below, that enhance cell attachment and several sequences which specifically accelerate nerve regeneration. The distribution of these active sites on laminin is shown in Figure 1.6. Further evidence of the activity of laminin in neuronal outgrowth include data obtained from nerve growth guide studies. Nerves grown in NGGs with

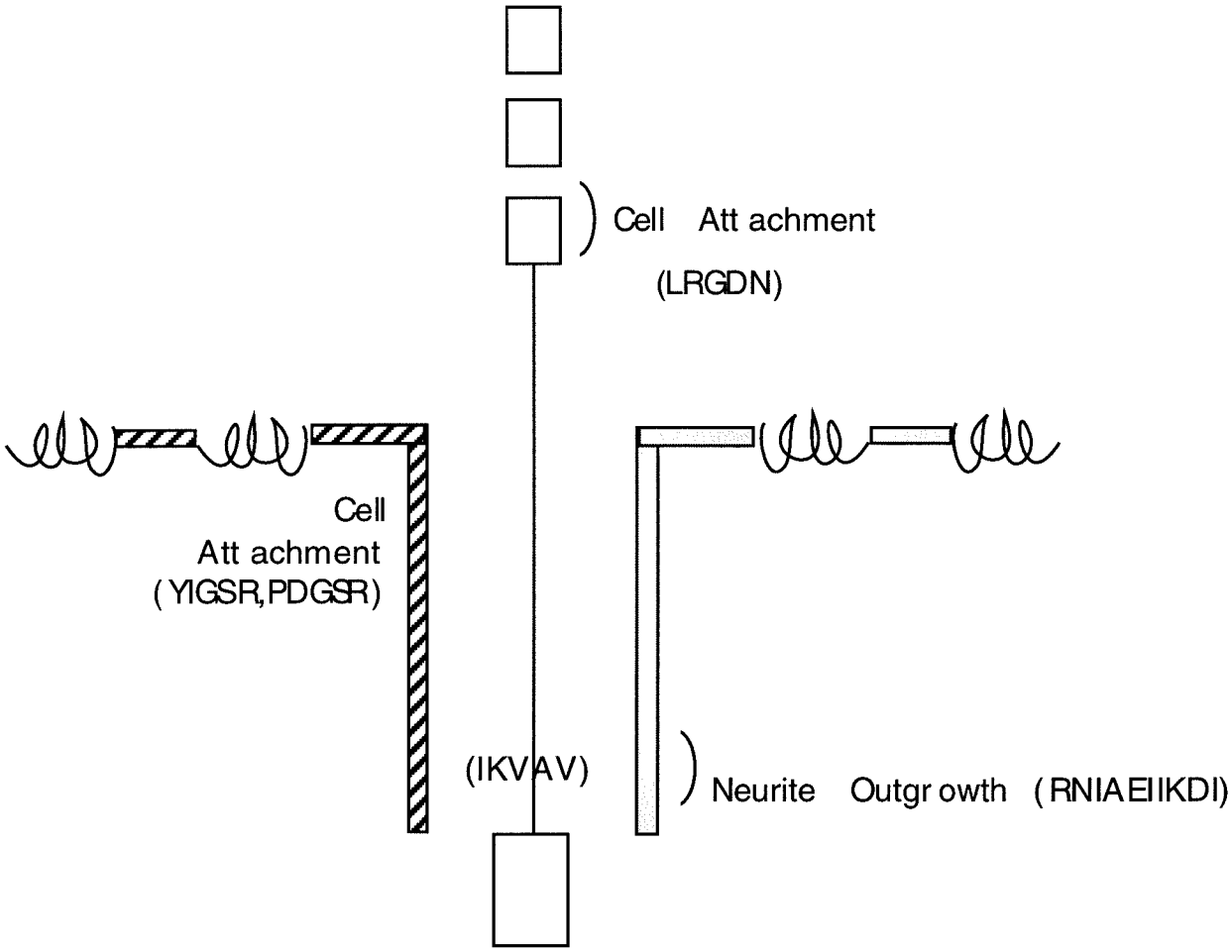

Figure 1.6 Structure of Laminin
Laminin consists of three chains held together by disulfide bonds. Each chain consists of several domains. The B1 chain has two neighboring sites active for cell attachment (YIGSR an PDGSR). The B2 chain has a site for cell attachment and neuronal outgrowth (RNIAEIIKDI). The A chain has the major cell attachment site RGD in addition to the IKVAV neuronal active site.

laminin have had a markedly increased extension over controls that lack laminin (Madison, Da Silva et al. 1985). Activity has been observed in collagen and fibronectin as well at picomolar amounts. It is known that collagen can interact with Schwann cells, as it has been shown that Schwann cells have the relevant $\alpha_2\beta_1$ integrin receptor present on the surface (Zutter and Santaro 1990). However, the level of activity associated with fibronectin or collagen has been shown to be lower than that observed with laminin.

These active ECM proteins provide the basis for many aspects of tissue engineering, allowing scientists to utilize their evolved activity. However, employing any of these large, bulky proteins often proves to be difficult, limiting their potential applications. In the following section, it will be shown how the activity of these proteins can be mimicked with short peptide sequences derived from within the larger protein structure and then how this can be applied to modify fibrin matrices.

1.6. BIOLOGY OF PEPTIDE/RECEPTOR INTERACTION

1.6.1 Short Peptide Sequences

Some of the specific sequences that interact with cell surface receptors and induce either adhesion, spreading or signal transduction have been identified. This means that the short active peptide sequences can be employed instead of the entire protein for both *in vivo* and *in vitro* experiments. In order to test the bioactivity of short peptide sequences, peptides have been either adsorbed onto a surface or incorporated into an otherwise inert biomaterial, and the subsequent neuroactivity and relevant cell-surface receptors have been elucidated.

Laminin, a large multidomain protein (Martin 1987), has been shown to consist of three chains with several receptor-binding domains. These receptor-binding domains include the YIGSR sequence of the laminin B1 chain (Graf, Iwamoto et al. 1987; Kleinman, Graf et al. 1989; Massia, Rao et al. 1993), LRGDN of the laminin A chain (Ignatius, Large et al. 1990) and PDSGR of the laminin B1 chain (Kleinman, Graf et al. 1989). Several other recognition sequences for neuronal cells have also been identified. These include IKVAV of the laminin A chain (Tashiro, Stephel et al. 1989) and the sequence RNIAEIIKDI of the laminin B2 chain (Liesi, Narvanen et al. 1989). These sequences and the general structure of laminin are shown in Figure 1.6. Short bioactive sequences have been identified in several other neuroactive proteins as well. One such protein is N-cadherin, a molecule found on the surface of both neurons and associated glial cells. Further research has shown that HAV is the minimum binding sequence in N-cadherin (Blaschuk 1990), and the activity of the whole protein can be partially mimicked with this short peptide. Another protein which has activity that can be mimicked with a short peptide sequence is collagen. One particular neuroactive sequence that has been identified is DGEA (Yamada and Kleinman 1992).

Much of the initial research in identifying these active domains of proteins is derived from competitive inhibitor studies. In these studies, the short peptide sequence is used as an inhibitor for the activity of the whole protein and the inhibition of protein activity is noted (Liesi, Narvanen et al. 1989). Additional research has been completed as well, where these individual bioactive domains were grafted onto an otherwise inert biomaterial, and it was demonstrated that the specific activity was transferred to the biomaterial. One particularly common bioactive domain, RGD, has been grafted onto biomaterials and has transformed an inert surface to an adhesive substrate for fibroblasts (Massia and Hubbell 1991), monocytes (Benelli, Mortarini et al. 1998) and many numerous mammalian cells (Ruoslahti and Pierschbacher 1987). In addition to work with RGD, it has been demonstrated that incorporation of YIGSR can lead to cell

adhesion and spreading (Massia, Rao et al. 1993), IKVAV mediates neuronal adhesion and outgrowth (Tashiro, Sephel et al. 1989) and PDGSR results in nerve attachment and outgrowth (Huber, Heiduschka et al. 1998). This ability to endow an inert biomaterial with functional properties of the donor protein through the grafting of a short peptide sequence is convincing evidence of the specific activity of these peptides and suggests that covalent incorporation of these same peptides within a fibrin matrix may transfer the specific bioactivity of these sequences to a three-dimensional fibrin matrix.

1.6.2 Receptors

The specific molecules involved in the interaction between these short active peptide sequences and the relevant cells are a series of transmembrane receptors located on the cell surface. A subset of cellular receptors that has shown to be responsible for much of the binding is the integrin superfamily (Rouslahti 1991). Integrins are protein heterodimers that consist of various combinations of α and β subunits. Some of the specific integrins that bind to neuronal active peptides have been identified. Previous work has shown that the tripeptide RGD binds to several $\beta 1$ and $\beta 3$ integrins (Yamada 1991; Hynes 1992), and DGEA, a collagen sequence, binds to the $\alpha 2\beta 1$ integrin (Zutter and Santaro 1990). Other sequences have been shown to bind more ambiguous receptors, where the molecular weight is known, but the relevant receptor family is not known. This series of peptide receptor combinations includes IKVAV, which binds to a 110 kDa receptor (Tashiro, Sephel et al. 1989; Luckenbill-Edds, Kaiser et al. 1995), and YIGSR, which binds to a 67 kDa receptor (Graf, Iwamoto et al. 1987). The receptor for the RNIAEIIKDI sequence is unknown at this time.

1.7 INCORPORATION OF PEPTIDES INTO FIBRIN

The mechanism employed to covalently incorporate bioactive factors into fibrin has been through the enzymatic activity of the transglutaminase, factor XIIIa. Through the activity of this enzyme, bi-domain peptides were cross-linked into fibrin matrices during coagulation. Here, the behavior of this enzyme is outlined, as well as the various native proteins that act as substrates. Finally the design of bi-domain peptides for enzymatic incorporation is shown.

1.7.1 Mechanism

Factor XIIIa is a transglutaminase enzyme that covalently cross-links a lysine residue with a glutamine residue in separate proteins (Pisano, Finlayson et al. 1968). Initially, factor XIII, the inactive zymogen, is present in the blood. It is subsequently converted into the active form by thrombin-induced cleavage. The newly formed enzyme then is able to cross-link various proteins, and the specific mechanism involved has been elucidated. In the initial step, factor XIIIa binds to a glutamine substrate, forming an acyl-enzyme intermediate (Folk 1969). The binding of the enzyme to the glutamine has been shown to be highly substrate specific (Gorman and Folk 1980). However, this acyl-enzyme intermediate is somewhat less stable, as it then reacts with any lysine present, completing the transamidation reaction (Figure 1.7). This lower specificity for the lysine substrate has been exploited in the study of substrates for testing enzymatic activity, as any large lysine containing molecules to be used as the recipient amine (Gorman and Folk 1981).

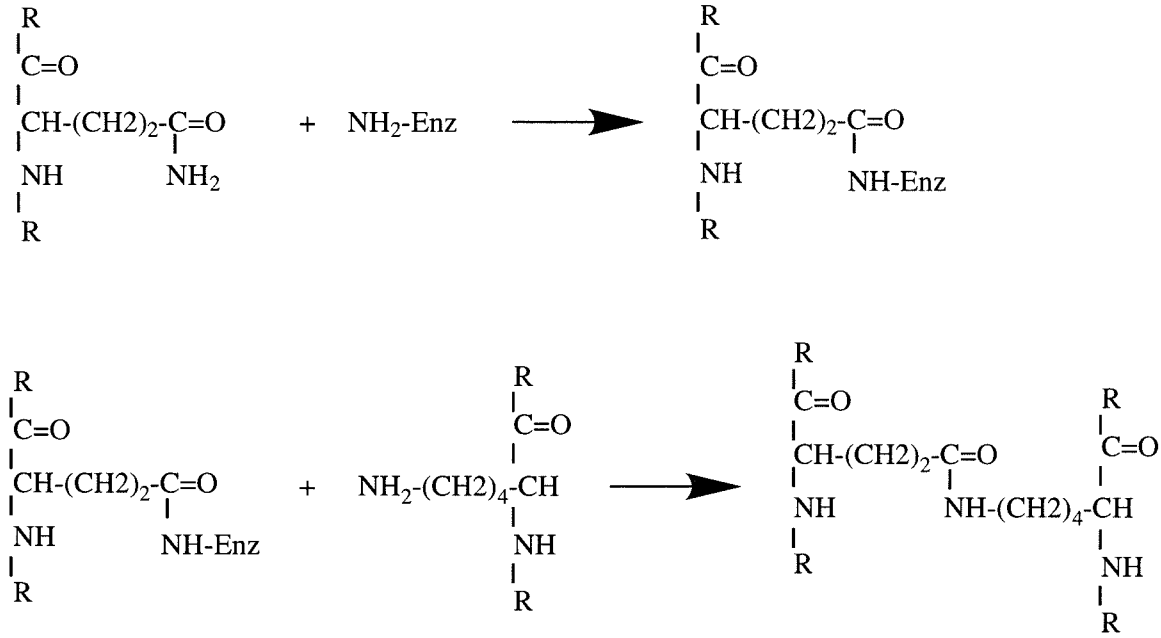

Figure 1.7 Molecular Mechanism of Factor XIIIa Activity
Initially, the enzyme attacks a glutamine residue forming an acyl-enzyme intermediate. This intermediate then reacts with the free amine of a lysine on a separate molecule to complete the transamidation reaction.

1.7.2 Factor XIIIa Activity

Factor XIIIa serves two main functions in coagulation, cross-linking of fibrin gels to provide stability from fibrinolysis (Doolittle, Chen et al. 1971) and immobilization of proteins within fibrin gels to achieve high local concentrations (Tamaki and Aoki 1981). During the initial formation of a fibrin gel, the intermolecular interactions are noncovalent in nature. This results in a gel that can be easily degraded, especially within a blood vessel where the shear forces are high. If the cross-linking of the fibrin gel does not occur, either through genetic defects or specific antibody blockage (Lukacova, Matsueda et al. 1991), the gel is quickly degraded. Furthermore,

if the gel is formed in the presence of high concentrations of factor XIIIa, the resulting gel is even more resistant to fibrinolysis than a normal gel (Francis and Marder 1988).

It has been shown that factor XIIIa cross-links α_2-plasmin inhibitor, plasminogen and fibronectin to fibrin. By cross-linking α_2-plasmin inhibitor to the gel, it provides high local concentrations within the fibrin, which serves to further inhibit the natural fibrinolysis process. By cross-linking collagen or fibronectin to the fibrin gel, the enzyme is incorporating proteins with significantly higher bioactivity than fibrinogen itself (Okada, Blomback et al. 1985). Fibronectin contains many bioactive signals, including heparin binding domains, an RGD and numerous other cell binding domains which can interact with many cell types, including platelets, endothelial cells and neurons (Pierschbacher and Ruoslahti 1984). Collagen contains many active signals as well, and the incorporation of these proteins serves to increase subsequent cell migration into the gel and associated protein production by these cells. Therefore, the action of factor XIIIa is able to significantly enhance the bioactive character of native fibrin gels.

1.7.3 Design for Incorporation of Bioactive Factors

The approach of this project is to utilize known factor XIIIa cross-linking sequences to develop bidomain peptides that could be enzymatically cross-linked into fibrin gels during gellation. Factor XIIIa cross-linking substrates were synthesized based on native substrates, such as α_2-plasmin inhibitor or fibrinogen, as well as nonnative substrates, including an oligomer of lysine. These substrates composed one domain of a peptide, whereby the other domain was a bioactive sequence. These bidomain peptides were then covalently cross-linked into fibrin gels and the resulting concentration and

Table 1.1 List of Factor XIIIa substrate sequences

Protein	Substrate Sequence
α_2-plasmin inhibitor	N**Q**EQVSPL
Fibrinogen	A**K**DV
Fibrinogen	TIGEGQ**Q**HHLGG
Fibronectin	AQ**Q**MV
Collagen	LG**Q**S
Nonbiological	**KKKK**

bioactivity was measured. Through this method the action of factor XIIIa is employed to improve the bioactivity of the fibrin gels while maintaining the necessary structural integrity of the matrix.

In the following chapters, the development of enzymatically-modified fibrin matrices will be outlined. In Chapter 2, the chemistry of peptide incorporation will be detailed. Here, the specific bidomain peptides synthesized and incorporated within the fibrin matrices during coagulation will be discussed with the resulting final peptide density and effect on fibrin structure and bioactivity explained. From this work, one specific factor XIIIa cross-linking sequence was identified for its ability to incorporate at high densities, and was used for all of the subsequent cell studies. These peptide-modified matrices were then employed in an academic study to probe the RGD-dependent effect on three-dimensional cell migration, with the results detailed in Chapter 3. Additionally, a series of biomaterials were developed that were selected for maximal *in vitro* neurite outgrowth, and this process is explained in Chapter 4. Through these studies, it was discovered that fibrin modified with combinations of bioactive peptides provided the greatest level of neurite outgrowth and these materials

were used in preliminary *in vivo* studies. The results of these *in vivo* studies are presented in Chapter 5. Finally, the future research applications for these materials is outlined in Chapter 6.

2. CHEMISTRY OF PEPTIDE INCORPORATION *

2.1. ABSTRACT

Bi-domain peptides with a factor XIIIa substrate in one domain and a bioactive peptide in another domain were covalently incorporated into fibrin gels during coagulation through the action of the transglutaminase factor XIIIa. The cross-linking characteristics were determined for two bi-domain peptides with factor XIIIa substrates based on fibrinogen, dYRGD*TIGEGQQHHLGG*-NH_2 and dLRGD*GAKDV*-NH_2, as well as one bi-domain peptide with a substrate sequence based on α_2-plasmin inhibitor, dL*NQEQVSPL*RGD-NH_2 , and another with a nonbiological, oligolysine substrate, dLRGD*KKKK*G-NH_2 (substrate domains in italic). Each of these peptides was able to cross-link into the fibrin gels during coagulation, with the peptide containing the factor XIIIa substrate based on α_2-plasmin inhibitor being incorporated at levels in excess of 8 mol/mol fibrinogen. The structural characteristics of these peptide-modified gels proved to be the same as those for a native fibrin gel. The bioactivity of the incorporated active factors was tested in a neuronal culture model with day 8 chicken dorsal root ganglia using two bioactive sequences, RGD and DGEA, and one inactive control sequence, RDG. Each of these peptides influenced the extension of neurites from the ganglia as expected, indicating that the incorporated factors retained their activity. With the use of soluble competitive inhibitors, it was shown that this effect was due to the covalently incorporated peptides. Through exploiting the role of factor XIIIa in coagulation, we have developed a method by which to impart the character of non-fibrin proteins, such as extracellular matrix proteins, to fibrin, a biological material with many potential therapeutic and academic applications.

* The contents of this chapter were published in Bioconjugate Chemistry 10 (1) 75-81.

2.2. INTRODUCTION

Fibrin is a biopolymeric gel that plays an important role in natural wound healing and medicine. Fibrin is formed by the enzymatic polymerization of fibrinogen at most sites of injury, and fibrin can be formed for therapeutic purposes either from purified fibrinogen or from processed blood plasma. Such therapeutic applications include use as an adhesive and sealant, and as a vehicle for the local delivery of drugs (Sierra 1993; Zarge, Husak et al. 1997). A special feature of fibrin, both formed naturally and therapeutically, is that it is degraded and remodelled by cell-associated enzymatic activity during cell migration and wound healing (Krystosek and Seeds 1984; Seeds, Haffke et al. 1992). As such, the material differs qualitatively from most synthetic biomaterials employed in medicine, in that there exists an active feedback from the cells in the healing response to the material (leading to degradation of the material) as well as from the material to the cells (providing a mechanical scaffold with biospecific adhesion sites for cell infiltration) (Francis, Bunce et al. 1993). The direct interest in fibrin relates to its use as a therapeutic material, and this report addresses methods by which to harness the enzymatic processes of coagulation to incorporate exogenous bioactive signals into the fibrin network. The processes by which fibrinogen is polymerized into fibrin and by which this fibrin is subsequently degraded have been well characterised. Initially, the protease thrombin cleaves the dimeric fibrinogen molecule at two symmetric sites. Once the fibrinogen is cleaved, a self-assembly step occurs in which the fibrinogen monomers come together and form a non-covalently cross-linked polymer gel via the proteolytic exposure of binding sites (Sierra 1993). Factor XIIIa, proteolytically activated from factor XIII by thrombin, may then covalently cross-link this polymer network (see below). Once a cross-linked fibrin gel

is formed, the subsequent degradation of the fibrin network is tightly controlled. One of the key molecules in controlling the degradation of fibrin is α_2-plasmin inhibitor (Aoki 1979). This molecule is cross-linked to the α chain of fibrin through the action of factor XIIIa (Sakata and Aoki 1980). By attaching to the gel, a high concentration of inhibitor can be localized to the gel. The inhibitor then acts by preventing the binding of plasminogen to fibrin (Aoki, Moroi et al. 1978) and by inactivating plasmin (Aoki 1979).

The enzyme factor XIIIa plays an important role in the formation and degradation of fibrin gels (Francis and Marder 1988). Factor XIIIa is a transglutaminase, and the specific cross-linking mechanism has been elucidated (Pisano, Finlayson et al. 1968; Folk 1969). Initially, the zymogen factor XIII is activated by thrombin cleavage to form the active transglutaminase factor XIIIa. This enzyme then proceeds to create primarily intermolecular cross-links between the γ chains in the fibrin network (Doolittle, Chen et al. 1971; Shainoff, Urbanic et al. 1991). These covalent cross-links provide the fibrin gel with significantly greater stability and resistance to protease degradation. Furthermore, factor XIIIa has been shown to cross-link other proteins, including fibronectin to both fibrin (Potts, Phan et al. 1995) and collagen (Mosher, Schad et al. 1979). For several proteins, the exact amino acid sequence of the factor XIIIa substrate domain has been elucidated, including for fibrin (Doolittle, Chen et al. 1971), fibronectin and α_2-plasmin inhibitor (Ichinose, Tamaki et al. 1983).

Fibrin is a very useful material in several biomedical applications. Its usefulness could be extended further, however, if one could alter the biological character of the fibrin by incorporating within it the biological activity of other proteins, such as those from the extracellular matrix including fibronectin, vitronectin, laminin and collagen (Yamada and Kleinman 1992; Engvall and Wewer 1996; Malinda and Kleinman 1996). In many cases, the bioactive domains, i.e., the regions of the

proteins that bind to cell-surface receptors, are known, and these domains can be mimicked with synthetic linear and cyclic peptides (Massia and Hubbell 1991; Yamada 1991). Two such sequences that were employed in this study were the tripeptide RGD, derived from many extracellular matrix proteins (Ignatius, Large et al. 1990), which binds to a variety of integrin receptors (Hynes 1992), and the tetrapeptide DGEA, derived from collagen, which has been shown to bind to the $\alpha_2\beta_1$ integrin (Zutter and Santaro 1990).

Bi-domain peptides were designed and evaluated, with a factor XIIIa substrate in the amino-terminal region and a bioactive region from fibronectin or collagen in the carboxyl-terminal region. The extent of incorporation of the exogenous peptide was determined, and the biological activity of such exogenous peptides was examined in the context of nerve regeneration. This technique has provided the ability to transfer biological activity from sources that are usually exogenous to fibrin gels, such as from extracellular matrix proteins, to create three-dimensional matrices with numerous medical and biological applications.

2.3. MATERIALS AND METHODS

2.3.1 Peptides and Proteins

Peptides were synthesized on solid resin using an automated peptide synthesizer (9050 Pep Plus Synthesizer, Millipore, Framingham, USA) with standard 9-fluorenylmethyloxycarbonyl chemistry (Fields and Noble 1990). All peptides were labeled with a fluorescent probe by including an α-dansylated leucine (Sigma, St. Louis, USA) at the amino terminus of the peptide, except for dYRGD*TIGEGQQHHLGG*-NH_2, which was dansylated off-line using dansyl chloride

(Varadarajan and Hawthorne 1991). All other reagents employed in peptide synthesis were purchased from Novabiochem (San Diego, USA). A series of bifunctional peptides were synthesized that contained an RGD sequence coupled to a factor XIIIa substrate sequence (italic). These peptides included: dYRGD*TIGEGQQHHLGG*-NH_2 (d=dansylated amino acid; sequence from fibrinogen), dLRGD*GAKDV*-NH_2 (sequence from fibrinogen), dL*NQEQVSPL*RGD-NH_2 (sequence from α_2-plasmin inhibitor), and dLRGD*KKKK*G-NH_2 (nonbiological sequence including an oligomer of lysine). An additional peptide was synthesized with the cross-linking domain from α_2-plasmin inhibitor and a bioactive domain from collagen, dL*NQEQVSPL*DGEA-NH_2. Hydrophobic scavengers and cleaved protecting groups were removed by precipitation of the peptide in cold diethyl ether and dissolution in deionized water. After lyophilization, peptides were redissolved in 0.03 M Tris-buffered saline (TBS, pH 7.0) and analyzed using HPLC (Waters; Milford, USA) on a size exclusion column with TBS.

Fibrinogen solutions were prepared by dissolving fibrinogen (Fluka; Ronkonkoma, USA) in deionized water at 8 mg/mL for 4 hr. Dialysis (Spectrum, 6000-8000 MWCO; Laguna Hills, USA) vs. 400 volumes of TBS, pH 7.4 for 24 hr was used to exchange salts present in the protein solution. The resulting solution was then sterilized by filtering through a 0.22 μm syringe filter. The final fibrinogen concentration was determined by measuring the absorbance of the protein solution at 280 nm (Dellenback and Chien 1970). TBS solutions at pH 7.0 that were made with either 50 mM calcium or without any calcium were sterilized by filtration though a 0.22 μm filter. Fresh thrombin solutions were made by dissolving thrombin (Sigma; St. Louis, USA) in TBS at pH 7.0 at a concentration of 20 units/mL.

2.3.2 Fibrin Formation and Peptide Incorporation

200 μL Fibrin gels were made by mixing the four components such that final concentrations of 3.6 mg/mL fibrinogen, 2.5 mM Ca^{++}, and 2 NIH Units/mL of thrombin were obtained with various amounts of peptide added. This solution was allowed to gel for 60 min at 37°C and 100% relative humidity. The gels were soaked in 0.3 M phosphate-buffered saline (PBS, pH 7.0) so that free peptide was allowed to diffuse out of the fibrin. In some experiments, the buffer was changed after the diffusion of free peptide reached equilibrium as indicated by spectrofluorimetric measurement of the dansyl signal.

To determine the amount of peptide incorporation, fibrin gels were degraded with 0.05 NIH units plasmin (Sigma, St. Louis, USA), and the resulting solution was analyzed using high performance liquid chromatography (Waters; Milford, USA). A size exclusion column (Shodex, polyhydroxymethylmethacrylate packing; New York, USA) was used that had a molecular weight range of 1,000-100,000. Samples were applied to the column and eluted in PBS. The resulting eluent was measured with both an inline ultraviolet and fluorescence detector to measure the presence of degraded fibrinogen and exogenous peptide (via the dansyl label) respectively.

2.3.3 Swelling

Fibrin gels were synthesized as described above with varying amounts of peptide. The gels were soaked in deionized water for 24 hr and then weighed. Each gel was added to a preweighed culture dish and placed in a vacuum oven to dry. After

all the water had been removed, the weight of the remaining gel was determined and the ratio of wet weight to dry weight was calculated.

2.3.4 Neurite Outgrowth

Dorsal root ganglia (DRG) were dissected from 8-day old white leghorn chicken embryos (Varon, Nomura et al. 1972). Ganglia were temporarily stored in modified neurobasal medium, consisting of insulin (5 μg/mL), transferrin (100 μg/mL), progesterone (6.3 ng/mL), putrascine (16.11 μg/mL), selenite (5.2 ng/ mL), fibronectin (5 μg/mL), L-glutamine (0.5 mM), L-glutamate (25 μM), murine mouse nerve growth factor (20 ng/mL), 0.2% bovine serum albumin and 1% antibiotic-antimycotic added to neurobasal medium (Gibco; Grand Island, USA). 400 μL fibrin gels were polymerized as described above in the bottom of flat 24-well culture plates, except that the fibrin was polymerized around a DRG, such that one DRG was embedded three-dimensionally inside each gel. The DRGs were cultured in 1 mL of modified neurobasal medium and the medium was changed at 3, 6, 9 and 24 hr post-seeding to wash out noncross-linked peptide.

Images were collected at 24 and 48 hr using light microscopy and digital image processing. At each time point, the average neurite length extending from each ganglion was measured as described elsewhere (Herbert, Bittner et al. 1996).

2.3.5 Statistics

Statistical analyses were performed using Statview 4.5. Comparative analyses were completed using analysis of variance (ANOVA) and an unpaired t-test at a 95% confidence level. Mean values and standard error are shown.

2.4. RESULTS

2.4.1 Calibration

Linear calibration curves were determined for both the incorporated peptides and the major components of the fibrin gels. Peptides were labeled with an α-dansylated amino acid, and this label was detected using a flow-through fluorescence detector. Fibrinogen was degraded with plasmin and the correlation between the UV_{280} absorbance signal and protein concentration was determined. It was found the UV_{280} signal was linearly proportional to fibrinogen concentration for both degraded and nondegraded fibrinogen.

2.4.2 Measurement of Peptide Incorporation

To determine the extent of incorporation of the various bi-domain peptides into the fibrin network during coagulation, gels were formed, free peptide was permitted to diffuse out of the network, the fibrin was degraded with plasmin and the degradation products were analyzed by HPLC to determine the amount of peptide (by fluorescence from the dansyl label) relative to fibrin (by UV absorbance) (Fig 2.1). Fibrin gels were synthesized and degraded as described above. The resulting solutions were applied to a size exclusion column with a separation range of 1 to 100 kDa. The chromatogram obtained from a degraded, native fibrin gel with UV detection contained a single peak that eluted at 18 min. The fluorescence signal was collected as well; however, the magnitude of the signal (from gels lacking exogenous peptide) was negligible. Fibrin gels modified with different factor XIIIa substrate peptides were formed. The UV signals collected from these gels were consistently identical to that produced by native

fibrin lacking exogenous peptides. However, for the peptide-modified gels, a large fluorescence signal appeared at an elution time later than the UV signal, appearing between 18 and 34 min. When soluble peptide was added to the degraded peptide-modified fibrin gel, the fluorescence signal seen between 18 and 34 min was essentially identical to that seen in the degraded gel alone. However, the fluorescence signal seen between 34 and 39 min was increased, indicating that the added free peptide

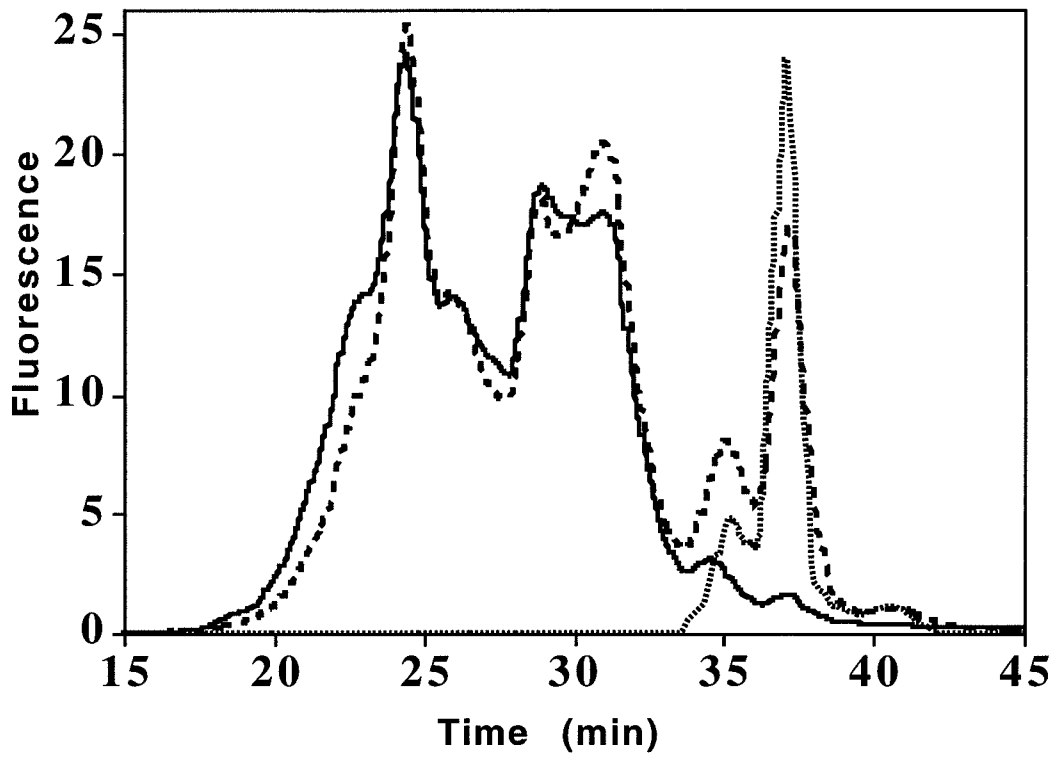

Figure 2.1 Fluorescence Detection of Chromatograms of Plasmin-degraded Gels.
Size exclusion chromatography of a degraded gel with the peptide dL*NQEQVSPL*RGD (—) incorporated is shown. The second chromatogram shown (---) is identical to the first, except for the addition of free peptide after degradation of the gel with plasmin. The free peptide was observed to elute at longer times, corresponding to a lower molecular weight than the peptide incorporated during coagulation, demonstrating that peptide incorporated via the factor XIIIa substrate was covalently bound to the fibrin network.

was eluting at the later time than did peptide which had been incorporated during coagulation (Figure 2.1, dashed trace). These extra peaks, between 34 and 39 min, correlated directly with those observed for free peptide analyzed alone, in the absence of fibrinogen or fibrin degradation products (Figure 2.1 dotted trace).

The incorporation of four different bi-domain peptides, each with a different factor XIIIa substrate domain but the same bioactive domain, was determined as a function of exogenous peptide concentration during coagulation (Figure 2.2). The amount of peptide that was cross-linked into each gel was measured as described above. The bi-domain peptide with the cross-linking domain from α_2-plasmin inhibitor reached a maximum incorporated concentration of 8.18 ± 0.09 mol peptide/mol of fibrinogen. It was possible to control the amount of peptide that was incorporated within the fibrin gel via the amount of peptide initially incorporated into the fibrinogen solution before coagulation. At initial peptide concentrations in excess of 135 mol peptide/mol fibrinogen, the ability of the peptide to cross-link into the gels decreased ($p<0.05$). The cross-linking of dLRGD*KADV* and dYRGD*TIGEGQQHHLGG*, the two peptides

Table 2.1 Efficiency and extent of incorporation of bi-domain peptides containing different factor XIIIa substrates

Peptide Sequence (Factor XIIIa substrate sequence in italics)	Maximum Cross-linking Density (mol peptide/mol fibrinogen)	Maximum Cross-linking Efficiency (%)
dL*NQEQVSPL*RGD	8.18 ± 0.09	14 ± 0.6
dLRGD*AKDV*	0.44 ± 0.03	0.6 ± 0.1
dLRGD*KKKK*G	1.18 ± 0.05	12 ± 5
dYRGD*TIGEGQQHHLGG*	1.53 ± 0.04	29 ± 0.6

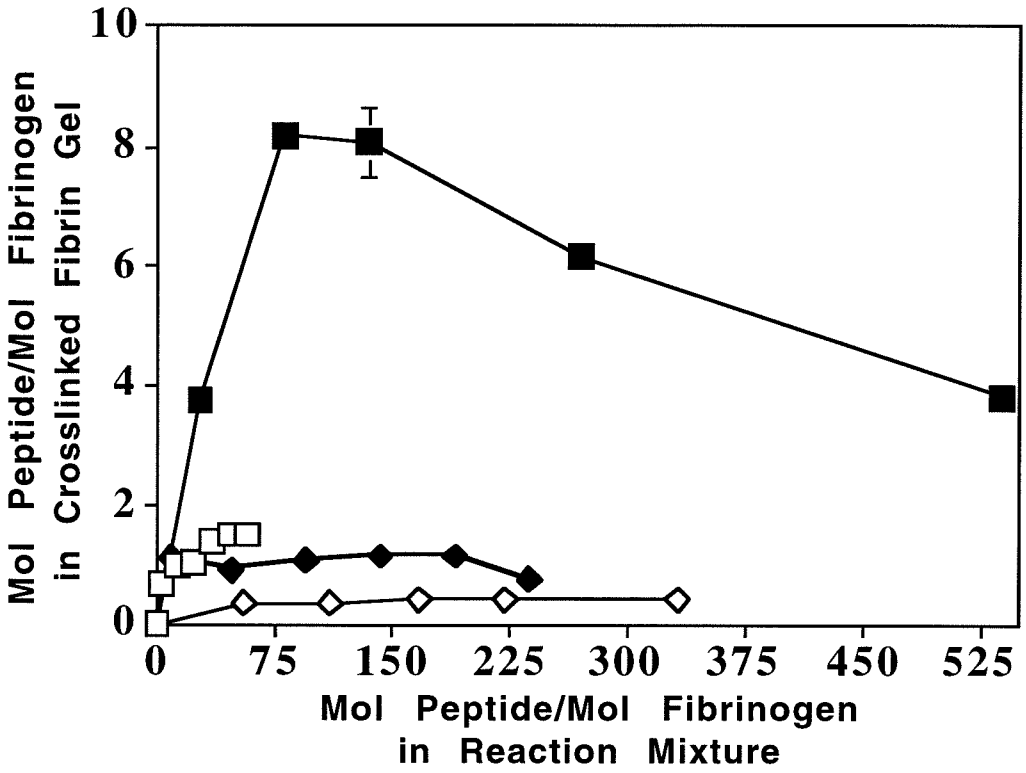

Figure 2.2 Incorporation of Four Bi-domain Peptides with Different Factor XIIIa Peptide Substrates (italics)

(black squares) dL*NQEQVSPL*RGD, from α_2-plasmin inhibitor, (empty diamonds) dLRGDAKDV, from fibrinogen, (empty squares) dYRGD*TIGEGQQHHLGG*, from fibrinogen, and (black diamonds) dLRGD*KKKK*G a nonbiological substrate, were each cross-linked into fibrin at a series of initial peptide concentrations, and the extent of incorporation is shown. Mean values and standard error of the mean are shown. The peptide dL*NQEQVSPL*RGD cross-linked more efficiently into the fibrin gel than the other three peptides, and achieved concentrations of up to 8.18 ± 0.09 mol peptide/mol fibrinogen.

with the fibrinogen factor XIIIa substrate domains, proved to be independent of initial peptide concentration, reaching levels of incorporation of 0.44 ± 0.03 and 1.53 ± 0.04 mol peptide/mol fibrinogen respectively. The lysine oligomer substrate, dLRGD*KKKK*G, displayed a similar cross-linking profile and reached a maximum cross-linking density of 1.18 ± 0.05 mol peptide/mol fibrinogen. When the average efficiency of incorporation (the fraction of exogenous peptide present in the coagulation mixture that was incorporated into the fibrin gel) was measured for each peptide, it was found that the factor XIIIa substrates with a glutamine at the active site were 3-30 times more efficiently incorporated than the lysine substrates (Table 2.1).

2.4.3 Swelling

As a measure of disruption of the fibrin network that might potentially occur as a result of the incorporation of exogenous peptide, the swelling of the fibrin gel was measured (Figure 2.3). Fibrin gels were polymerized as described above and modified separately with two factor XIIIa substrate peptides. The two substrates chosen were dLRGDG*KADV*, based on a fibrinogen substrate site, and d*LNQEQVSPL*RGD, based on the α_2-plasmin inhibitor substrate site. The influence of the incorporation of these peptides on the swelling of the resulting fibrin matrix was determined by comparing swelling to that of unmodified gels as well as to the initial ratio of water-to-protein, 190. When an unmodified fibrin gel was created, the ratio of wet weight to dry weight, 141.8 ± 20.7, was lower than the initial value in the fibrinogen solution before coagulation ($p<0.05$). The swelling of peptide-modified gels proved to be similar to the swelling of unmodified fibrin gels and was independent of cross-linked peptide density for both peptides tested; no value for either peptide tested was statistically different than that for the unmodified gel ($p>0.1$).

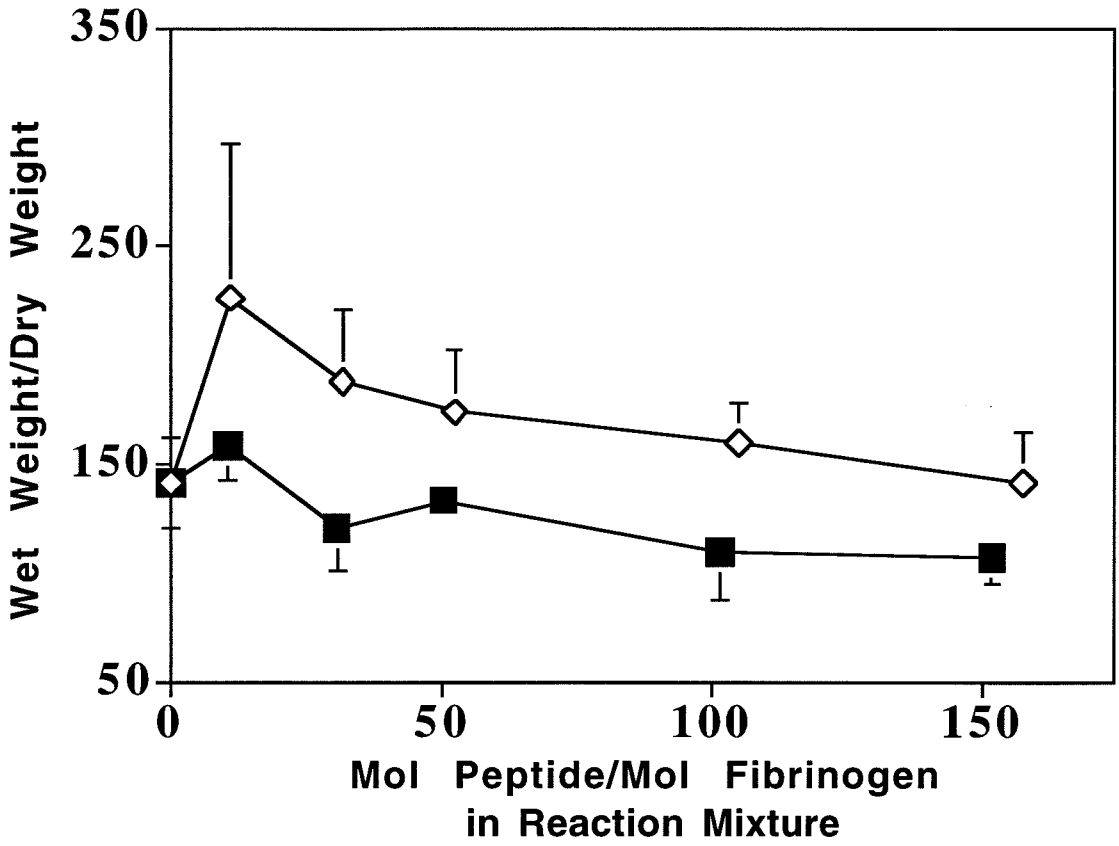

Figure 2.3 Swelling of the Peptide Cross-linked Gels

(black squares) dL*NQEQVSPL*RGD and (empty diamonds) dLRGDAKDV, were cross-linked into fibrin gels at a series of concentrations and the amount of swelling, determined by comparing the dry weight to the wet weight, is shown. Based upon the initial volume and amount of material used, the swelling ratio at the time of synthesis before any contraction was 190. Mean values and standard error of the mean are shown. The change in swelling induced by incorporation of these two peptides was statistically insignificant ($p>0.10$).

2.4.4 Neurite Extension

As a measure of the bioactivity of the exogenous peptide, incorporated via the factor XIIIa substrate domain of the bi-domain peptide, a neuronal cell culture model was employed in which neurites extend from dorsal root ganglia taken from day 8 chick embryos cultured within a three-dimensional fibrin network (Figure 2.4). Three peptides were individually cross-linked into fibrin and tested for their ability to interact with growing neurites *in vitro*. Each was a bi-domain peptide that contained the α_2-plasmin inhibitor sequence *NQEQVSPL* as the cross-linking substrate. Two active sequences, RGD and DGEA, and one control sequence, RDG, were attached to this factor XIIIa substrate. By incorporating 8 mol peptide/mol fibrinogen, statistically different neurite growth was obtained for both of the active sequences. At 24 and 48 hr of culture, the incorporation of RGD into the fibrin reduced outgrowth ($p<0.05$) relative to native fibrin by 21 ± 5.2 % at 24 hr and 13 ± 4.3 % at 48 hr. DGEA-modified gels proved to enhance neurite growth, increasing neurite length at 48 hr of culture by 22 ± 7.0% ($p<0.05$) relative to that in native fibrin. The cross-linking of the control peptide, RDG, into the fibrin did not affect the extent of neurite extension ($p=0.22$). As a final control, peptides were added in the soluble phase in the culture medium as well as cross-linked into the gel to serve as competitive inhibitors. The resulting level of neurite growth returned to the level attained in native fibrin alone in all cases ($p>0.21$).

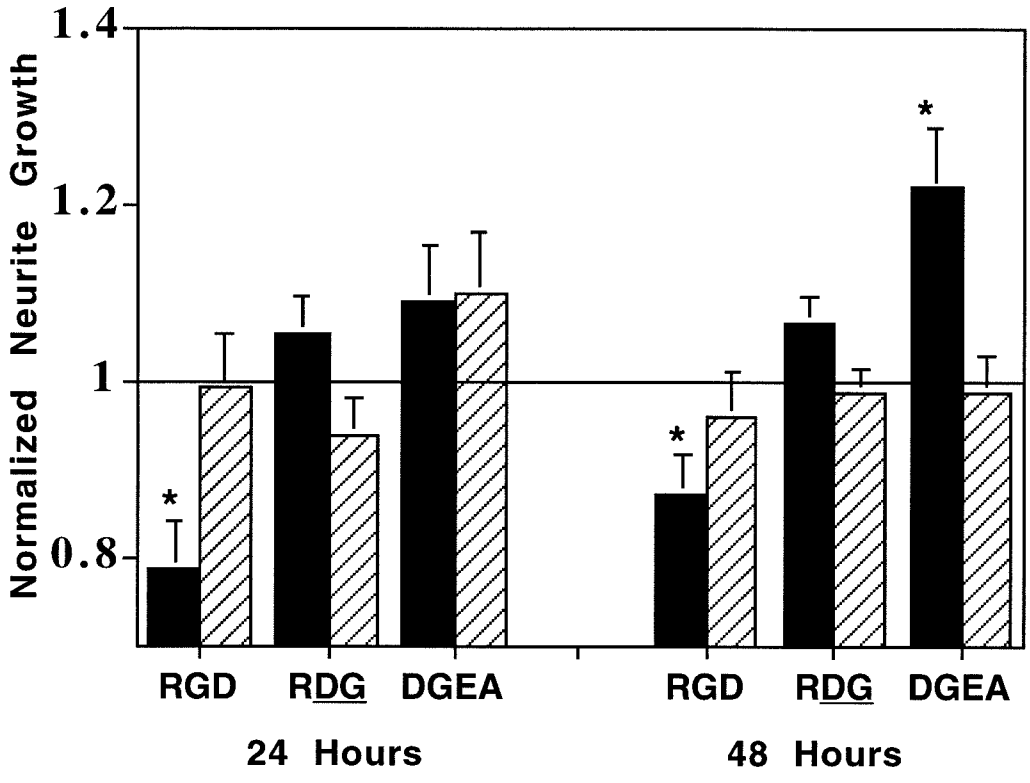

Figure 2.4 Efficacy of Cross-linked Bidomain Peptides

The effect of incorporated peptide at 8 mol peptide/ mol fibrinogen on neurite length within three-dimensional fibrin matrices with (hatched bars) and without (solid bars) soluble peptide added to the culture medium. Neurite growth from day 8 DRGs was normalized to growth in unmodified fibrin at 24 and 48 hr culture time. All cross-linked peptides contained the substrate sequence d*LNQEQVSPL* and the active sequence as shown. Experiments were performed in triplicate with 6 DRGs and mean values and standard error of the mean are shown; * denotes $p<0.05$ compared to unmodified fibrin. Addition of RGD statistically lowered neurite extension, DGEA increased neurite extension and the inactive control peptide RDG had no effect on neurite extension. In each case, the effect of the incorporated peptide was neutralized by the addition of soluble peptide as a competitive inhibitor.

2.5. DISCUSSION

Multiple peptide substrates were able to cross-link into a fibrin gel and the incorporation of dL*NQEQVSPL*RGD provided an efficient vector that could covalently incorporate active factors into fibrin gels. The chromatography provided information about the characteristics of the plasmin-mediated fibrin degradation in addition to proof of covalent incorporation of the bi-domain peptides. When peptide-modified and native fibrin gels were degraded by the fibrinolytic enzyme plasmin, multiple fragments of differing molecular weights were created. This resulted in several peaks in both the UV and fluorescence detection signals. The majority of the UV absorption signal was present in a major peak that eluted around 18 min. Based on the column specifications, this indicates that most of the fragments were larger than the cutoff of 100 kDa, which is in good agreement with previous work (Pizzo, Schwartz et al. 1973). When soluble peptide was eluted alone or in the presence of degraded gel, the associated fluorescence peaks appeared between 34 to 39 min, after the fluorescence signal had appeared for a peptide modified gel. Later elution times signify smaller fragments, indicating that most of the fluorescence from a degraded peptide modified gel was associated with molecules larger than the peptide alone. The fibrinogen does not contain significant fluorescence at this excitation and emission wavelengths. Therefore, the signal must be indicative of fibrin fragments that were covalently bound to peptide.

HPLC of fibrin modified with the bi-domain peptide dL*NQEQVSPL*RGD also indicated that the incorporated peptides were localized within the fibrin. Chromatograms of fibrin modified with the dL*NQEQVSPL*RGD peptide had a UV detection signal that was similar to the other peptide modified gels. In contrast to the signal obtained from fibrin gels modified with the other three peptides, the fluorescence signal obtained with dL*NQEQVSPL*RGD was observed at a later elution time, indicating that the peptide was bound to smaller protein fragments. These small

fragments were primarily degradation products of the α chain of fibrinogen (Ständler, Sillard et al. 1995), proving that the incorporated peptide is localized to the α chain of fibrin. Previous work has shown that α_2-plasmin inhibitor only cross-links to one of the three chains in fibrinogen, the α chain (Tamaki and Aoki 1981). This demonstrates that the peptide substrate is binding in a manner similar to the native protein.

The ability of each peptide to incorporate into fibrin can be correlated to both the activity of the specific cross-linking substrate and the intermolecular interactions that occur during gelation. Two substrate sequences were selected that mimicked cross-linking domains on the γ chain of fibrinogen, one that contains a lysine substrate and one that contains a glutamine substrate. The lysine based fibrinogen substrate had a very low maximum cross-linking concentration of 0.5 mol/mol fibrinogen and the incorporation was independent of initial peptide concentration. The glutamine based sequence could cross-link into the fibrin rather well, at three times the density of the first substrate, but this peptide easily saturated as well. The ability of these peptides to incorporate was dependent on the specific amino acid at the cross-linking site, with the glutamine being much more efficient. However, the final cross-linking density was limited by the mechanism through which both of these peptides were incorporated into the fibrin. The sites at which these peptides cross-linked into the fibrin gel were the same that are used for intermolecular fibrin cross-linking. Therefore, the peptides had to compete directly with the polymerizing fibrin gel for both the factor XIIIa and cross-linking sites. During the formation of a fibrin gel, precursor fibrils become ordered, placing the two fibrinogen cross-linking substrates into close proximity. This alignment enhances cross-linking of the fibrils and prevented these two peptides from efficiently competing for cross-linking sites. Next, a polylysine oligomer was tested. The incorporation of this peptide reached a useful concentration of 1 mol/mol fibrinogen, but it was both inefficient and independent of peptide concentration. Factor XIIIa is a highly specific enzyme, preferentially forming intermolecular fibrinogen

cross-links. The specificity of the nonspecific sequence that contained only the active amino acid for cross-linking was not high enough to compete with the fibrinogen for the factor XIIIa. Each of these three peptides were unable to compete with fibrin for cross-linking. Therefore, the final peptide density in the gel was dependent on the number of free sites available after intermolecular cross-linking. The peptide that contained the glutamine substrate was more efficient, and this probably resulted in the observed differences in final cross-linking concentration. The final factor XIIIa substrate selected was a mimic of the glutamine based α_2-plasmin inhibitor cross-linking domain. α_2-plasmin inhibitor has several beneficial properties that make it an excellent candidate for cross-linking into fibrin. The active cross-linking site α_2-plasmin inhibitor contains a glutamine, which was previously shown to be more active than lysine for cross-linking. The native protein is also highly efficient at cross-linking onto fibrin gels, attaching at 17 separate sites on the α chain that are not used for intermolecular fibrinogen cross-links (Ichinose, Tamaki et al. 1983). Therefore, a peptide with this substrate would not need to compete with the fibrin for cross-linking sites. Additionally, α_2-plasmin inhibitor is known to cross-link onto the fibrin matrix after the formation of the gel, allowing the fibrin gel to form unimpeded. Because of these natural traits, the peptide was able to cross-link into the gel at much higher concentrations and with a better efficiency than the other substrates tested. Furthermore, this was accomplished without noticeably disrupting the natural cross-linking of the fibrils.

The incorporated peptides had little effect on the swelling characteristics of the fibrin gels, and this was the result of the amount of peptide covalently bound to the fibrin as well as the specific binding characteristics. Two peptides were tested; a bi-domain peptide with the factor XIIIa substrate from fibrinogen, dLRGDGAKDV, and the factor XIIIa substrate from the α_2-plasmin inhibitor sequence, dL*NQEQVSPL*RGD. The amount of water absorbed by the gels was not significantly

higher for gels cross-linked when either of these peptides were incorporated than for a native fibrin gel. The first peptide tested had very low incorporation levels due to its inability to compete with the fibrin gel for enzymatic activity. Therefore, the composition of the gel was essentially unchanged from native fibrin and had similar structural characteristics. In contrast, the second peptide cross-linked into the gel at a high concentration. However, the swelling of these gels remained unchanged as well. The role of α_2-plasmin inhibitor is to prevent proteolytic degradation of the fibrin gel by factor XIIIa mediated cross-linking to the fibrin. Therefore, the cross-linking of this protein to the gel is critical for gel stability and has been optimized to minimize disruption of the gel structure. This property has been transferred to the peptide substrate as well, allowing it to cross-link at high densities without affecting the physical character of the fibrin.

Fibrin gels modified with bioactive peptides provided significantly different neurite growth from unmodified fibrin, with the associated active sequences providing the expected activity. Gels modified with the RDG control sequence resulted in statistically identical neurite extension to that observed in native fibrin. The RDG sequence does not have any associated bioactivity. Therefore, the only possible associated effect would be derived from physical changes to the fibrin gel. Incorporation of this peptide did not affect neurite extension, indicating that any physical changes to the gel must have a negligible effect in this cell model. The incorporation of RGD into fibrin gels resulted in significantly shorter neurites at both 24 and 48 hours. RGD modifies cell migration by acting as a adhesion site (DiMilla, Barbee et al. 1991). Fibrin naturally contains two RGD domains, so this modification led to the formation of gels with 10 mol RGD/mol fibrinogen. By creating a material with so much adhesive character, it became more difficult for the advancing neurite to migrate through the gel (this topic is addressed in more detail in Chapter 3 of this thesis). This led to the slower growth that was observed. The last active peptide

sequence tested was DGEA, another motif borrowed from a basement membrane protein. This collagen based sequence was able to enhance the level of neurite growth, as previously demonstrated (Lander 1989).

The activity associated with the incorporated peptides can be attributed to the covalently bound peptides, with any soluble peptide present acting as a competitive inhibitor. When peptides were cross-linked into fibrin at 8 mol/mol fibrinogen, the efficiency of cross-linking was about 10%. Therefore, the majority of the peptide remained in the soluble phase and was removed during the initial phase of neurite growth. When ganglia were cultured under these conditions, it becomes impossible to distinguish the activity of the covalently bound peptides from the activity of the free peptide. For each of the three sequences tested above, neurites were cultured in peptide modified fibrin gels with the respective free peptide added to the media for the entire experiment. The addition of the free peptide served to inhibit the activity of the peptide attached to the matrix, resulting in growth statistically identical to native fibrin. This demonstrated that it was necessary for the active sequence to be anchored to the matrix for the peptide to alter neurite extension. Furthermore, by preventing the covalently bound bi-domain peptides from interacting with neuronal receptors, changes in the structure of the fibrin became the only influence on the ganglia. Since the growth under these conditions was identical to growth in unmodified fibrin, this proved that the mechanical properties of gel were not altered significantly by peptide incorporation.

3. ROLE OF RGD IN MODULATING THREE-DIMENSIONAL MIGRATION

3.1. ABSTRACT

Three-dimensional neurite outgrowth rates within fibrin matrices with variable amounts of RGD peptides incorporated were shown to depend on adhesion site density and affinity. Bi-domain peptides with a factor XIIIa substrate in one domain and a RGD sequence in the other domain were covalently incorporated into fibrin gels during coagulation through the action of the transglutaminase factor XIIIa, and the RGD-dependent effect on neurite outgrowth was quantified, employing chick dorsal root ganglia cultured two- and three-dimensionally within the modified fibrin. Two separate bidomain peptides were synthesized, one with a lower affinity-binding linear RGD domain and another with a higher affinity-binding cyclic RGD domain. Both peptides were cross-linked into fibrin gels at concentrations up to 8.2 mol peptide/mol fibrinogen and their effect on two- and three-dimensional neurite outgrowth was measured. Both two- and three-dimensional neurite outgrowth demonstrated a biphasic dependence on RGD concentration for both the linear and cyclic peptides, with intermediate adhesion site densities yielding maximal neurite extension and higher densities inhibiting outgrowth. The adhesion site density that yielded maximal outgrowth depended strongly on adhesion site affinity in both two and three dimensions, with lower densities of the higher affinity ligand being required (0.8-1.7 mol/mol for the linear peptide versus 0.2 mol/mol for the cyclic peptide yielding maximum neurite outgrowth rates in three-dimensional cultures).

3.2. INTRODUCTION

Cell adhesion and migration in two and three dimensions play important roles in a host of physiological phenomena, including angiogenesis (Beck and D'Amore 1997) and immune responses (Benelli, Mortarini et al. 1998). The adhesion of cells within their environment is controlled primarily by the binding of cell surface receptors to short, exposed protein domains in the extracellular matrix. An important receptor family involved in adhesion and migration is the integrins, a class of heterodimeric receptors that interact with both extracellular matrix and cell-surface ligands (Hynes 1992; Yamada 1997). Each unique dimeric receptor binds to a particular subset of ligands, which can often be mimicked with immobilized short peptides (Massia and Hubbell 1991). One such short peptide that binds to integrins and influences subsequent cell adhesion and migration is RGD, a motif present in many proteins, including fibronectin, laminin, collagen and fibrinogen (Yamada 1991). This sequence has been shown to be recognized by $\alpha_5\beta_1$, $\alpha_v\beta_3$ (Pierschbacher and Ruoslahti 1984) and several other integrin heterodimer pairs. Even though RGD is present in multiple proteins, it is able to maintain specificity through its conformation and associated flanking sequences in the various protein ligands. Therefore, two common methods to alter both RGD binding strength and specificity in synthetic peptides include adding these endogenous flanking sequences (Yamada 1991), and cyclizing the RGD domain (Koivunen, Wang et al. 1995). Cyclization can constrain the RGD into a conformation that mimics a beta turn (Jois, Tambunan et al. 1996), allowing the peptide to partially mimic this common secondary structure in which RGD is naturally presented (Dickinson, Veerapandian et al. 1994).

Adhesion and migration upon a surface are distinct processes that depend on related molecular mechanisms. Cell adhesion involves the static interaction between

cell surface receptors and adhesion ligands on the supporting matrix (Lander 1989; Rouslahti 1991), with the strength of this attachment being related to the concentration of cell-surface receptors, the concentration of ligands available, and the specific binding affinity of these ligands (Palecek, Loftus et al. 1997). Migration of cells upon a material is an analogous phenomenon, with the rate of cell migration depending on both ligand density and binding strength as well as the density of corresponding cell surface receptors. As cells move within their environment, they must constantly detach at the trailing edge and reattach on the leading edge to nearby ligands (Lauffenburger and Horwitz 1996). The corresponding rate of cell migration upon a surface depends on the simultaneous detachment and attachment to nearby ligands, causing extreme ligand densities (both high and low) to inhibit one of these two processes and subsequently limit cell migration rates.

The outgrowth of neurites upon a surface is analogous to cell migration, with the advancing neuronal growth cone controlling migration. Much of the interaction between an extending neurite and its environment occurs at the leading edge of the neurite, the neuronal growth cone (Bixby and Harris 1991). As the growth cone migrates within its environment, it constantly binds to adhesion ligands at the leading edge and detaches from similar ligands at the trailing edge (Schmidt, Dai et al. 1995). This dependence on simultaneous ligand binding and detachment is similar to the model proposed by DiMilla et al. for migrating smooth muscle cells upon a surface (DiMilla, Stone et al. 1993). Furthermore, when neurites migrate upon an adhesive fibronectin coated surface, it has been demonstrated that the growth cones form focal contacts (Gomez, Roche et al. 1996) in a manner similar to other migrating cells.

Cell migration within a three-dimensional matrix involves a more complex balance of cellular activity than the analogous cell migration upon a two-dimensional surface, with cell-associated proteolysis playing a key role in three-dimensional migration. When cells are present inside a fibrin matrix, for example, the porosity of

the fibrous network is significantly smaller than the average cell and even neurite size. Therefore, in order for cells or neurites to migrate within fibrin, it is necessary to remodel the surrounding matrix, and this is accomplished through the action of the protease plasmin (Herbert, Bittner et al. 1996). While adhesion signals probably play an important role in three-dimensional migration, the dependence on proteolysis complicates a one-to-one correlation between two- and three-dimensional cell migration.

The focus of this work was neurite migration within a fibrin matrix with controlled adhesive characteristics. Through the action of the transglutaminase, factor XIIIa, bi-domain peptides were incorporated within a three-dimensional fibrin matrix at precise and predetermined concentrations (Schense and Hubbell 1999). Briefly, bi-domain peptides were synthesized that contained a factor XIIIa substrate from α_2-plasmin inhibitor (Ichinose, Tamaki et al. 1983) in one domain and an RGD sequence in the other domain. These peptides could be cross-linked into the fibrin matrices at very high concentrations, reaching 8.2 mol/mol. A biphasic response was observed in both two- and three-dimensional neurite outgrowth, with intermediate RGD adhesion site densities leading to fastest outgrowth. Outgrowth rates depended strongly on binding affinity with a lower number of higher affinity binding sites leading to a similar response as a higher number of lower affinity ligands.

3.3. MATERIALS AND METHODS

3.3.1 Peptides and Proteins

d*LNQEQVSPL*RGD-NH_2 and d*LNQEQVSPL*RDG-NH_2 (d=dansyl; for clarity, the factor XIIIa substrate domain is shown in italics) were synthesized on solid resin using an automated peptide synthesizer (9050 Pep Plus Synthesizer, Millipore, Framingham, USA) with standard 9-fluorenylmethyloxycarbonyl chemistry (Fields and Noble 1990). These peptides were labeled with a fluorescent probe by including an α-dansylated leucine (Sigma, Buchs, Switzerland) at the amino terminus of the peptide. Hydrophobic scavengers and cleaved protecting groups were removed by precipitation of the peptide in cold diethyl ether and dissolution in deionized water. After lyophilization, these two peptides were redissolved in 0.03 M Tris-buffered saline (TBS, pH 7.0) and analyzed using HPLC (Waters; Milford, USA) on a size exclusion column with TBS, pH 7.0 as the running buffer. The cyclic peptide, NH_2-L*NQEQVSP*D[CRGDNRC] (cyclic ring shown in brackets), was purchased from Genemed (South San Francisco, USA) at greater than 85% purity.

Fibrinogen solutions were prepared by dissolving fibrinogen (Fluka; Buchs, Switzerland) in deionized water at 8 mg/mL for 4 hr. Dialysis (Spectrum, 6000-8000 MWCO; Laguna Hills, USA) vs. 400 volumes of TBS, pH 7.4 for 24 hr was used to exchange salts present in the protein solution. The resulting solution was then sterilized by filtering through a 0.22 μm syringe filter. The final fibrinogen concentration was determined by measuring the absorbance of the protein solution at 280 nm (Dellenback and Chien 1970). TBS solutions at pH 7.0 that were made with either 50 mM Ca^{++} or without any Ca^{++} were sterilized by filtration though a 0.22 μm filter. Fresh thrombin solutions were made by dissolving thrombin (Sigma; Buchs, Switzerland) in TBS at pH 7.0 at a concentration of 20 units/mL.

3.3.2 Fibrin Formation

Fibrin gels were made in flat-bottomed 24-well culture plates by mixing the four components such that final concentrations obtained were (1) 3.6 mg/mL fibrinogen, (2) 2.5 mM Ca^{++}, (3) 2 NIH Units/mL of thrombin, and (4) various amounts of added peptide.

3.3.3 DRG Dissection

Dorsal root ganglia (DRGs) were dissected from 8-day old white leghorn chicken embryos (Varon, Nomura et al. 1972). Ganglia were temporarily stored in modified neurobasal medium, consisting of insulin (5 μg/mL), transferrin (100 μg/mL), progesterone (6.3 ng/mL), putrascine (16.11 μg/mL), selenite (5.2 ng/ mL), fibronectin (5 μg/mL), L-glutamine (0.5 mM), L-glutamate (25 μM), murine mouse nerve growth factor (20 ng/mL), 0.2% bovine serum albumin and 1% antibiotic-antimycotic added to neurobasal medium (Gibco; Grand Island, USA).

3.3.4 Competitive Inhibition of Three-Dimensional Neurite Outgrowth

DRGs were cultured within 400 μL unmodified fibrin gels, with the gels being polymerized in the presence of a DRG such that one ganglion was embedded three-dimensionally within the fibrin gel during polymerization. After polymerization, 1 mL of modified media was added to the matrices. In three series of experiments, the media added after fibrin gel polymerization was further supplemented with either of the RGD peptides or the control RDG peptide added as soluble competitive inhibitors. In the first experiment, the linear RGD peptide was added to the medium at concentrations of 0, 0.34, 0.67, and 1.68 mg/mL. The peptide-supplemented medium was changed at 3, 6, and 9 hr after seeding, and the migration of neurites at 24 hr was quantified and

normalized to that observed when 0 mg/mL soluble peptide was added. A second series of experiments was performed in which the cyclic RGD peptide was added as a competitive soluble inhibitor at the concentrations of 0, 0.15, 0.3 or 0.75 mg/mL. Finally, a third series of experiments was performed with the linear RDG peptide, where the peptide was added to the media at concentrations of 0, 0.34, 0.67 and 1.68 mg/mL. The washing and analysis procedures were identical to those used with the linear RGD peptide, with the peptide-supplemented medium changed at 3, 6 and 9 hr. Neurite outgrowth was measured as described below.

3.3.5 Two-dimensional Neurite Outgrowth Assay

DRGs were cultured upon fibrin gels without added exogneous peptides (all values were normalized to this level of neurite extension within native fibrin), or with cross-linked d*LNQEQVSPL*RGD-NH_2 , d*LNQEQVSPL*RDG-NH_2 or L*NQEQVSP*D[CRGDNRC]. 300 μL gels were synthesized with various amounts of peptide and washed at 3, 6, 9 and 24 hr after gellation with TBS to remove noncross-linked peptide. The peptides employed were cross-linked at different densities. The linear RGD and RDG were incorporated separately in a series of final concentrations that corresponded to 0.8, 1.7, 4.4 and 8.2 mol peptide/mol fibrinogen, obtained by polymerization of fibrinogen in the presence of 55, 110, 220, 275 and 330 nmol/mL peptide (Schense and Hubbell 1999). The cyclic RGD was incorporated at 0.2, 0.4, 4.4 and 8.2 mol peptide/mol fibrinogen, obtained by polymerization of fibrinogen in the presence of 13.75, 27.5, 275 and 330 nmol/mL peptide. DRGs were placed on the surface of the fibrin gel and allowed to adhere for 2 hr before media was added, and then the ganglia were cultured on the surface of the matrix for 24 hr. The neuronal media used was further modified by the addition of 1 μg/mL aprotinin to prevent the

ganglia from detaching from the surface due to protease activity (Herbert, Bittner et al. 1996). Neurite extension was measured at 24 hr after seeding as described below.

3.3.6 Three-dimensional Neurite Outgrowth Assay

DRGs were cultured within 400 μL fibrin gels without added exogneous peptides (all values were normalized to this level of neurite extension within native fibrin), or with cross-linked d*LNQEQVSPL*RGD-NH_2 , d*LNQEQVSPL*RDG-NH_2 or L*NQEQVSP*D[CRGDNRC]. These gels were polymerized in the presence of a DRG such that one ganglion was embedded three-dimensionally within the fibrin gel during polymerization. All peptides were incorporated separately in a series of final concentrations that corresponded to 0.8, 1.7, 4.4 and 8.2 mol peptide/mol fibrinogen, obtained by polymerization of fibrinogen in the presence of 55, 110, 220, 275 and 330 nmol/mL peptide (Schense and Hubbell 1999). The cyclic peptide was cross-linked into the gel at two additional concentrations of 0.2 and 0.4 mol peptide/mol fibrinogen, obtained from the addition of 13.75 and 27.5 nmol/mL of peptide during polymerization. The fibrin gels with embedded ganglia were cultured in 1 mL of modified neurobasal medium and the medium was changed at 3, 6, 9 and 24 hr after seeding to wash out noncross-linked peptide. Neurite outgrowth was measured at 24 hr and 48 hr after seeding as described below.

3.3.7 Imaging

Images of the entire DRG and the outgrowing neurites were collected using phase contrast microscopy with a 4.0 x objective (Zeiss, Zurich Switzerland). At each time point, the average neurite length extending from each DRG was measured by digital image processing as described in detail elsewhere (Herbert, Bittner et al. 1996).

3.3.8 Statistics

Statistical analyses were performed using Statview 4.5 (Abacus, Berkeley USA). Comparative analyses were completed using analysis of variance (ANOVA) with Fisher's protective t-test at a 95% confidence level. Mean values and standard errors of the mean are shown.

3.4. RESULTS

3.4.1 Competitive Inhibition of Three-Dimensional Neurite Outgrowth

As a measure of the relative binding affinity of the two RGD peptides employed, the effect of each peptide as a soluble competitive inhibitor of three-dimensional outgrowth of neurites from day 8 chick DRGs within fibrin was studied. When the linear peptide, d*LNQEQVSPL*RGD, was added to the culture media, after fibrin polymerization, as a competitive inhibitor in a concentration series, neurite outgrowth decreased in a dose dependent manner (Figure 3.1). At the highest concentration tested, 1.68 mg/mL, the peptide decreased neurite outgrowth by 25 ± 7% at 24 hr relative to the outgrowth within unmodified fibrin in the absence of soluble peptide inhibitor ($p<0.05$). The cyclic peptide L*NQEQVSP*D[CRGDNRC] decreased neurite outgrowth to a greater extent compared to the linear peptide, e.g., by 46 ± 6% at 24 hr relative to that seen within unmodified fibrin in the absence of soluble peptide inhibitor when 0.75 mg/mL peptide was added to the medium. Furthermore, at every concentration point, the cyclic RGD peptide inhibited the growth of neurites to a greater extent than did the linear RGD peptide ($p<0.05$). The control sequence, RDG, proved to have no effect on three-dimensional neurite outgrowth over the entire concentration range tested ($p>0.52$).

3.4.2 Neurite Extension in Two-dimensions with Cross-linked RGD Sites

Fibrin gels were modified by cross-linking various concentrations of either the linear RGD peptide, the cyclic RGD peptide or the linear control RDG peptide, and a biphasic RGD-dependent effect on two-dimensional neurite outgrowth was observed (Figure 3.2). When the linear RGD was incorporated over the range of 0-8.2 mol peptide/mol fibrinogen, the cross-linking of 1.6 mol linear RGD/mol fibrinogen proved to provide the greatest enhancement of neurite outgrowth, increasing the level of neurite outgrowth by 23 ± 9% at 24 hr ($p<0.05$). The neurite outgrowth decreased for each of the higher peptide densities such that when 8.2 mol linear RGD/mol fibrinogen was incorporated, the level of neurite outgrowth decreased by 16 ± 6% of the level seen in unmodified fibrin matrices ($p<0.05$). The incorporation of the cyclic RGD peptide over the same concentration range demonstrated a similar biphasic effect on neurite outgrowth, with the maximal enhancement occurring at a lower cross-linked peptide density. When 0.2 mol cyclic RGD/mol fibrinogen was incorporated, the level of enhancement proved to be 26 ± 7% compared to unmodified fibrin. Once again, at each of the higher peptide densities, the level of neurite outgrowth decreased such that at 8.2 mol cyclic RGD/mol fibrinogen, the level of neurite outgrowth was reduced by 22 ± 5% of the level observed in unmodified fibrin. The control sequence, RDG, proved to not have an effect on neurite outgrowth over the entire concentration range tested, with the level of outgrowth similar to that in unmodified fibrin ($p>0.74$).

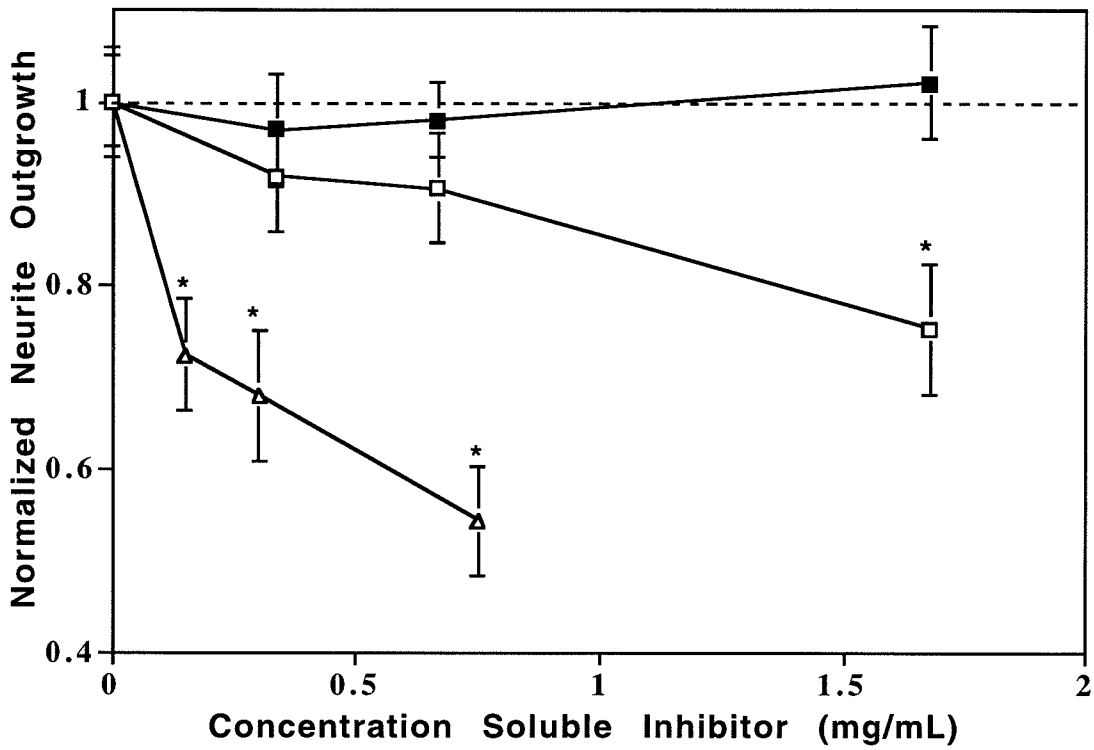

Figure 3.1 RGD Peptides as Soluble Inhibitors

The effect of soluble cyclic (triangles) or linear (empty squares) RGD peptides and a linear (black squares) RDG peptide on neurite outgrowth within three-dimensional fibrin matrices. Peptide was added in the culture media as a soluble competitive inhibitor for endogenous RGD in the fibrin matrix. Neurite outgrowth from day 8 DRGs was measured and normalized to outgrowth in unmodified fibrin at 24 hr culture time. Experiments were performed in triplicate with 6 DRGs per replicate, and mean values and standard errors of the mean are shown; * denotes $p<0.05$ compared to unmodified fibrin. At each concentration point, the cyclic peptide statistically inhibited neurite growth to a greater extent than did the linear peptide, demonstrating that the cyclic RGD is a higher affinity ligand than the linear RGD. The incorporation of the inactive control, RDG, did not affect neurite outgrowth at any of the concentrations tested.

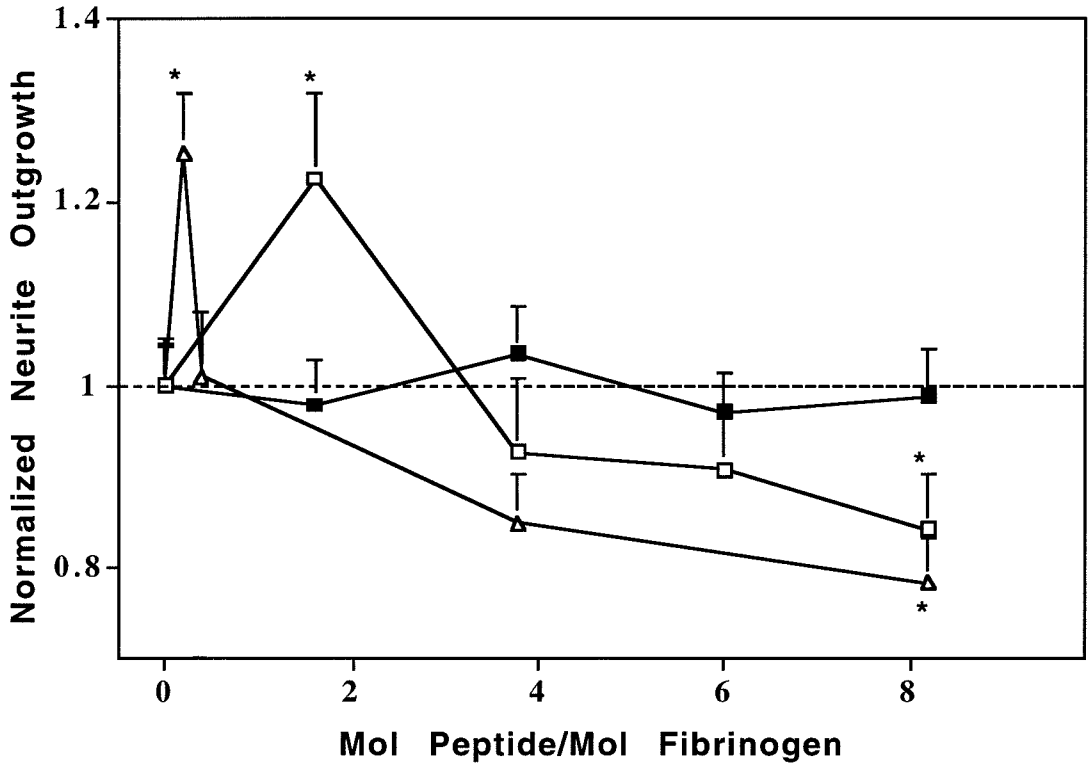

Figure 3.2 Effect of RGD on 2D Neurite Outgrowth

The effect of incorporated cyclic (triangles) or linear (empty squares) RGD peptides and a linear (black squares) RDG peptide on neurite outgrowth upon two-dimensional fibrin matrices. Neurite outgrowth from day 8 DRGS was measured and normalized to outgrowth in unmodified fibrin at 24 hr culture time. Experiments were performed in triplicate with 6 DRGs per replicate, and mean values and standard errors of the mean are shown; * denotes $p<0.05$ compared to unmodified fibrin. The correlation between two-dimensional neurite outgrowth and RGD density showed a biphasic effect, with moderate densities of exogenous peptide eliciting the greatest neurite outgrowth. The incorporation of the inactive control peptide, RDG, did not affect two-dimensional neurite outgrowth at any of the concentrations tested.

3.4.3 Neurite Extension in Three-dimensions with Cross-linked RGD Sites

Fibrin gels were modified by cross-linking various concentrations of linear or cyclic RGD or linear RDG peptide into the gel, and the resulting effect on neurite outgrowth was measured at 24 and 48 hr. A biphasic response was observed, with the cross-linking of 0.8 mol linear RGD peptide/mol fibrinogen enhancing the level of outgrowth by 23 ± 4% at 24 and 9 ± 2% at 48 hr and the cross-linking of 1.6 mol peptide/mol fibrinogen enhancing the level of neurite outgrowth by 23 ± 7% at 24 and 14 ± 5% at 48 hr ($p<0.05$) (Figure 3.3). The incorporation of 4.4 or 8.2 mol of linear RGD peptide/mol fibrinogen decreased the level of neurite outgrowth, such that it was lower than the outgrowth observed within both unmodified fibrin as well as the fibrin modified with lower concentrations of cross-linked peptide ($p<0.05$). At the highest incorporated adhesion density of 8.2 mol/mol, the level of neurite outgrowth was reduced by 21 ± 5% at 24 and 13 ± 4% at 48 hr relative to outgrowth within unmodified fibrin.

The incorporation of the higher affinity-binding cyclic peptide, L*NQEQVSP*D[CRGDNRC], into fibrin gels had a similar biphasic effect on the outgrowth of neurites, with an overall effect that was shifted to lower peptide densities relative to the effect for the lower affinity linear RGD peptide. Incorporation of 0.2 mol/mol of cyclic peptide enhanced the outgrowth of neurites by 17 ± 7% at 24 and 14 ± 3% at 48 hr compared to outgrowth within unmodified fibrin ($p<0.05$). Each of the higher cross-linking concentrations tested resulted in a decrease in neurite outgrowth, such that at 0.8 mol/mol, outgrowth was reduced by 25 ± 7% at 24 and 16 ± 4% at 48 hr, and at 8.2 mol/mol, neurite outgrowth was reduced by 51 ± 5% at 24 and 25 ± 5% at 48 hr relative to outgrowth within unmodified fibrin ($p<0.05$). Incorporation of the RDG peptide had no statistically significant effect on neurite outgrowth at either 24 or 48 hr at any of the concentrations tested ($p>0.36$).

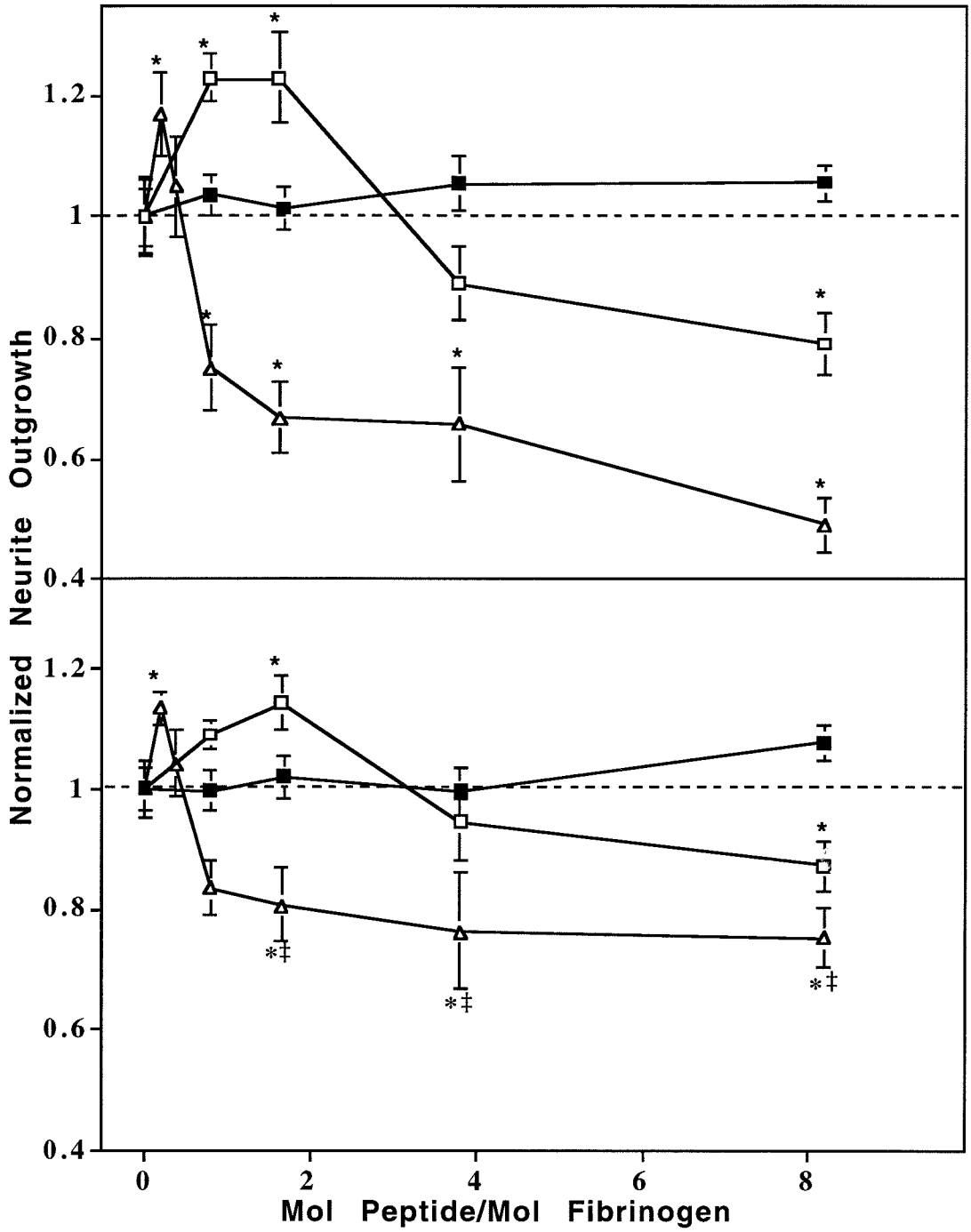
Normalized Neurite Outgrowth
1.2
1
0.8
0.6
0.4
0
2
4
6
8
Mol Peptide/Mol Fibrinogen

Figure 3.3 Effect of RGD on 3D Neurite Outgrowth

The effect of incorporated cyclic (triangles) or linear (empty squares) RGD peptides and a linear (black squares) RDG peptide at various concentrations on neurite outgrowth within three-dimensional fibrin matrices. Neurite outgrowth from day 8 DRGs was measured and normalized to outgrowth in unmodified fibrin at 24 (A) and 48 hr (B) culture time. Experiments were performed in triplicate with 6 DRGs per replicate and mean values and standard error of the mean are shown; * denotes $p<0.05$ compared to unmodified fibrin. ‡ denotes points that are statistically identical. The correlation between neurite outgrowth and cross-linked linear RGD peptide showed a similar biphasic effect as seen in two-dimensional RGD-dependent cellular migration. The incorporated cyclic RGD peptide showed a similar effect, with the curve shifted consistently with its higher binding affinity. The incorporation of the inactive peptide, RDG, did not affect neurite outgrowth at any of the concentrations.

3.5. DISCUSSION

The migration of cells within a three-dimensional matrix is an important component of many cellular processes both in vivo and in vitro. However, the lack of three-dimensional materials in which the biochemical characteristics can be readily and reproducibly controlled prevents complete understanding of the relevant factors involved. Through the factor XIIIa-mediated modification of fibrin matrices, we have developed a method by which the bioactive character of a degradable matrix can be reproducibly manipulated. Using these matrices, the role that exogenous RGD sequences have on the outgrowth of neurites within a three-dimensional environment was studied, with the effect on neurite outgrowth shown to exhibit a biphasic dependence on RGD concentration and binding affinity.

The relative binding affinities of the peptides employed were characterized by competitive inhibition experiments in three-dimensional culture. RGD is found endogenously in fibrin, at an active concentration of 2 mol/mol fibrinogen (Ruoslahti and Pierschbacher 1987). The addition of either of the RGD peptides as a soluble

inhibitor blocked the activity of these endogenous sequences, resulting in a decrease in neurite outgrowth within the fibrin matrix. A high concentration of the linear RGD peptide (1.68 mg/mL) was required to decrease the outgrowth of neurites, corresponding to approximately a 110-fold molar excess of competitive peptide to endogenous adhesion site density. The addition of the cyclic RGD peptide as a soluble competitive inhibitor proved to be much more potent in slowing neurite outgrowth, with a molar excess of approximately 10-fold demonstrating inhibition. At each tested concentration, the cyclic peptide inhibited neurite outgrowth to a greater extent than did the linear peptide. This greater binding affinity for cyclic RGD peptides over linear RGD peptides has been demonstrated previously, with the enhanced binding affinity due to the forced secondary structure occurring from the ring (Jois, Tambunan et al. 1996). It has been shown that cyclic RGD peptides adopt a conformation similar to the turn between strands in a β sheet, forcing cyclic peptides to mimic a common structural motif for native RGD sequences.

Two-dimensional neurite outgrowth demonstrated a biphasic dependence on RGD site density, consistent with the outgrowth of neurites being a form of cell migration. Much experimental and modeling work has been completed showing that the effect of adhesion site surface concentration on the motility of cells, such as smooth muscle cells, displays a biphasic response (DiMilla, Barbee et al. 1991; DiMilla, Stone et al. 1993).

The extent of the biphasic response in two dimensions depends on the adhesion site and receptor surface concentrations, and the degree to which cell surface receptors are polarized toward the leading edge (Palecek, Loftus et al. 1997). Evidence suggests that the migration of the neuronal growth cone, and therefore the overall neurite outgrowth rate, represents a similar phenomenon (Schmidt, Dai et al. 1995). By incorporating RGD into a fibrin matrix, it has been demonstrated that the neurite outgrowth from chick DRGs displays an identical dependence on RGD density, with a moderate peptide

density eliciting the highest outgrowth rate. Therefore, these results are consistent with the migration of neuronal growth cones, and therefore neurite outgrowth, being directly analogous to the migration of individual cells.

RGD peptides cross-linked into fibrin gels demonstrated a biphasic adhesive-like character in three-dimensions. The striking similarity between the results in three-dimensional and two-dimensional outgrowth experiments suggest that the rate-controlling phenomenon are similar between the two cases. This is very interesting, because in two-dimensional cultures proteolysis plays a minor role, whereas in the three-dimensional cultures proteolysis is required for neurite outgrowth (Herbert, Bittner et al. 1996). The similarity in results suggests that proteolysis and remodeling of the fibrin matrix is not the rate-controlling phenomenon, but rather that migration biomechanics control the overall outgrowth rate. The results also suggest, although indirectly, that the density of exogenous RGD sites did not influence neurite outgrowth rates primarily by modulating local protease activity. If they had done so, then the site density-dependent biphasic response that was observed in three-dimensional outgrowth (where proteolysis is requisite) would not have been observed in two-dimensional outgrowth (where proteolysis is not requisite; (Herbert, Bittner et al. 1996)).

A strong dependence of both two-dimensional and three-dimensional neurite outgrowth upon adhesion site binding affinity was observed. The phenomena observed for the two RGD peptides employed were similar in both form and extent, but with the response curve being markedly shifted to lower concentrations for the higher affinity cyclic RGD peptide. It is likely that this lower density of the stronger binding cyclic peptide represents a total adhesive character similar to the higher density of the weaker binding linear peptide, resulting in matrices with identical adhesive character and leading to similar migration rates. This is further supported in that the level of enhancement of neurite outgrowth for these two formulations are of similar intensities. Within the concentration series tested, both RGD peptides demonstrated a similar

adhesive-like character in three-dimensional migration, and this character corresponds directly to effects seen in previous two-dimensional studies.

It has been recently demonstrated that integrin number in the growth cone is regulated by receptor occupancy such that at high occupancy (where growth cone migration would be inhibited), the number of receptors is down-regulated (to thus maintain growth cone migration) (Condic and Letourneau 1997). This compensatory mechanism is hypothesized to enable the neurite to migrate over a very wide range of adhesion site densities. This compensatory mechanism may be less at play in the fibrin model employed, as a clear response of outgrowth rate to the adhesive characteristics of the matrix was observed. It also may be possible that this compensatory effect is even less at play in three-dimensional outgrowth, since high immobilized concentrations of the high affinity cyclic RGD ligand inhibited neurite outgrowth much more extensively in three-dimensional culture than in two-dimensional culture, approximately two-fold more.

The addition of exogenous adhesion sites within a fibrin matrix is sufficient for enhancing two- and three-dimensional neurite outgrowth. The overall process of neurite outgrowth involves the simultaneous coordination of many individual processes, including binding to both adhesion and other bioactive sites, polymerization of actin, formation of new membrane and intracellular migration of proteins within the axon (Bisby and Tetzlaff 1992; Fu and Gordon 1997; Luckenbill-Edds 1997). While each of these components are necessary for neurite migration, it is possible that modulating a single factor might not be sufficient to increase the overall rate of neurite migration. By cross-linking various concentrations of exogenous RGD peptides within a fibrin matrix, materials have been developed whereby the only change is in the adhesive character of the material. These materials were able to modestly increase the level of neurite outgrowth, demonstrating that adhesion of the migrating neurite growth

cone is a significant and rate controlling factor, even if it constitutes only one part of a complicated process.

The use of biomaterials for tissue regeneration, such as the promotion of nerve healing, is a promising field of research. By developing materials with specific adhesive characteristics, it is possible to control and enhance the level of cell migration within the material. This may lead to more effective forms of therapy in many areas of tissue engineering, as integration of biomaterials into the surrounding tissues and many forms of tissue regneration are often limited by the migration of cells into the area of interest.

4. FORMULATIONS THAT ENHANCE *IN VITRO* NEURITE OUTGROWTH*

4.1. ABSTRACT

Fibrin is a naturally forming biomaterial that plays an important role in wound healing and regeneration, and it enjoys widespread use in surgery and tissue engineering. The enzymatic activity of factor XIIIa, a transglutaminase involved in coagulation, was employed to covalently incorporate exogenous bioactive peptides within fibrin during coagulation. Fibrin gels were formed with incorporated peptides from the extracellular matrix protein, laminin (RGD, IKVAV, YIGSR, and RNIAEIIKDI) and the cell-cell adhesion protein N-cadherin (HAV) alone and in combination at concentrations up to 8.2 mol peptide/mol fibrinogen. Neurite extension from dorsal root ganglia from embryonic chicks was measured. The peptides IKVAV, YIGSR and RNIAEIIKDI induced enhanced neurite extension in a manner that increased with the concentration of the incorporated peptide. When the peptides RGD and HAV were incorporated, a biphasic response was observed, in which intermediate concentrations of incorporated peptide induced maximal neurite extension. When the four laminin peptides were co-immobilized, each at 1.7 mol/mol, a 75% enhancement in neurite extension was observed. These results demonstrate that it is possible to enhance the biological activity of this important, natural biomaterial by enzymatically incorporating within it exogenous bioactive domains of morphoregulatory proteins involved in development and remodeling.

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* The contents of this chapter have been submitted to Nature Biotechnology.

4.2. INTRODUCTION

Biomaterials play an important role in a variety of clinical applications in wound healing, regeneration and tissue engineering (Langer and Vacanti 1993; Peppas and Langer 1994; Hubbell 1995). Important features of such materials include the ability to display a variety of adhesive ligands that directly bind to cell surface receptors to provide adhesive and morphogenetic cues as well as the ability to be remodelled and replaced by the proteolytic molecules produced by cells during migration and invasion. Fibrin has developed an important role in a host of clinical applications as it readily fulfills the second requirement, being degraded by cell-derived proteases (Pittman and Buettner 1989; Herbert, Bittner et al. 1996). The focus of this work was the first requirement, the ability to endow fibrin with adhesion and morphogenetic signals that are not naturally present within the material. A large number of extracellular matrix and cell-surface proteins that regulate development and healing have been identified, and we sought to explore the incorporation of oligopeptide mimetics of these regulatory proteins to engineer the bioactivity of fibrin, both as a biomaterial and also as it forms spontaneously *in vivo*. The context of this research, as an example, was the induction of neurite extension, toward materials for use in promoting nerve regeneration. A culture model was selected for this exploration, namely neurite extension from dorsal root ganglia (DRGs) of embryonic chicks; this culture model permitted the study of structure-function relationships in a very large number of material formulations. Importantly, the work was carried out in three-dimensional cultures, in which the neurites were forced to migrate through a three-dimensional fibrin network, as would be carried out clinically if such materials were employed in nerve repair.

Many extracellular matrix and cell-surface proteins have been shown to play a role in inducing and guiding neurite extension, although mostly in two-dimensional models (Lander 1989). One such very important protein, a component of the

extracellular matrix, is laminin (Williams 1987; Williams, Danielson et al. 1987). This large, multidomain protein has been shown to consist of three chains with several receptor-binding domains (Martin 1987). The specific amino acids involved in receptor interaction with several of the binding sites have been identified, including LRGDN of the laminin A chain (Ignatius, Large et al. 1990), IKVAV of the laminin A chain (Tashiro, Stephel et al. 1989), YIGSR of the laminin B1 chain (Graf, Iwamoto et al. 1987; Kleinman, Graf et al. 1989; Massia, Rao et al. 1993), and RNIAEIIKDI of the laminin B2 chain (Liesi, Narvanen et al. 1989). The receptors that bind to these laminin sites have been identified for many of the above sequences (Graf, Iwamoto et al. 1987; Zutter and Santaro 1990; Yamada 1991; Hynes 1992), and an extensive backdrop of knowledge exists about the receptor-ligand interactions and signaling that results from such binding. Cell-cell contacts also play an important role in induction and guidance of neurite extension. Once such important protein is N-cadherin, present on the surface of axons and associated glial cells, such as Schwann cells (Letourneau, Shattuck et al. 1990). The sequence HAV has been shown to be involved in this homophilic interaction and capable of mimicking the binding site of one member of the homophilic pair (Blaschuk 1990).

It has been previously shown that exogenous peptides can be readily incorporated into fibrin matrices by harnessing the enzymology of coagulation. Specifically, bi-domain peptides are synthesized with the bioactive domain of interest in one domain and a substrate for the coagulation transglutaminase factor XIIIa substrate in the second domain. By employing the factor XIIIa substrate from α_2-plasmin inhibitor (Ichinose, Tamaki et al. 1983), exogenous peptides could be incorporated at controllable concentrations up to 8.2 mol peptide/mol fibrinogen (Chapter 2 of this thesis; Schense and Hubbell 1999). Utilizing this system, peptides from the two neuroactive proteins, laminin, as an example of extracellular matrix protein, and N-cadherin, as an example of a cell-surface adhesion protein, were incorporated at various

concentrations and combinations so that novel biological activities could be incorporated into this very flexible biomaterial platform, fibrin.

4.3. MATERIALS AND METHODS

4.3.1 Peptides and Proteins

Peptides were synthesized on solid resin using an automated peptide synthesizer (9050 Pep Plus Synthesizer, Millipore, Framingham, USA) with standard 9-fluorenylmethyloxycarbonyl chemistry (Fields and Noble 1990). All peptides were labeled with a fluorescent probe by including an α-dansylated leucine (Sigma, St. Louis, USA) at the amino terminus of the peptide. All other reagents employed in peptide synthesis were purchased from Novabiochem (San Diego, USA). Hydrophobic scavengers and cleaved protecting groups were removed by precipitation of the peptide in cold diethyl ether and dissolution in deionized water. Several bifunctional peptides were synthesized that contained the factor XIIIa cross-linking domain from α_2-plasmin inhibitor, namely residues 1-8 of the human sequence (for clarity, the factor XIIIa substrate domains are always shown in italics), and active sequences from various proteins involved in cell adhesion, namely, four sequences from laminin, dL*NQEQVSPL*RGD-NH_2, dL*NQEQVSPL*IKVAV-NH_2, dL*NQEQVSPL*YIGSR-NH_2, and dL*NQEQVSPL*RNIAEIIKDI-NH_2, one from N-cadherin, dL*NQEQVSPL*HAV-NH_2 and the bi-domain peptide, dL*NQEQVSPL*RDG-NH_2, a biologically inactive control sequence. After lyophilization, peptides were redissolved in 0.03 M Tris-buffered saline (TBS) at pH 7.0 and analyzed using HPLC (Waters; Milford, USA) on a size exclusion column with TBS as the solvent and detection by absorbance.

Fibrinogen solutions were prepared by dissolving fibrinogen (Fluka; Buchs, Switzerland) in deionized water at 8 mg/mL for 4 hr. Dialysis (Spectrum, 6000-8000 MWCO; Laguna Hills, USA) vs. 400 volumes of TBS, pH 7.4 for 24 hr was used to exchange salts present in the protein solution. The resulting solution was then sterilized by filtration through a 0.22 μm syringe filter. The final fibrinogen concentration was determined by measuring the absorbance of the protein solution at 280 nm (Dellenback and Chien 1970). TBS solutions at pH 7.0 that were made with either 50 mM Ca^{++} or without any Ca^{++} were sterilized by filtration though a 0.22 μm filter. Fresh thrombin solutions were made by dissolving thrombin (Sigma; St. Louis, USA) in TBS at pH 7.0 at a concentration of 20 units/mL.

4.3.2 Fibrin Synthesis

A series of 400 μL fibrin gels was made in flat 24 well culture plates by mixing the four components such that the final concentrations obtained were (1) 3.6 mg/mL fibrinogen, (2) 2.5 mM Ca^{++}, (3) 2 NIH Units/mL thrombin, and (4) various amounts of added peptide. In each case, peptide was incorporated at 8.2 mol/mol fibrinogen, which was obtained by polymerization of fibrinogen in the presence of 330 nmol/mL of peptide (Schense and Hubbell 1999). Experiments were conducted such that gels were soaked in medium in the presence and absence of soluble peptide at a concentration of 0.67 mg/mL as a competitive inhibitor.

Another series of 400 μL fibrin gels was made identically, except that all bi-domain peptides were incorporated separately at a series of final concentrations that corresponded to 1.7, 3.8, 6.0 and 8.2 mol/mol, obtained by polymerization of fibrinogen in the presence of 110, 220, 275 and 330 nmol/mL peptide, except for RGD

which was incorporated at final concentrations of up to 0.8, 1.7, 3.8 and 8.2 mol/mol, where the value of 0.8 mol/mol was obtained from 55 nmol/mL peptide.

A final series of 400 μL fibrin gels was made in flat 24 well culture plates by mixing the four components such that final concentrations of fibrinogen, calcium and thrombin were identical to those listed above. Prior to polymerization, peptides were added pairwise such that they were co-cross-linked into the fibrin matrices at various concentrations (the sum of which could not exceed 8.2 mol/mol). In each experiment, the active peptide was co-cross-linked into the fibrin gels at the concentration which resulted in the highest level of neurite outgrowth from the concentration series experiments. Specifically, RGD and HAV were incorporated at 1.7 mol/mol fibrinogen, obtained by polymerizing in the presence of 110 nmol/mL peptide. Each of the other peptides were incorporated at a concentration of either 3.8 or 6.0 mol/mol, obtained by polymerization in the presence of 220 or 275 nmol/mL peptide. Finally, the four laminin-derived peptides were co-cross-linked into a series of fibrin gels at equimolar concentrations of 1.7 mol/mol. This was obtained by adding each peptide at 110 nmol/mL.

4.3.3 Neuronal Outgrowth

DRGs were dissected from 8-day old white leghorn chicken embryos (Varon, Nomura et al. 1972). Ganglia were temporarily stored in modified neurobasal medium, consisting of insulin (5 μg/mL), transferrin (100 μg/mL), progesterone (6.3 ng/mL), putrascine (16.11 μg/mL), selenite (5.2 ng/ mL), fibronectin (5 μg/mL), L-glutamine (0.5 mM), L-glutamate (25 μM), murine mouse nerve growth factor (20 ng/mL), 0.2% bovine serum albumin and 1% antibiotic-antimycotic added to neurobasal medium (Gibco; Grand Island, USA). 400 μL fibrin gels were polymerized as described above

in the bottom of flat 24-well culture plates, in which the fibrin was polymerized around a DRG, such that one DRG was embedded three-dimensionally within each gel. The DRGs within the fibrin gels were cultured in 1 mL of modified neurobasal medium and the medium was changed at 3, 6, 9 and 24 hr post-seeding to wash out any non-cross-linked peptide. DRGs were cultured in fibrin gels without added exogenous peptides (all values were normalized to this level of neurite extension in native fibrin), or with added soluble peptide used as a competitive inhibitor as described above.

Images were collected at 24 and 48 hr using phase contrast microscopy with a 4.0 x objective. At each time point, the average neurite length extending from each ganglion was measured by digital image processing as described elsewhere (Herbert, Bittner et al. 1996), and the neurite length was normalized to the value observed in unmodified fibrin gels.

4.3.4 Statistics

Statistical analyses were performed using Statview 4.5. Comparative analyses were completed using analysis of variance (ANOVA) and an unpaired t-test at a 95% confidence level. Mean values and standard error of the mean are shown.

4.4. RESULTS

4.4.1 Peptides Incorporated Individually

DRGs were cultured in fibrin modified with 8.2 mol peptide/mol fibrinogen and the rate of neurite outgrowth was measured at 24 (Figure 4.1a, open bars) and 48 hr (Figure 4.1b, open bars) and normalized to the outgrowth in unmodified fibrin at the appropriate time point. Each gel was modified with a different bi-domain peptide that

Figure 4.1 Effect of Peptide at 8.2 mol/mol on DRG

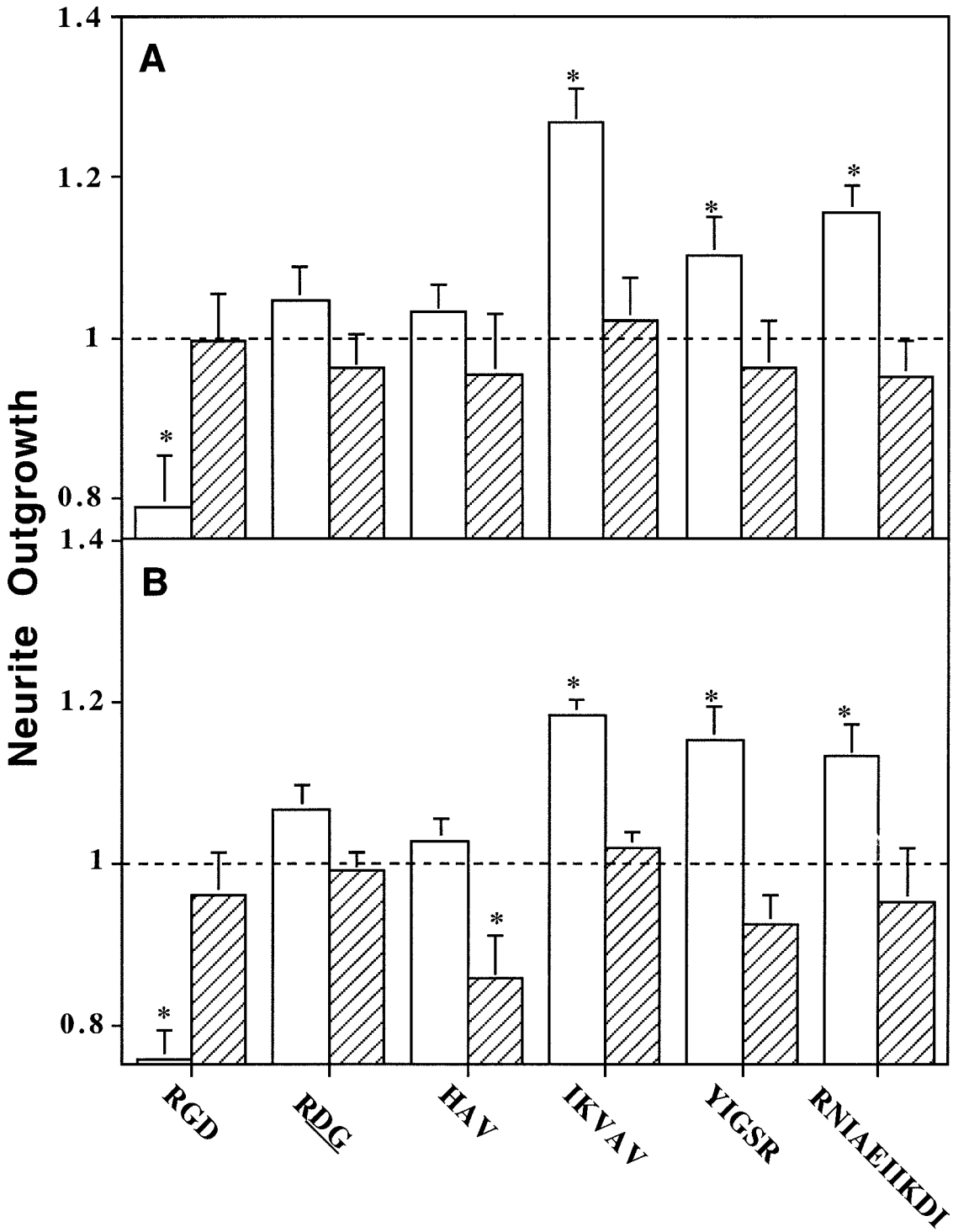


Figure 4.1 Effect of Peptide at 8.2 mol/mol on DRG

The effect of incorporated peptide at 8.2 mol/mol on neurite length within three-dimensional fibrin matrices without (open bars) and with (hatched bars) soluble peptide added to the culture medium as competitive inhibitor. Neurite outgrowth from day 8 DRGs was normalized to outgrowth in unmodified fibrin at 24 (A) and 48 hr (B) culture time. All cross-linked peptides contained the factor XIIIa substrate sequence dL*NQEQVSPL* and the active sequence as shown. Experiments were performed in triplicate with 6 DRGs per replicate, and mean values and standard error of the mean are shown; * denotes $p<0.05$ compared to unmodified fibrin. At 24 hr, the addition of RGD, IKVAV, RNIAEIIKDI or YIGSR statistically altered the extension of neurites, with RGD decreasing neurite outgrowth and the other peptides increasing neurite outgrowth. The addition of HAV or the inactive control peptide RDG did not affect the extension of neurites, resulting in outgrowth that was statistically identical to unmodified fibrin. The results at 48 hr were similar to those seen at 24 hr. In each case, the effect of the incorporated peptide was neutralized by the addition of soluble peptide as a competitive inhibitor.

contained the factor XIIIa substrate sequence in one domain and a different bioactive peptide in the other domain, four derived from the extracellular matrix protein laminin (RGD, IKVAV, YIGSR and RNIAEIIKDI), and one from the cell-cell adhesion protein N-cadherin (HAV). Covalent incorporation of RGD at 8.2 mol/mol decreased neurite outgrowth relative to unmodified fibrin, lowering neurite extension by 21% at 24 and 24.4% at 48 hr. When the inactive control peptide RDG was incorporated, the level of outgrowth was statistically similar to that in unmodified fibrin ($p>0.37$). When the N-cadherin adhesion peptide, HAV, was tested at this concentration, it did not induce a statistically significant change at 24 or 48 hr ($p>0.31$). The remainder of the peptides explored induced an increase in neurite extension at this concentration of incorporation: for IKVAV by 27% at 24 and 18% at 48 hr; for YIGSR by 11% at 24 and 15% at 48 hr; and for RNIAEIIKDI by 16% at 24 and 13% at 48 hr.

4.4.2 Peptides Incorporated with Competitive Soluble Inhibitors

As a control experiment to the studies described above, a study was undertaken in which an identical series of modified fibrin gels was synthesized; however, the

cross-linked bi-domain peptide was also added to the culture medium as a competitive soluble inhibitor. At the 24 hr timepoint, in all cases the extent of neurite extension in the presence of competitive inhibitor was not statistically different from that in the control unmodified fibrin (Figure 4.1a, hatched bars). This was also the case at 48 hr for all peptides except HAV, with which a 14% reduction was observed (Figure 4.1b, hatched bars).

4.4.3 Peptide Incorporated over a Range of Concentrations

The bi-domain peptides were cross-linked into fibrin over the concentration range of 0-8.2 mol/mol and each, except the RDG control, proved to enhance the level of neurite outgrowth compared to unmodified fibrin at some concentration. When the peptide RGD was tested in a concentration series, it demonstrated a biphasic influence on neurite outgrowth (Figure 4.3a). At low concentrations of cross-linked peptide, the rate of neurite outgrowth increased compared to unmodified fibrin. Neurite extension reached a maximum enhancement of 24% at 24 and 15% at 48 hr for RGD at the concentration of 1.7 mol/mol. At higher cross-linked peptide concentrations, the rate of neurite outgrowth decreased with increasing peptide concentration. The incorporation of the inactive control peptide, RDG resulted in outgrowth statistically unchanged compared to unmodified fibrin over the entire concentration range tested (Figure 4.3b). The incorporation of HAV also resulted in a biphasic response identical to that seen with RGD, with the maximum enhancement of 23% at 24 and 14% at 48 hr at a concentration of 1.7 mol/mol. Finally, for IKVAV, YIGSR, and RNIAEIIKDI, a monotonic increase in neurite outgrowth was observed (Figure 4.2d, e and f).

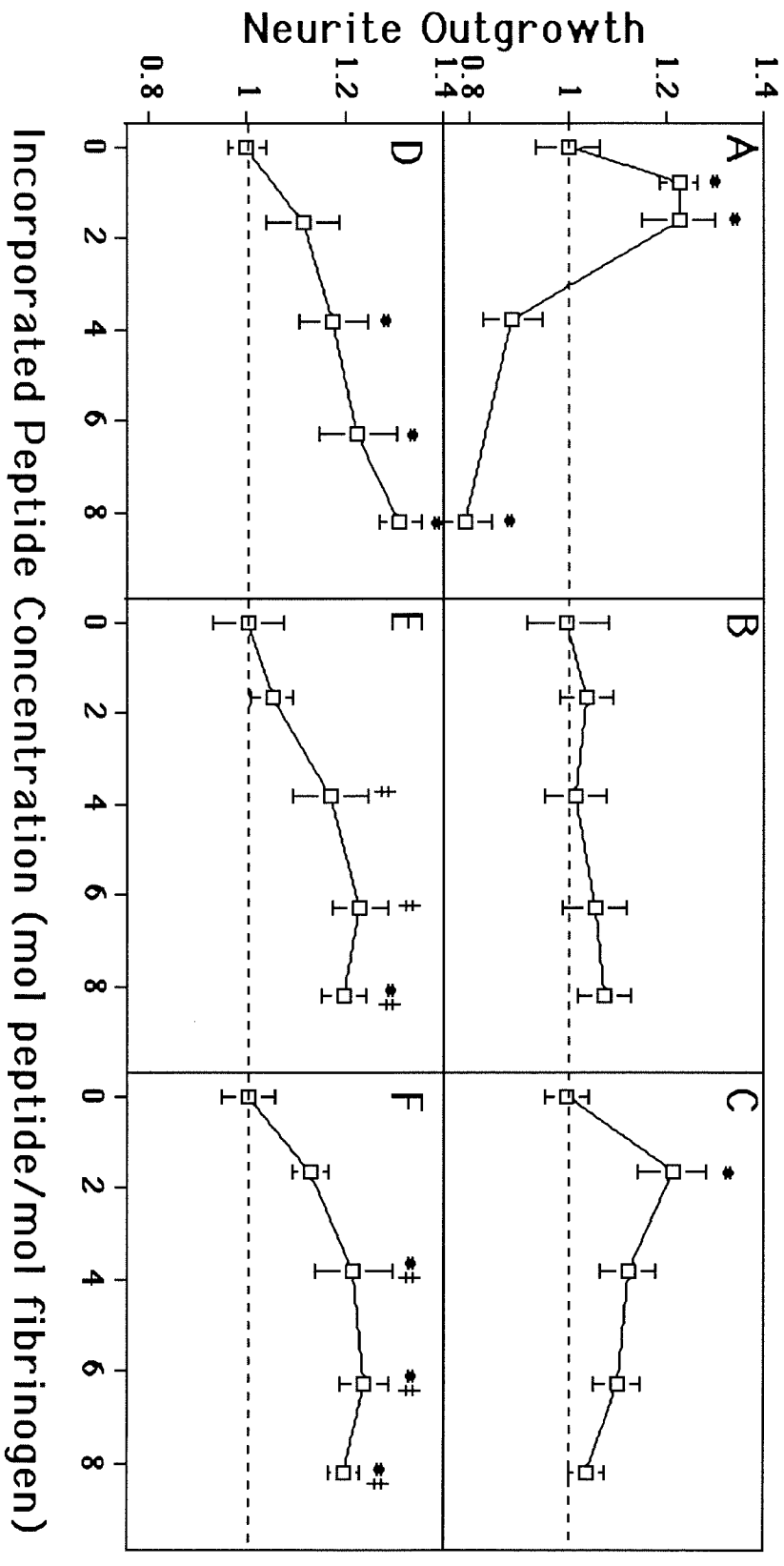

Neurite Outgrowth
0.8
1
1.2
1.4
A
B
C
D
E
F
0
2
4
6
8
Incorporated Peptide Concentration (mol peptide/mol fibrinogen)

Figure 4.2 Peptide Concentration Effect

The effect of peptide incorporated in a concentration series on neurite length within three-dimensional fibrin matrices. Matrices were modified with bi-domain peptides containing the factor XIIIa substrate sequence dL*NQEQVSPL*, and the active domain RGD (A), HAV (C), IKVAV (D), YIGSR (E) or RNIAEIIKDI (F) in a range between 0 and 8.2 mol/mol. Additionally, the inactive control peptide RDG (B) was tested within the same concentration range. Neurite outgrowth from day 8 DRGs was measured and normalized to outgrowth in unmodified fibrin at 24 hr culture time. Experiments were performed in triplicate with 6 DRGs per replicate and mean values and standard error of the mean are shown. The dependence of neurite outgrowth on RGD (A) and HAV (C) density showed an adhesive-like effect, with a maximal enhancement of outgrowth occurring at a low density of cross-linked peptide, 1.7 mol/mol. When IKVAV (D) was cross-linked in a concentration series, a monotonic correlation between peptide density and neurite length was established. The incorporation of YIGSR (E) or RNIAEIIKDI (F) led to a similar monotonic dependence, but demonstrated saturation at approximately 7 mol/mol for YIGSR and 5 mol/mol for RNIAEIIKDI. * denotes measurements that are statistically identical to each other ($p<0.05$).

4.4.4 Peptides Incorporated in Combination

In the subsequent series of experiments, pairs of peptides were co-cross-linked into the fibrin gels. Each possible pairwise combination was explored, with the peptides RGD and HAV always incorporated at a density of 1.7 mol/mol (at which their positive effect was maximal) and the other peptides incorporated at either 3.8 or 6.0 mol/mol, as indicated (also at the level of maximal effect). Three types of behavior were observed. In some peptide combinations, an inhibitory effect was observed, in which the extent of neurite extension was statistically less than when each peptide had been incorporated alone (Table 4.1a). This list includes all the possible pairwise combinations of RGD, HAV and IKVAV. A second type of behavior was observed, in which an additive effect was observed, i.e., the extent of neurite extension was

Table 4.1a Peptide combinations that exhibited an inhibitory effect.

Sample*	Normalized Neurite Outgrowth		Normalized Neurite Outgrowth When Tested in Combination‡
	Alone‡	Sum	
RGD (1.7) IKVAV (6.0)	1.24 ± 0.07 1.23 ± 0.08	1.47 ± 0.08	1.07 ± 0.04
RGD (1.7) HAV (1.7)	1.24 ± 0.07 1.21 ± 0.07	1.45 ± 0.07	0.99 ± 0.05
HAV (1.7) IKVAV (6.0)	1.21 ± 0.07 1.23 ± 0.08	1.44 ± 0.08	0.95 ± 0.10

* Incorporated peptide concentrations shown in units of mol/mol

‡ In Table 4.1a the statistical basis for comparison is between the neurite outgrowth for the peptides co-cross-linked compared to each of the individual peptides cross-linked alone.

Table 4.1b Peptide combinations that exhibited an additive effect.

Sample*	Normalized Neurite Outgrowth		Normalized Neurite Outgrowth When Tested in Combination‡
	Alone	Sum‡	
RGD (1.7) YIGSR (6.0)	1.24 ± 0.07 1.23 ± 0.06	1.47 ± 0.09	1.55 ± 0.08
HAV (1.7) RNIAEIIKDI (6.0)	1.21 ± 0.07 1.23 ± 0.05	1.44 ± 0.06	1.38 ± 0.12
HAV (1.7) YIGSR (6.0)	1.21 ± 0.07 1.23 ± 0.06	1.44 ± 0.07	1.43 ± 0.15

* Incorporated peptide concentrations shown in units of mol/mol

‡ In Table 4.1b, the statistical basis for comparison is between the neurite outgrowth for the peptides co-cross-linked compared to the sum of the outgrowth from when each peptide was cross-linked alone.

Table 4.1c Peptide combinations that exhibited a synergistic effect.

Sample*	Normalized Neurite Outgrowth		Normalized Neurite Outgrowth When Tested in Combination‡
	Alone	Sum‡	
RGD (1.7)	1.24 ± 0.07	1.53 ± 0.05	1.77 ± 0.12
YIGSR (1.7)	1.05 ± 0.04		
IKVAV (1.7)	1.11 ± 0.07		
RNIAEIIKDI (1.7)	1.13 ± 0.02		

* Incorporated peptide concentrations shown in units of mol/mol

‡ In Table 4.1c, the statistical basis for comparison is between the neurite outgrowth for the peptides co-cross-linked compared to the sum of the outgrowth from when each peptide was cross-linked alone.

Table 4.1d Peptide combinations that exhibited a non-interactive effect.

Sample*	Normalized Neurite Outgrowth		Normalized Neurite Outgrowth When Tested in Combination
	Alone‡	Sum	
RGD (1.7) RNIAEIIKDI (6.0)	1.24 ± 0.07 1.23 ± 0.05	1.47 ± 0.06	1.15 ± 0.07
IKVAV (3.8) RNIAEIIKDI (3.8)	1.18 ± 0.07 1.21 ± 0.08	1.39 ± 0.08	1.18 ± 0.07
RNIAEIIKDI (3.8) YIGSR (3.8)	1.21 ± 0.08 1.17 ± 0.08	1.38 ± 0.06	1.10 ± 0.09
IKVAV (3.8) YIGSR (3.8)	1.18 ± 0.07 1.17 ± 0.08	1.35 ± 0.08	1.14 ± 0.09

* Incorporated peptide concentrations shown in units of mol/mol

‡ In Table 4.1d, the statistical basis for comparison is between the neurite outgrowth for the peptides co-cross-linked compared to each of the individual peptides cross-linked alone.

Table 4.1 The effect of peptides co-cross-linked in various combinations on neurite length within three-dimensional fibrin matrices. Each peptide was co-cross-linked at a density near that which had been shown to elicit the maximal enhancement of neurite outgrowth. Neurite outgrowth from day 8 DRGs was measured and normalized to outgrowth in unmodified fibrin at 24 hr culture time. Experiments were performed in triplicate with 6 DRGs per replicate and mean values and standard error of the mean are shown. When peptides were grafted in combination, four types of effects on neurite outgrowth were observed. (A) Some peptide combinations appeared to inhibit neurite outgrowth, with the resulting outgrowth being lower than that seen for either of the two peptides studied alone. (B) Some peptides had additive effects, the neurite extension for co-cross-linked peptides being statistically the same as the sum of the values obtained for the two peptides cross-linked alone. (C) Other peptide combinations had synergistic effects, where the enhancement was greater than the sum of the values for the peptides cross-linked alone. (D) Finally some combinations of peptides seemed to have little interaction, the resultant outgrowth being statistically the same as that seen for the best peptide tested alone.

statistically identical to the sum of the individual enhancement of the two peptides (Table 4.1b). This series included the following pairs: HAV and YIGSR, HAV and RNIAEIIKDI, and RGD and YIGSR. Other combinations, demonstrated no additive effect (Table 4.1d) with the result as tested in combination being equal to that obtained when each individual peptide was tested alone.

4.4.5 Laminin Mimic

In a final series of experiments, the four laminin-derived peptides, namely RGD, IKVAV, YIGSR and RNIAEIIKDI, were co-cross-linked into the fibrin gels in equimolar concentrations of 1.7 mol/mol. The extent of neurite outgrowth within these gels was enhanced by 77% at 24 and 58% at 48 hr relative to unmodified fibrin (Table 4.1c). The resulting influence on enhancement in neurite outgrowth demonstrated a fourth type of behavior, synergism, in that neurite outgrowth was statistically greater

than that for both unmodified fibrin as well as the sum of the responses to the four peptides when they were tested alone at identical concentrations.

4.5. DISCUSSION

The goal in this research was to demonstrate that peptide adhesion factors covalently incorporated three-dimensionally within fibrin can significantly modulate the bioactive character of this important matrix. The immobilization of these peptides via substrates for factor XIIIa provided a simple and effective approach to functionalize the fibrin. The outgrowth of neurites from DRGs was used as a model system for investigation, and compositions were developed that augmented outgrowth by as much as 75% relative to the level seen in native fibrin matrices. Thus, it is apparent that by modifying fibrin matrices with bioactive peptides, it is possible to enhance the bioactivity, as indicated by neurite outgrowth, of an important biomaterial matrix with applications in wound healing, regeneration and tissue engineering.

The covalent incorporation of a single short bioactive peptide into a three-dimensional fibrin matrix can enhance the outgrowth of neurites. Previous work has shown that not only can large proteins, such as laminin, collagen and fibronectin, enhance two-dimensional neurite outgrowth, but that this activity can be partially mimicked by adsorbing or covalently attaching onto a surface the short peptide sequences that directly bind to the relevant receptors (Graf, Iwamoto et al. 1987; Massia and Hubbell 1991). However, for neuro-inductive proteins or peptides to have clinical applicability, they must be immobilized within a three-dimensional matrix that is subject to the proteolytic remodeling influences of the neurite growth cone. In this series of experiments, short active peptide sequences were cross-linked into fibrin, a matrix that is permissive to neurite outgrowth (Herbert, Bittner et al. 1996) and nerve

regeneration (Williams, Danielson et al. 1987). When DRGs were entrapped within matrices modified with the peptide RGD, HAV, IKVAV, YIGSR or RNIAEIIKDI, an enhancement in outgrowth of approximately 20% was observed. This enhancement is rather modest, demonstrating that while a short peptide can enhance neurite outgrowth, it is difficult for one single peptide to dramatically affect such a complicated process. The mechanisms involved in three-dimensional neurite outgrowth are significantly different from two-dimensional outgrowth; in the three-dimensional case, a delicate balance is struck between effects due to adhesion signals, growth factors and protease activity (Herbert, Bittner et al. 1996). Nevertheless, it is apparent that by incorporating exogenous adhesion sequences into the fibrin, the biological activity of non-fibrin proteins can be conferred.

The short active peptide sequences enhanced neurite outgrowth through specific receptor-mediated interaction with cells in the implanted ganglia. When an active peptide was incorporated into the fibrin at 8.2 mol/mol, it enhanced the outgrowth of neurites. In contrast, the biologically inactive peptide RDG did not affect neurite outgrowth over the entire concentration range, demonstrating that the neurite enhancement is not a function of structural changes to the fibrin matrix. Furthermore, the specific enhancement of the various exogenous incorporated bioactive peptides could be blocked by adding the same peptide to the medium as a soluble competitive inhibitor. Two of the incorporated peptides are not exogenous to the culture system, RGD and HAV. The RGD domain is present natively within fibrinogen, the main constituent of fibrin, and the HAV domain is present in N-cadherin, a molecule on the surface of both neurons and associated glial cells (Blaschuk 1990; Letourneau, Shattuck et al. 1990). In the case of addition of RGD as a soluble competitive inhibitor, it would appear that a fortuitous concentration of soluble inhibitor was selected, sufficient to overwhelm the effect of the exogenously added peptide, but insufficient to inhibit interaction with the endogenous domain. Higher concentrations

of soluble inhibitor do indeed inhibit neurite extension (Schense and Hubbell, unpublished results). In the case of HAV, it would appear that some of the endogenous cell-cell N-cadherin mediated interactions were inhibited, and this inhibited neurite extension at 48 hr.

When studied in a concentration series, some peptides enhanced neurite outgrowth monotonically throughout the range of 0-8.2 mol/mol and others acted in a biphasic fashion, indicating a difference in either affinity or mode of interaction. The two peptides RGD and HAV would seem to be more high affinity binders, exhibiting a maximum enhancement at intermediate levels of peptide density. Such behavior has been observed with cell migration in two dimensions, i.e., a maximal rate of migration at some intermediate surface concentration of adhesion ligands, and this behavior was demonstrated to be more prevalent at higher receptor-ligand binding affinity (Palecek, Loftus et al. 1997). When the RGD and the HAV ligands were co-immobilized in fibrin gels, an inhibitory interaction was observed; this may be indicative of rendering the three-dimensional matrix too adhesive for maximal rates of neurite extension and migration. Interestingly, all pairwise combinations of RGD, HAV and IKVAV demonstrated this inhibitory behavior. The IKVAV sequence when singly immobilized over a range of concentrations did not exhibit this bimodal behavior. This may indicate that its inhibitory interaction when co-immobilized is due to some other mode of action, or it may be that a biphasic behavior really does exist, just at IKVAV concentrations in excess of the experimental maximum of 8.2 mol/mol.

The co-cross-linking of some peptides provided additive and even synergistic effects on neurite outgrowth, resulting in materials with important therapeutic potential. By co-cross-linking specific pairs of peptides, it was possible to enhance neurite outgrowth at much higher levels than with individual short peptides. While the mechanisms involved in these additive and synergistic effects are not addressed by the experimental design of this study, the results suggest that the activity of

morphoregulatory proteins such as laminin can be better mimicked with multiple short peptide sequences. This is most clearly demonstrated by the co-cross-linking of the four laminin peptides (Table 4.1c). The co-cross-linking of RGD, IKVAV, YIGSR and RNIAEIIKDI, each at 1.7 mol/mol provided a synergistic level of enhancement, i.e., enhancement greater than the sum of the responses observed with the four peptides tested individually. Clearly the three-dimensional structure of the overall protein laminin has not been mimicked, but by adding multiple active sequences from the same protein, a material with the greatest bioactivity was identified, enhancing neurite extension by 75%.

The application of fibrin as a sealant, and ingrowth matrix has current widespread clinical use. Through the enzymatic incorporation of exogenous factors into fibrin, procedures have been developed whereby the bioactive character can be precisely and reproducibly controlled and augmented. These matrices provide scientists with a tool for studying the activity of various factors in a more realistic three-dimensional environment, as well as enhancing the properties of an important material for clinicians. In the context of the latter, fibrin plays a unique role in the body, being the initial wound repair matrix in almost all tissues, often inducing rapid healing in the form of a scar. By the approach described in this report, it may be possible to employ the molecular cues that are normally involved in development and to confer these upon a material that normally functions in wound healing.

5. *IN VIVO* NEURONAL ACTIVITY*

5.1. ABSTRACT

Fibrin-based biomaterials have enormous potential for application in *in vivo* nerve regeneration as both unmodified fibrin and enzymatically modified fibrin are conducive for the regeneration of severed nerves. The development of biomaterials for *in vivo* applications requires testing of both the general toxicity as well as the efficacy of the material. These were tested for fibrin to a preliminary extent, by using it as a filler material in silicone nerve growth guides (NGGs) for short gap studies in the sciatic and dorsal root nerve models. Fibrin gels were synthesized *in situ* with and without exogenous bidomain peptides added. For gels with exogenous peptides added, sufficient peptide was added such that 1.7 mol peptide/mol fibrinogen was incorporated for each of four separate bidomain peptides that contained the factor XIIIa substrate sequence from α_2-plasmin inhibitor in one domain and a bioactive sequence from laminin, RGD, IKVAV, YIGSR or RNIAEIIKDI, in the other domain. When saline filled and peptide-modified fibrin filled NGGs were tested on resected rat sciatic nerves with a 6 mm gap, a similar number of axons was observed at the midpoint. Furthermore, the use of fibrin, with or without enzymatic modification, proved to be effective in enhancing nerve regeneration in resected dorsal root nerves. When a 4 mm gap was created in the dorsal root nerves of rats, the number of axons at the midpoint increased sequentially with saline, fibrin and finally with enzymatically modified fibrin being the highest. The use of fibrin as a filler for nerve growth guides appears to have great potential, as the structural properties appear to enhance cellular ingrowth and this

* The animal surgeries and histology were performed by Michael Papaloizos and Jocelyne Bloch.

is further augmented by the endogenous and exogenous biochemical nature of the matrix.

5.2. INTRODUCTION

The *in vivo* development of biomaterials involves two distinct and equally crucial steps, testing the general toxicity of the material and its subsequent associated degradation products and testing the efficacy of the biomaterial through a relevant model system. The materials created in this project were designed as fillers for nerve growth guides attached to severed nerves. Therefore, the relevant models were the sciatic nerve for the toxicity test and the dorsal root nerve for the efficacy test.

The goal of this work has been to develop biomaterials for application as a filler material in NGGs for severed peripheral nerves. Therefore, the material with the greatest enhancement of neurite outgrowth from chick dorsal root ganglia in the *in vitro* model was to be chosen as the candidate material for filling nerve growth guides. The incorporation of individual bioactive peptides proved to have a limited ability in enhancing neurite outgrowth. However, when these peptides were tested in combination, some of them exhibited additive effects on neurite outgrowth. The material with the greatest potential proved to be fibrin matrices modified with each of the four laminin-derived bioactive domains, RGD, IKVAV, YIGSR and RNIAEIIKDI, at equimolar concentrations of 1.7 mol peptide/mol fibrinogen. In *in vitro* tests using the chick DRG model, these modified materials enhanced neurite outgrowth by 77% over unmodified fibrin (Chapter 4 of this thesis).

The initial step in the *in vivo* development of enzymatically-modified fibrin matrices was to perform a toxicity test on fibrin and its associated degradation products. In order to determine if the material of interest has a negative impact on nerve

regeneration, it is necessary to test the material in a highly permissive environment for nerve regeneration, leading to the choice of the short gap model in the sciatic nerve. Even though the sciatic nerve model is ideal for toxicity testing, it continues to serve as the primary model for entubulation efficacy studies of severed peripheral nerves. This is for several primary reasons. First, the technical difficulty associated with this procedure is quite low, enabling relatively large numbers of animals to be operated on in a short time period. Furthermore, the sciatic nerve is readily accessible, leading to a low mortality rate during surgery. Finally, the level of natural regeneration in this model is relatively high, allowing nerve regeneration to be detected in materials with low regeneration capabilities. During the initial studies, sciatic nerves were severed and empty silicone guides were inserted with a short gap, around 4 or 5 mm, created between the proximal and distal stumps. Even with an empty growth guide, a large amount of regeneration was observed, such that within a few weeks, thousands of axons crossed this short gap (Le Beau, Ellisman et al. 1988). In subsequent experiments, similar silicone tubes were filled with either matrix precursors, such as saline or fibrinogen-containing plasma (Williams 1987), with bioactive materials, such as collagen or laminin (Klavjin and Madison 1991), or with related glial cells, such as Schwann cells (Ansselin, Fink et al. 1997). In each of these models, it was possible to transverse a gap on the order of 10-14 mm between the proximal and distal stumps of the severed nerves with thousands of axons. At this point, a physical limit has been reached, preventing the insertion of a larger gap. Therefore, it is impossible to use this model for distinguishing between highly bioactive materials. However, as this model provides a high level of natural regeneration with a short gap, it can continue to serve as a useful model for toxicity tests.

The continual progress in developing better methods and materials for peripheral nerve regeneration has resulted in the use of the dorsal root, a less permissive system, as a model for peripheral nerve regeneration. The use of the dorsal

root nerve has several distinct disadvantages compared to the sciatic model. The dorsal root ganglion lies close to the spinal cord and resecting the nerve requires the performance of a laminectomy prior to nerve resection. This procedure requires a higher level of technical skill and leads to a higher intraoperative mortality rate. Furthermore, the time required for each procedure is significantly longer than for the sciatic surgery, limiting the number of animals used in the study. However, due to the limited natural regeneration capabilities of the dorsal root, it is possible to distinguish between the relative activities of various highly permissive matrices using this model. Using this model to test regeneration through modified matrices has met with little success due to the low permissivity of this model; however, it has been used to demonstrate that the release of NGF, even in the absence of a supporting exogenous structural matrix, has a dramatic effect on regenerating nerves over short gaps (Bloch and Aebischer, unpublished results).

The application of these enzymatically modified fibrin matrices to an *in vivo* system followed the natural *in vivo* development of materials. Initially, materials with the highest regeneration capacities during *in vitro* testing, i.e., those that contain the four laminin-derived bioactive peptides, RGD, IKVAV, YIGSR and RNIAEIIKDI, were selected for incorporation into nerve growth guides. As exogenous fibrin gels derived from purified fibrinogen have not been tested *in vivo*, the first *in vivo* test involved probing the toxicity of these matrices. 6 mm gaps in the rat sciatic nerve were resected with growth guides filled with saline, fibrin or IKVAV modified fibrin. When these materials prove to be nontoxic, then a similar study was conducted in the dorsal root model, using empty guides or guides filled with either unmodified fibrin or fibrin with the four laminin peptides incorporated to fill a 4 mm gap to test the relative efficacy of each matrix.

5.3. MATERIALS AND METHODS

5.3.1 Peptides and Proteins

Peptides were synthesized on solid resin using an automated peptide synthesizer (9050 Pep Plus Synthesizer, Millipore, Framingham, USA) with standard 9-fluorenylmethyloxycarbonyl chemistry (Fields and Noble 1990). All peptides were labeled with a fluorescent probe by including an α-dansylated leucine (Sigma, St. Louis, USA) at the amino terminus of the peptide. All other reagents employed in peptide synthesis were purchased from Novabiochem (San Diego, USA). Hydrophobic scavengers and cleaved protecting groups were removed by precipitation of the peptide in cold diethyl ether and dissolution in deionized water. Several bifunctional peptides were synthesized that contained the factor XIIIa cross-linking domain from α_2-plasmin inhibitor, namely residues 1-8 of the human sequence (for clarity, the factor XIIIa substrate domains are always shown in italics), and one of four bioactive sequences from laminin, dL*NQEQVSPL*RGD-NH_2, dL*NQEQVSPL*IKVAV-NH_2, dL*NQEQVSPL*YIGSR-NH_2, and dL*NQEQVSPL*RNIAEIIKDI-NH_2. After lyophilization, peptides were redissolved in 0.03 M Tris-buffered saline (TBS) at pH 7.0 and analyzed using HPLC (Waters; Milford, USA) on a size exclusion column with TBS as the solvent and detection by absorbance.

Fibrinogen solutions were prepared by dissolving fibrinogen (Fluka; Buchs, Switzerland) in deionized water at 8 mg/mL for 4 hr. Dialysis (Spectrum, 6000-8000 MWCO; Laguna Hills, USA) vs. 400 volumes of TBS, pH 7.4 for 24 hr was used to exchange salts present in the protein solution. The resulting solution was then sterilized by filtration through a 0.22 μm syringe filter. The final fibrinogen concentration was determined by measuring the absorbance of the protein solution at 280 nm (Dellenback and Chien 1970) and converting to a concentration using the absorbtion coefficient for

human fibrinogen of 1.55 (cm*ml/mg). TBS solutions at pH 7.0 that were made with either 50 mM Ca^{++} or without any Ca^{++} were sterilized by filtration though a 0.22 μm filter. Fresh thrombin solutions were made by dissolving thrombin (Sigma; St. Louis, USA) in TBS at pH 7.0 at a concentration of 20 units/mL.

A series of 10 μL fibrin gels were polymerized *in situ* in silicone tubes that were sutured onto the proximal stump of the severed nerve by mixing the four components such that final concentrations obtained were (1) 3.6 mg/mL fibrinogen, (2) 2.5 mM Ca^{++}, (3) 2 NIH Units/mL thrombin, and (4) various amounts of added peptide. Fibrin gels were synthesized without exogenous peptide or with the four laminin derived peptides co-cross-linked. When the IKVAV-modified gels were tested in the sciatic model, the peptide was incorporated at a density of 8.2 mol peptide/mol fibrinogen, obtained by adding 330 nmol/mL of peptide. When the four laminin-derived peptides were co-cross-linked into this series of fibrin gels for the efficacy test, they were incorporated at equimolar concentrations of 1.7 mol peptide/mol fibrinogen, which was obtained by adding each peptide at 110 nmol/mL.

5.3.2 Sciatic Nerve Surgeries

Adult male Wistar Albino Rats (250-350 g) were anesthetized by intraperitoneal injection of pentobarbital. The operative site was shaved and cleaned with an iodophore (Betadine®). Silicon nerve growth guides having an internal diameter of 1.47 mm were cut to 8 mm and sterilized with ethylene oxide at 30°C and allowed to degas for at least three days. The right sciatic nerve was exposed and severed. Silicone nerve growth guides were attached to the proximal stump of the sciatic nerve with a single stitch through the epinurium. The NGGs were filled with saline or fibrin *in situ*, and the distal stumps were secured with 10-0 nylon sutures. The stumps were positioned 1 mm from the channel ends, so that a 6 mm gap was left. Muscle

approximation and closure was achieved with 6-0 polylactic acid sutures. A total number of ten animals were used in this study, with five having saline filled growth guides and five having IKVAV-modified fibrin filled growth guides. Nerve regeneration was studied after a time period of four weeks. Animals were caged in pairs and housed in a controlled environment with 12 hour on-off light cycles. Food and water was provided ad libitum.

For retrieval of the implant, animals were deeply anesthetized with an intraperitoneal injection of pentobarbital and perfused transcardially with 200 mL heparinized physiologic saline followed by 200 mL of a cold 4% paraformaldehyde, 2.5% glutaraldehyde solution in PBS at pH 7.4. The operative site was reopened and the guidance channel was removed. The specimens were post-fixed, dehydrated and embedded in epoxy resin. Semi thin sections (1 μm) were cut with a Zeiss microtome for light microscopical determination of regenerated myelinated axons and stained with toluidine-blue. The cross-sectional area of the regenerated nerve cable was analyzed at the midpoint and the number of regenerated myelinated axons was determined.

5.3.3 Dorsal Root Surgeries

Adult male Wistar Albino Rats (250-350 g) were anesthetized by intraperitoneal injection of pentobarbital. The operative site was shaved and cleaned with an iodophore (Betadine®). A 5 cm dorsal midline incision was made using the iliac crests as a landmark. Surgery was performed aseptically under a Zeiss operating microscope. Mobilization and retraction of the peraspinous muscles exposed the L3 to L6 spinous processes and laminae. Bilateral laminectomies from L4 to L6 were performed. The spinal roots were exposed through a longitudinal midline incision in the dura matter. The left L5 ventral and dorsal root were identified using anatomical landmarks and then

isolated from neighboring roots. A 2 mm segment of the root was resected 7-10 mm from the root's exit of the spinal canal. The proximal and distal stumps were secured 4 mm apart in a 6 mm long PVC (Florio®) channel using 10-0 nylon sutures placed through the root. Muscle approximation was achieved with 6-0 monofilament nylon suture (Ethilon®). Skin closure was accomplished using 4-0 Dermalon suture. A total number of 23 animals was divided into three groups. Each group had a different matrix within the implanted growth guide. The three filler materials tested were empty tubes (control, 6 animals), unmodified fibrin (7 animals) and fibrin modified with the four laminin peptides, RGD, IKVAV, YIGSR and RNIAEIIKDI, in equimolar amounts of 1.7 mol peptide/mol fibrinogen (10 animals). Nerve regeneration was studied after a time period of four weeks. Animals were caged in pairs and housed in a controlled environment with 12 hour on-off light cycles. Food and water was provided *ad libitum.*

For retrieval of the implant, animals were deeply anesthetized with an intraperitoneal injection of pentobarbital and perfused transcardially with 200 mL heparinized physiologic saline followed by 200 mL of a cold 4% paraformaldehyde, 2.5% glutaraldehyde solution in PBS at pH 7.4. The operative site was reopened and the guidance channel was removed. The specimens were post-fixed, dehydrated and embedded in epoxy resin. Semi thin sections (1 μm) were cut with a Zeiss microtome for light microscopical determination of regenerated myelinated axons. The cross-sectional area of the regenerated nerve cable was analyzed at the midpoint and the number of regenerated myelinated axons was determined.

5.3.4 Statistics

Statistical analyses were performed using Statview® 4.5. Comparative analyses were completed using analysis of variance (ANOVA) and an unpaired t-test at

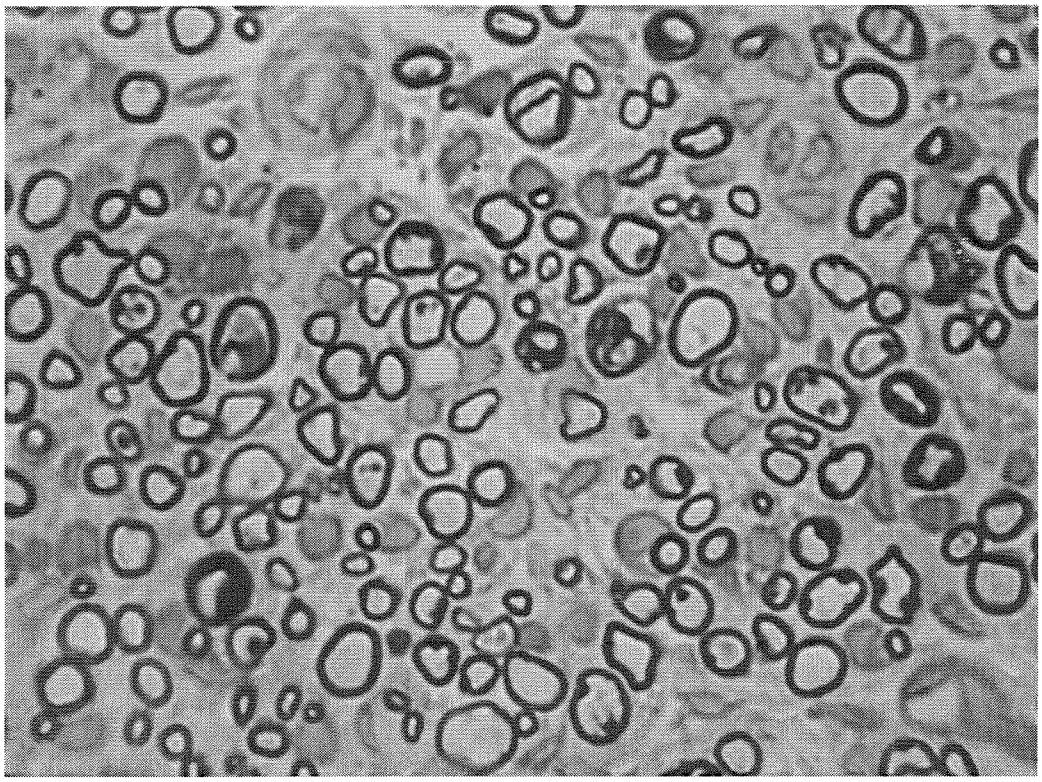
a 95% confidence level for the *in vitro* experiments. Mean values and standard error of the mean are shown. Mann-Whitney U tests were performed for the *in vivo* results.

5.4. RESULTS

5.4.1 Toxicity Test

Fibrin, derived from purified human fibrinogen, as a base matrix for filling nerve growth guides to bridge severed nerves is nontoxic for nerve regeneration. When sciatic nerves were severed and resected with silicone guides filled with saline, the number of axons at the midpoint was 6800 ± 2500. Fibrin-filled growth guides

A



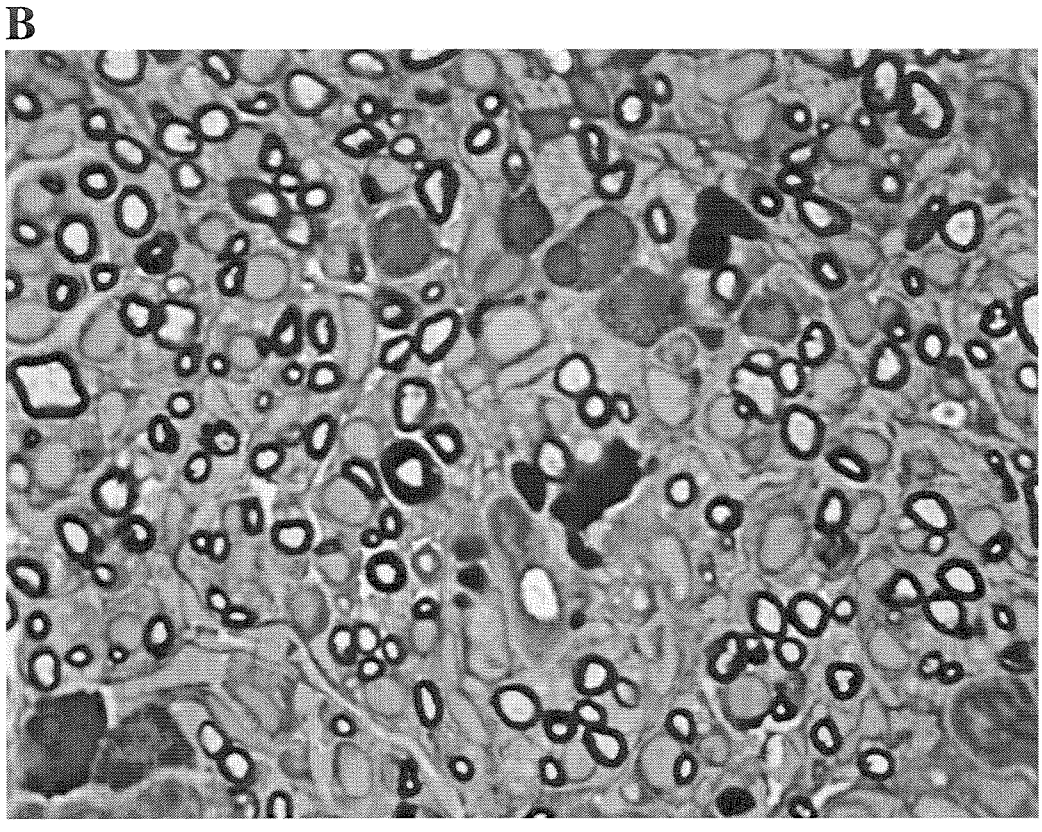


Figure 5.1 Cross-sectional Image of Axons
After retrieval of the NGGs at 4 weeks, the regenerating axons were fixed and stained with toluidine blue. High resolution images were obtained at 100x and a random count of the number of axons was performed with an example shown of samples taken from saline filled NGGs (A) and IKVAV-modified fibrin filled NGGs (B). The number of axons, axon diameter and neurite density are similar for both series of samples.

modified with IKVAV gave slightly higher, although not significantly higher, numbers of axons at the midpoint with 7800 ± 3800 at the midpoint. The morphology of the axons, including axon diameter and density, were similar for both series of sample (Figure 5.1). At the time of removal, very little fibrin remained inside the tube.

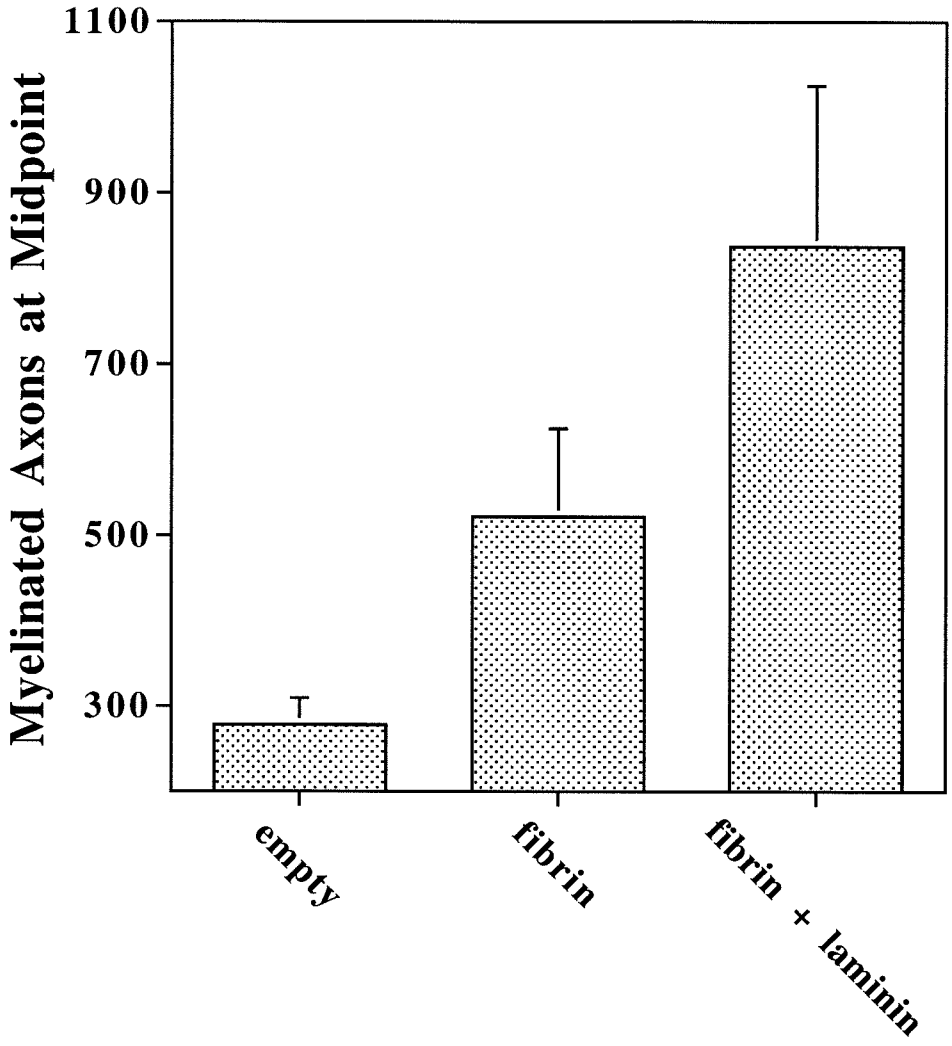


Figure 5.2 Efficacy of Fibrin *in vivo*

Number of axons obtained when fibrin matrices were used as filler for NGGs in the dorsal root model. The dorsal root nerve was transected and attached to a 6 mm silicone NGG, creating a 4 mm gap between the stumps. The guides were either empty, or filled with unmodified fibrin or peptide-modified fibrin polymerized *in situ*. After four weeks of regeneration, the number of axons at the midpoint were quantified. Use of unmodified fibrin resulted in a greater number of axons than were present in empty tubes, and peptide-modified fibrin had a further increase in the number of axons, with greater regeneration levels than that observed in both empty guides and guides filled with unmodified fibrin ($p<0.05$).

5.4.2 Efficacy Test

Fibrin and peptide-modified fibrin were used as filler material for nerve growth guides in the dorsal root model and both provided higher numbers of axons than empty growth guides. Six animals were given empty nerve growth guides spanning a 4 mm gap in the dorsal root nerve, and the resulting number of axons at the midpoint at 4 wk was 282 ± 30. When the same model was used, but instead the guide was filled with unmodified fibrin derived from purified fibrinogen, the subsequent number of axons at the midpoint increased to 522 ± 105. An even greater increase was noted in the guides filled with the peptide-modified fibrin matrices. These matrices resulted in 840 ± 185

Empty (# axons at midpoint)	Fibrin (# axons at midpoint)	Peptide-modified Fibrin (# axons at midpoint)
179	455	308
402	658	551
259	394	1301
269	359	200
280	304	720
302	387	586
	1097	755
		862
		2260
		861
Mean ± SEM	Mean ± SEM	Mean ± SEM
282 ± 30	522 ± 105	840 ± 185

p values

empty vs. fibrin	p = 0.02
empty vs. peptide-modified fibrin	p = 0.01
fibrin vs. peptide-modified fibrin	p = 0.17

Table 5.1 In vivo Dorsal Root Regeneration

The number of axons present at the midpoint for empty, fibrin filled and peptide-modified filled nerve growth guides attached to severed rat dorsal root nerves. The individual values for each animal are shown in the table with the mean and standard error of the mean shown. Addition of exogenous fibrin polymerized *in situ* proved to enhance nerve regeneration and the use of the fibrin modified with the four laminin peptides enhanced the effect even further.

axons at the midpoint. The number of axons in the guides filled with fibrin was statistically higher than the empty guides (p=0.02) and the peptide-modified fibrin provided even greater regeneration, with the number of axons being statistically greater than both the empty (p=0.01) and fibrin filled guides (p=0.17) (Figure 5.2, Table 5.1). When the morphology of the regenerated axons was studied, little deviation between the samples was observed. With all three experimental conditions, the average axon diameter, number of axons per bundle and percent of myelinated axons were similar. Interestingly, some proteinaceous areas were observed in the two fibrin treatments that did not appear in the saline treatment, with these areas corresponding to cross sections that did not contain bundles of axons.

5.5. DISCUSSION

Through the use of two separate model systems, the sciatic and the dorsal root nerve, this new preliminary data has demonstrated that the use of fibrin, derived from purified fibrinogen, either unmodified or enzymatically modified with exogenous bioactive peptides is potentially both nontoxic and can enhance the regeneration of nerves across a gap through nerve growth guides.

Peptide-modified fibrin as filler matrix for growth guides is nontoxic to regenerating nerves, including both the base matrix and the subsequent degradation products. Sciatic nerves in rats were severed and short gaps were established between the two stumps. When these gaps were bridged with a saline filled silicone growth guide, the level of regeneration was extremely high, leading to thousands of axons at the midpoint. This result is well established, as previous groups have shown this natural level of regeneration in the sciatic model. When the peptide-modified fibrin matrix was used instead, the level of regeneration was similar with slightly higher

numbers of axons at the midpoint. This high level of regeneration clearly demonstrates that the presence of the fibrin gel does not inhibit the nerve regeneration and is therefore nontoxic for regenerating nerves in the rat. The nontoxicity of exogenous fibrin is further supported in that for neither experimental condition, with or without an exogenous fibrin matrix, induced inflammation or scar formation. As human fibrinogen is the primary precursor component, it was possible that inflammation could occur. However, this study indicates that these exogenous fibrin matrices do not induce a potent immune response. It should be noted that the details of immune interactions between the human fibrin and the rat immune system were not the topic of this study, and it could be that some immune response was present; however, any response that did occur was apparently not inhibitory to nerve regeneration in this model. Furthermore, there was not significant fibrin induced fibroblast infiltration, as the histological examination of each sample indicated that the cellular components within each tube were similar. Altogether, these results indicate that the exogenous fibrin matrix is nontoxic and potentially beneficial for nerve regeneration.

Another potential toxic effect could be derived from the degradation products of the exogenous fibrin matrix. It was observed that much of the initial matrix that was polymerized inside the tube had been degraded during the process of regeneration, through either local or global proteolysis, resulting in significant amount of fibrin degradation products. These products did not affect the level of regeneration either, indicating that the subsequent degradation fragments are not toxic for the regenerating nerves as well. Previous work has shown that these fibrin degradation products can interact with various cell types. It has also been demonstrated that much of this effect is directed towards cells of the immune system, with these degradation products affecting lymphocyte activity (Edgington, Curtiss et al. 1985). These products also can affect the growth of smooth muscle cells (Ishida and Tanada 1982) and protease activator inhibitor production in fibroblasts (Hagood, Olman et al. 1996). It is likely, however,

that the level of interaction induced by the fibrin degradation products is limited and does not have a direct effect on nerve regeneration.

The use of fibrin or enzymatically modified fibrin has significant potential as an ingrowth matrix for regenerating nerves, increasing the number of myelinated axons within a short gap model. The second important test for the applicability of this work to future animal or clinical trials involves testing the efficacy of the materials developed. The most suitable model for this study is the short gap within the dorsal root nerve. When empty growth guides were used to bridge the gap between the distal and proximal stumps of severed dorsal root nerves, the level of regeneration was consistently low, even for short gaps. However, the polymerization of unmodified fibrin inside the lumen of the guide *in situ* resulted in higher numbers of axons at the midpoint. Previous work has shown that when an empty guide is used, the lumen is initially filled with endogenous fibrin as a conduit for nerve regeneration and that nerve regeneration through similar conduits can be enhanced by prefilling the guide with fibrinogen-containing plasma. However, it has never been demonstrated that preforming an exogenous fibrin matrix from purified components would enhance nerve regeneration. Through this work, it has been demonstrated that these exogenous fibrin matrices do enhance nerve regeneration and the use of this material as a base matrix is suitable, as even the unmodified material is neuroinductive. Finally, the ability of the exogenous fibrin matrix to enhance nerve regeneration was further enhanced by the enzymatic incorporation of the four laminin-derived peptides into the fibrin matrix. This material induced even greater nerve regeneration than the unmodified fibrin, demonstrating that this potent matrix can be further enhanced with the incorporation of adhesion peptides.

One important point is to analyze the results from this study within the context of other work in the field. In comparison with the gold standard of the nerve autograft, it is likely that a peptide-modified fibrin matrix does not yet provide the same level of

functional regeneration. However, other matrices have been tested in this model and provide another benchmark for comparison. The use of agarose, functionalized with some of the same bioactive peptides as utilised in this study, which met with significant success in inducing regeneration in the sciatic nerve, had little success in regenerating the dorsal root nerve, resulting in levels of regeneration similar to that seen with saline. (Aebischer, unpublished results) Clearly then, it is observed that the dorsal root regeneration model in the rat is a much more difficult regeneration model than the sciatic nerve regeneration model. Furthermore, it is clear that the use of fibrin and modified fibrin matrices has shown a superior level of regeneration to other forms of matrix employed. Another area of treatment is the application of growth factors instead of matrix factors within the tube. Experiments using delivery of the growth factor NGF in a four mm gap in the dorsal root has shown a significantly higher level of regneration than the matrices employed in this study, inducing twice as many myelinated axons at the midpoint. (Bloch and Aebischer, unpublished results) It may be that the use of both a growth factor delivery device, either from the matrix of the polymer tube, in conjunction with the peptide-modified fibrin matrices may provide the closest levels of regeneration and functional recovery to that seen with an autograft.

The development of methods toward clinical applicability in the repair of severed peripheral nerves has led to research on nerve growth guides filled with bioactive material. While some improvements have been gained through the design of the guide itself, the greatest potential for enhancement lies in the development of the filler material for the guide. Through this research, it has been shown that fibrin and enzymatically modified fibrin hold an exciting potential for this application. The materials used were clearly nontoxic to the regenerating nerves, as indicated by the level of regeneration in the transected sciatic nerve model. Furthermore, the fibrin material was effective in improving nerve regeneration both as an unmodified matrix and to an even greater extent with bioactive peptides covalently cross-linked into the matrix. The

future potential of these materials is even greater, as the incorporation of both adhesion peptides and highly active soluble growth factors within the material or guide could lead to an exciting therapeutic application.

6. SUMMARY AND RECOMMENDATIONS

6.1 SUMMARY

Utilization of factor XIIIa to enzymatically cross-link bi-domain peptides into fibrin provides a method to develop materials with significant therapeutic potential for many clinical applications. Through this project, it has been demonstrated that bi-domain peptides containing a factor XIIIa substrate sequence from α_2-plasmin inhibitor in one domain and a bioactive sequence in the other domain, can be cross-linked into fibrin at very high concentrations. Using the endogenous factor XIIIa present in the fibrinogen preparation, it was possible to incorporate up to 8.2 mol peptide/mol fibrinogen in a highly controllable fashion. Furthermore, using a swelling assay, it was determined that the incorporation of these peptides within this concentration range had little effect on the structural integrity of the fibrin matrices. These materials were then tested with a three-dimensional *in vitro* cell culture model, neurite extension from the chick dorsal root ganglion Briefly, individual ganglia were entrapped inside fibrin matrices modified with 8.2 mol peptide/mol fibrinogen during polymerization and the average neurite length was measured and compared to that observed in unmodified fibrin. The level of outgrowth was statistically greater for fibrin modified with many of the bioactive peptides, demonstrating that they retain some of their activity. Additionally, through the use of the inactive control peptide, RDG, and the active peptides as competitive soluble inhibitors, it was shown that the enhancement was derived from specific interaction between covalently bound peptides and cell-surface receptors.

The enzymatic process of covalently incorporating these bi-domain peptides is highly reproducible and controllable, allowing each of these peptides to be studied either in a concentration series or co-cross-linked into fibrin gels. Through this work, the mechanistic nature of the peptides of interest was further revealed and many formulations with therapeutic potential were discovered. When peptides were incorporated in a concentration series, two distinct effects were observed. For RGD and HAV, the effect on neurite outgrowth was biphasic, with the maximal enhancement occurring at moderate peptide densities. Then, at higher peptide densities, the enhancement decreased and even became negative. This effect has been seen and well described in two-dimensional cell migration with RGD, where the RGD sequence activity has been modeled as an adhesive site. This work demonstrates that a similar adhesion mechanism is probably involved in three-dimensional RGD-dependent neurite migration as well. This similar adhesive-like character of the N-cadherin binding domain, HAV, is novel, further elucidating the activity of this neuronally active sequence. In contrast, the other three peptides, IKVAV, YIGSR and RNIAEIIKDI, increased neurite outgrowth monotonically with peptide density, indicating that different mechanisms are involved. However, one peptide that provided conflicting results is the laminin-derived sequence, IKVAV. During the co-cross-linking experiments, RGD or HAV were incorporated at the density that corresponded to their maximal enhancement of neurite outgrowth. If additional adhesive peptides were cross-linked into the fibrin, the enhancement level should decrease, which was observed when the two known adhesive sequences RGD and HAV were co-cross-linked. However, a similar behavior was observed when the second peptide was IKVAV. Therefore, it may be that the activity of IKVAV is biphasic as well, whereby the second phase occurs at densities higher than those tested, i.e., 8.2 mol peptide/mol fibrinogen. Finally additional combinations of peptides were tested that had additive or synergistic effects. These were able to enhance neurite outgrowth at levels up to 77%

greater than unmodified fibrin alone and served as the basis for those materials to be used in future *in vivo* testing. The one material which provided the greatest outgrowth and was therefore selected for *in vivo* trials contained the four laminin derived peptides, RGD, IKVAV, YIGSR and RNIAEIIKDI, incorporated in equimolar amounts of 1.7 mol peptide/mol fibrinogen.

In vivo testing of fibrin and enzymatically-modified fibrin matrices demonstrated that both of these materials are nontoxic and furthermore, are able to enhance nerve regeneration, with the modified matrices providing even greater numbers of new axons. IKVAV-modified fibrin matrices were tested for toxic effects in the sciatic nerve model. In this model, the natural level of regeneration over a short gap is extremely high, and using this system, it was demonstrated that the level of regeneration was similar for nerve growth guides filled with either saline or the modified fibrin matrix. This supports the conclusion that neither the modified fibrin matrix nor the associated degradation products are toxic to the regenerating nerve. The short gap model with the dorsal root nerve was used to test the efficacy of the various materials. With this model, it was observed that the number of axons was greater when unmodified fibrin was used to fill the nerve growth guide instead of an empty guide and was enhanced even further when the fibrin was modified with the four laminin-derived peptides. Clearly then, fibrin is a permissive matrix for nerve regeneration that can be further augmented through the use of cross-linked exogenous peptides.

Through this project a novel biomaterial has been invented and studied in both a relevant *in vitro* system as well as a relevant *in vivo* system. It was clearly shown that this matrix has enhanced bioactivity *in vitro* and that this benefit is transferred to the *in vivo* model.

6.2 RECOMMENDATIONS FOR FUTURE WORK

The completed project points to many new avenues where these materials could either be enhanced or redirected for different applications. Some possible enhancements of these materials include incorporation of different active factors, use of different fibrinogen sources or addition of exogenous factor XIIIa. With this new series of materials, the project could then be expanded to many other areas of wound healing and tissue engineering.

While improvements in nerve regeneration have been made using these adhesive factors incorporated into purified fibrin gels, several alterations could prove to enhance the applicability and activity of these materials. First, it may be possible to reach higher incorporated peptide densities by adding exogenous factor XIIIa to the system. Previous work has shown that the highest possible level of incorporation is 34 mol peptide/mol fibrinogen (Ichinose, Tamaki et al. 1983), significantly higher than the levels achieved in this study. It may be that the concentration of factor XIIIa limited the final peptide density, and adding exogenous factor XIIIa to the system would lead to a similar density observed by Ichinose. Since many of the effects did not saturate within the concentration range tested, increasing the peptide density would clearly be beneficial. Furthermore, it would be potentially beneficial to cross-link bioactive peptides into fibrin gels derived from other sources. Two such sources would be clinically approved fibrin glue, a cryoprecipitate that forms a high density fibrin gel, or actual cross-linking into fibrin gels from endogenous plasma. Fibrin glue is very beneficial, as it would provide a matrix with a much higher density of incorporated sites, as the matrix is approximately thirty times denser, as well as providing a matrix with slower degradation rates. Red blood cell free plasma derived matrices have the additional benefits of low cost as well as providing autologous cells in the matrix. Furthermore, both of these materials have established clinical applications, and

endowing them with exogenous peptides would have a direct path to clinical work.

Finally, the choice of bioactive factor incorporated could be changed to increase the activity of the matrix. One potential application would be to incorporate heparin binding domains which could be used to sequester heparin binding growth factors into the matrix through a heparin bridge, and this work is already underway (Sakiyama and Hubbell, unpublished results). Additional work could be done on adding factors that interact through different mechanisms (heparin sulfate binding) or from different extracellular matrix proteins, such as collagen or fibronectin. It may also be interesting to apply new formulations of these materials that contain both adhesive factors as well as soluble growth factors. Finally, there is a potential application to the repair of lesions in the central nervous system.

Another area that can be explored is the application of these materials to different clinical models. While the materials described in this dissertation have been tailored to enhance peripheral nerve regeneration, it is possible to incorporate bioactive factors that specifically interact with other cell types, leading to a wide variety of applications that extend well beyond the field of neuroscience. Other areas of application outside of neuroscience include the use as a matrix for enhancement of skin regeneration, use as a filler material to fix bone defects, repair of cartilage or any of the numerous sealant applications for which fibrin glue is presently used. In many of these applications, unmodified fibrin glue is presently used clinically as a suture or sealant, and it may be possible to enzymatically modify the fibrin to create a more inducive environment.

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