CHAPTER 12

Fibronectin, Collagen, Fibrin - Components of Extracellular Matrix for Nerve regeneration

A. Alovskaya*, T. Alekseeva, J.B. Phillips, V. King and R. Brown

Summary

There are key differences in the extent to which the peripheral (PNS) and central (CNS) nervous systems recover function following damage. In each case there is a balance between factors that inhibit and promote neuronal regeneration. In the CNS this balance is skewed toward inhibition while in the PNS it is skewed towards promotion of neuronal growth. Following damage the CNS environment is generally hostile to neuronal growth. However, axonal regeneration does occur under certain conditions. In this review, various strategies for promotion of neuronal growth are explored including the use of tissue engineered grafts incorporating extracellular matrix proteins, synthetic materials, electrically active materials, coupled with biomolecular and cellular – based strategies. Development of biosynthetic conduits carrying extracellular matrix molecules and cells (Schwann cells, olfactory ensheathing glia or stem cells) expressing neurotrophic growth factors represents a novel and promising strategy for spinal cord and peripheral nerve repair. Native matrix scaffolds (e.g. collagen, fibrin, fibronectin) have been produced with appropriate biomimetic 3D mesoscale structures for improving nervous system repair. The structure, composition, biomechanical properties and effectiveness of such implants in supporting experimental PNS and CNS repair are reviewed and discussed in this chapter.

Key words: Extracellular matrix protein, biodegradable scaffolds, fibronectin, collagen, fibrin, spinal cord, repair

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Introduction

One of the fundamental tenets of neuroscience for many decades has been that the peripheral nervous system (PNS) can regenerate following damage whereas the central nervous system (CNS) lacks this capacity. However, recent clinical research has begun to question the truth of this even though many spinal cord injuries (SCIs) lead to permanent disabilities. When spinal injury is incomplete, involving only part of the cross-section, there is frequently a period of functional improvement [1]. This is seen in many species including humans [2, 3]. A significant part of this spontaneous functional recovery occurs 2-6 months after injury, and is closely linked to intensive rehabilitative treatment. Early studies demonstrated the capacity for functional compensation of descending pathways following injury. Compensatory plasticity of rubrospinal and bulbospinal tracts was found to mediate the recovery of forepaw function following corticospinal tract lesion in cats. Following dorsal column injury in rats, there was spontaneous recovery of "reaching ability" apparently due to compensation by the other sensorimotor pathways [4, 5]. Interest in neural regeneration has heightened with increased public awareness of issues surrounding patients with spinal cord injury and Parkinson's disease. This interest has stimulated a plethora of basic science research with promising clinical potential. The primary recent areas of interest include nerve repair in the CNS and PNS, the use of axonal guidance channels to improve regeneration, alternatives to conventional nerve grafting and chronic nerve injury. Various strategies for providing growth-promoting signals have been explored. The physical guidance of axons has made use of autologous and nonautologous grafts, materials containing extracellular matrix (ECM) proteins (e.g. silicon tubes filled with laminin, fibronectin and collagen) [6, 7], mats of fibronectin [8-15], synthetic materials (polylactic acid) [16], or electrically active materials such as piezoelectrics [17], and conducting polymers such as polypyrol (pPy) [17, 18].

Biomolecular–based therapies use neurotrophins: nerve growth factors (NGF), brain derived neurotrophic factor and neurotrophin -3 to enhance neuron survival and to promote neurite outgrowth and nerve regeneration [10, 15, 19, 20]. Cellular – based therapies for treating nerve injury often use macrophages to clear debris and glial cells to secrete neurotrophic factors [21, 22]. Development of biosynthetic conduits carrying ECM molecules and cells (Schwann cells, OECs or stem cells) expressing neurotrophic growth factors represents a novel and promising strategy for spinal cord and peripheral nerve repair. Native matrix scaffolds (e.g. collagen, fibrin, fibronectin) have been produced with appropriate biomimetic 3D mesoscale structures for improving nervous system repair. The aim here is to

review the structure, composition, biomechanical properties and effectiveness of such biomimetic implants in supporting experimental PNS and CNS repair. Clearly, though, where the aim is to mimic a biological structure at the microscale, it is useful first to understand the function and interplay of structure in the native tissues.

ECM in the nervous system

In peripheral nerves, flattened, basal lamina-lined perineurial cells [23] delineate the nerve fascicles, providing a barrier to movement of ionic compounds and macromolecules between non-neural tissues and the endoneurium [24]. The most abundant cells of the endoneurium are Schwann cells; fibroblasts constitute roughly 10% of total cells within the endoneurium [25], and are far less common in the CNS. The fibroblasts produce collagens, a family of extracellular structural proteins characterized by a triple α-helical configuration and a high content of hydroxyproline, proline and glycine. Collagens are present in the endoneurium both as fibrils of interstitial collagen and as a non fibrillar component of the basal lamina surrounding the processes of Schwann cells and perineurial cells, in addition to lining the outer aspect of endoneurial capillaries. Whilst the bulk of fibrillar interstitial collagen is synthesized by fibroblasts, Schwann cells and perineurial mesothelial cells secrete the more heavily glycosylated and non-fibrillar type IV collagen of basal lamina. Fibroblasts augment production of interstitial collagen both within a traumatized nerve segment and distal to it. This increases the tensile strength of the damaged nerve and provides the collagenous framework required for axonal ensheathment by Schwann cells but can also impair regeneration where dense scarring occurs [26]. As regeneration proceeds, axonal growth cones in repairing peripheral nerve penetrate the scar and extend into the tubular Schwann cell aggregates [27]. Extracellular accumulation of collagen in the injured PNS contrasts sharply with the astrogliosis and increased intracellular glial fibrillary acidic protein (GFAP) elicited by trauma in the CNS.

The ECM in brain tissue is mainly composed of protein-based aggregates, particularly proteoglycans, with the expression of basement membrane components (type IV collagen, laminins and fibronectin) [28]. Besides acting as a barrier to macromolecules and cells, the basement membrane also provides a substratum for some cell types, supporting spreading, differentiation, and migration [29]. During development, interactions of individual ECM components with specific cell surface molecules (integrin receptors) and proteoglycans

initiate a cascade of signal transduction leading to varied short-term or persistent cellular responses. Fibronectin (FN) is expressed in a very specific spatial and temporal manner important both for neuronal migration and outgrowth during development. It is one of the several ECM components that have been found in the embryonic nervous system [28]. Early in cortical development, FN is found through the telencephalic wall [28], where it is closely associated with cell bodies in the proliferating neuroepithelium and processes of the radial glial cells [28]. The close association of FN with radial glia and with preplate/subplate neurons suggests that one or both of these cell types may be producing FN during cortical development [30]. FN also participates in remodelling of injured brain tissue [29, 31, 32], promoting nerve regeneration and converting quiescent astrocytes to a proliferating phenotype in culture.

Spinal cord anatomy

The human spinal cord is encased in vertebral bone and segmentally organised into four major divisions: cervical (C; 8 segments), thoracic (T; 12 segments), lumbar (L; 5 segments) and sacral (S; 5 segments). Each spinal segment makes connections with discrete body regions through projections running through the sensory and motor spinal roots. An injury to the spinal cord has devastating consequences owing to the disruption of these signals passing between brain and body, resulting in loss of sensation or control of motor function immediately below the injury level. Therefore, the higher the injury is the more severe the debilitation results. For example, injuries occurring at the lumbar level can result in paraplegia, as well as sexual and bladder dysfunction; cervical injuries can result in quadriplegia; and high cervical injuries (such as the injuries of level C2) can impair breathing function and lead to dependence on a ventilator [1].

Spontaneous neuronal regeneration and CNS repair strategies

After an injury to the central nervous system (CNS), neurons are not able to regenerate their axons and most of them die by necrosis or apoptosis. The inability of axons to regrow is a characteristic of the mammalian CNS that was acquired late during evolution [1]. The majority of neurons in the peripheral nervous system (PNS) and the CNS of the lower vertebrates, newts and salamanders, can regenerate after injury. Very young mammals, birds and certain amphibians are also often capable of substantial CNS reparation [33, 34]. This observation suggests that CNS neurons might retain some inherent regenerative capacity.

Indeed, CNS neurons are able to elongate their axons through peripheral nerve grafts and other permissive substrates after injury [35, 36]. The molecular mechanisms involved in the regulation of axon growth are still not fully understood. It is known that the surrounding extracellular matrix (ECM) can promote or inhibit the elongation of neurites. In the PNS, laminin and heparin sulphate proteoglycans, are able to promote axon growth [37]. In contrast, these ECM substitute components do not enhance axon elongation in the injured CNS, suggesting that there may be additional interfering inhibitory systems blocking their action and ability to promote axon growth [38]. Over the past few years several molecules with such blocking effects on axonal growing have been identified, both in the myelin surrounding CNS axons and the glial scar that forms after a CNS injury. The outer myelin membrane contains non-permissive molecules, such as Nogo and myelin associated glycoprotein. Disruption of myelin following injury could expose such normally concealed inhibitory proteins to regenerating axons [33]. Alternatively, the glial scar which forms after CNS injury is known to act as a barrier to growth of new axons. Indeed, it was demonstrated by Davies *et al*. [39] that even PNS neurons, which will grow along normal CNS white matter tracts, cannot cross a glial scar substrate. This strong inhibitory effect is mediated by two distinct species of ECM molecule, tenascins and chondroitin sulphate proteoglycans (CSPGs) [40].

Spinal cord regeneration

Traumatic SCI results in a series of reactive changes. Damage to spinal axons is commonly the most significant factor that determines the extent of injury. The spinal cord is segmentally arranged and the sensory, motor and autonomic functions of each segment depend crucially on connections with supraspinal sites for all conscious or voluntary actions. Damage to these connections leaves spinal segments which are caudal to the lesion site partially or wholly isolated from the neuronal cell bodies. These retract from postsynaptic neurons and undergo Wallerian degeneration and although the proximal segment typically survives, it does not spontaneously regenerate. Damaged axons encounter a series of inhibitory cues within the injured site, which further prevent a successful regenerative response [1].

Strategies aimed at overcoming such inhibitory environmental cues have resulted in significant regeneration of lesioned axons through and beyond sites of damage in models of SCI. These strategies include neutralization by myelin inhibitors [41] and therapeutic degradation of inhibitory components of the glial scar such as CSPGs and Nogo [42, 43]. Axon regeneration after SCI has also been demonstrated following the administration of

growth-promoting molecules, which make the lesion environment more permissive to growth. This includes the provision of exogenous neurotrophic factors [44], [45] or the manipulation of pro-regenerative neuronal signalling pathways, as well as several cellular transplantation strategies such as genetically modified fibroblasts, Schwann cell bridges, OECs and stem cells [1]. Furthermore, combination therapies aimed at targeting multiple factors have also been shown to act synergistically to enhance the extent of axon regeneration after SCI [1].

Scaring and the role of ECM components

In traumatic brain injury and SCI, the lesion scar is comprised of a fibrous scar in the lesion core and a glial scar in the surrounding parenchyma [46-48].

Inhibitory molecules in the glial scar

Although the "glial scar" is a well known pathological hallmark of CNS injury, its formation, molecular composition, and function are only partially understood. Studies in several CNS experimental lesion models demonstrate that scarring is a multicomponent process consisting of glial reactivity, alteration in the ECM and, in some cases, collagen deposition. This reaction is the result of a multicellular response to injury involving astrocytes, microglia, macrophages, oligodendrocyte progenitors, fibroblasts, leptomeningeal cells, and Schwann cells [49-53], which produce molecules inhibitory to axon growth including CSPGs [54-57]. Many CSPG core proteins are up-regulated in the injured CNS [58] and various experiments have shown that the glycosaminoglycan (GAG) chains attached to CSPGs directly inhibit axon growth [59-62]. The enzymatic removal of these chains from the CSPGs improves nerve fibre regeneration *in vivo* after nigrostriatal tract lesions [52] and in the spinal cord after dorsal column transection [42]. The same treatment in the adult visual cortex degrades the CSPGs that form perineuronal nets around neurons and dendrites [63] reactivating their plasticity after the critical period [64].

NG2 is a major component of CSGP expression after SCI, thereby potentially exerting an important role in limiting axonal regeneration. It is a high molecular weight CSPG that was first identified as a cell surface antigen of neural tumour–derived cell lines that had properties intermediate between neurons and glial cells. Adult Schwann cells, which do not express NG2, support the extensive growth of neurites from adult DRG cells. By contrast, Schwann cells transfected to express NG2 on their surfaces are not very supportive of neurite growth and cells extend mainly short branching neurites with stubby brunches [54]. This

suggests that, after injury, centrally projecting axons of damaged sensory neurons would be unable to grow through accumulated NG2. Oligodendrocyte progenitors are among several cell types that react rapidly and express NG2 after injury. Macrophages have also been identified as another cell type which contributes strongly to NG2 deposition after spinal injury [65].

Cellular and molecular composition of the fibrous scar

Traumatic injury of the CNS results in the formation of a collagenous basement membrane-rich fibrous scar in the lesion site. Accumulation of growth inhibitory molecules makes the lesion scar a major impediment for axon outgrowth. The ECM consists of a dense collagen IV meshwork, which serves as a binding matrix for numerous other ECM components and inhibitory molecules such as CSPG and semaphorins, as well as growth-promoting factors. Inhibition of collagen matrix formation in brain and spinal cord lesions leads to permissive axonal regeneration and functional recovery, although collagen IV is not inhibitory to axonal outgrowth [66]. However, successful blockade of collagen type IV and basal lamina formation with 2,2'-bipyridine injections failed to enhance corticospinal axon regeneration or sprouting. These results suggest either that collagen and basal lamina formation after CNS injury do not contribute to corticospinal axonal growth failure or, more likely, that molecules in addition to collagen and basal lamina contribute to axonal growth failure and must be collectively blocked to promote corticospinal regeneration [67].

Tissue engineering for nerve regeneration

Development of biosynthetic conduits carrying extracellular matrix molecules and cell lines expressing neurotrophic growth factors represents a novel and promising strategy for both spinal cord and peripheral nerve repair. Native matrix scaffolds (e.g. collagen, fibrin, hyaluronic acid and fibronectin) have been produced with appropriate biomimetic 3D mesoscale structures for cell/tissue growth. However, limitations in the ability to control assembly of biomimetic structures at this scale, particularly, using native proteins and living cells, has restricted progress. This is partly due to limited knowledge of natural protein polymer 3D assembly, making it necessary to rely on cells to carry out the necessary assembly/remodelling processes. Here, we will focus on work using scaffolds made from endogenous proteins, in particular Type I collagen, fibrin and fibronectin.

Collagen, structure, fibrillogenesis

Collagens comprise a family of proteins (26 molecular species having been identified to date) which share a triple helical structure in the form of an extended rod. This monomer structure is dependent upon a highly characteristic sequence of amino acids, with glycine in every 3rd position and a high density of proline and hydroxyproline. This structure also contributes to the key functional property for structural collagens, namely aggregation into fibrils (fibrillogenesis) [68].

The self assembly of fibril-forming collagen types such as type I are similar to that occurring in other aggregating proteins. Loss of solvent molecules from the surface of the protein results in circular cross-section assemblies, with minimum surface area/volume ratio. Fibril-forming collagens are synthesized as soluble procollagens, which are converted into collagens by specific enzymatic cleavage of terminal propeptides by the procollagen metalloproteinases. Without these proteinases the aggregation of collagen fibrils would not occur. Collagen fibrils can be formed *in vitro* from acid soluble collagen in cell-free systems, usually generating unipolar, D- periodic nano-fibril aggregates [68]. The most important non-fibril forming collagen type is type IV, the principal component of basement membrane. This has been used in crude form, as a scaffold for neuronal growth in the form of matrigel, though this is rich in a wide range of other basement membrane components [69]. The application of type IV collagen as a nerve growth substrate is based on its interaction with Schwann cells [70] though this is a feature associated with mature, functional nerve structures rather than the transient scaffold which cells utilise during repair.

Collagen implants for tissue repair

Collagen, principally type I constituting approximately 30% of mammalian musculoskeletal tissues, is one of the oldest natural polymers to be used as a biomaterial. Collagen is used for tissue repair in two general ways: the first follows a “top-down approach” derived from whole connective tissue producing a complex collagen-rich matrix with some features of the required architecture. Commonly this scaffold is fabricated from cadaver or animal tissues from which all cells have been removed. The second is a “bottom-up approach” in which purified collagen is aggregated and shaped into scaffolds for cell delivery [71]. There are

three important groups of devices based on collagen scaffolds with distinct 3D microstructure: fibrous constructs, hydrogels and foams [71].

Hydrogels

Neutralisation of native acid soluble collagen generates a hydrogel scaffold within a few minutes by collagen fibrillogenesis. These gels represent very low density three dimensional (3D) lattices of collagen nano-fibrils (approx. 0.2 to 0.5% of wet weight) with no inherent orientation, containing a large excess (>98%) of fluid [72, 73]. This lattice also forms a substrate for cells through physical entrapment, producing a cell-seeded collagen gel scaffold. The resident cells form stable integrin-mediated attachments with the fibrils and generate significant contractile forces, eventually leading to limited compaction (contraction) of collagen hydrogel [7]. When a rectangular collagen lattice is tethered at its opposite ends the cell force generates a uniaxial strain in the gel along which cells and collagen fibrils become aligned [74, 75]. Such aligned, fibrilar/cellular collagen hydrogel implants have been used successfully to guide peripheral nerve repair *in vivo* [7].

Several investigators, with varying success, have applied collagen as a nerve guide tube for peripheral nerve [76] and spinal cord reconstruction [3]. Injecting collagen hydrogel into a rat model of spinal cord injury resulted in very dense collagen hydrogel with large cavities one week post-surgery. These dense areas were generally devoid of axonal growth or cellular infiltration. By four weeks, these dense areas had expanded to occupy most of the injection site [77].

It seems likely that neuronal regeneration is improved in terms of 3D organisation when the filling ECM provides some forms of micro or nano fibrillar alignment along the tube axis. Contact guidance of growth cones at neurite tips in response to the aligned fibres causes neurite projection to be axially directed, leading to improved structural alignment of regenerated axons. Alternative approaches to alignment of collagen fibrils have used high strength magnetic fields [78, 79], microwave irradiation for cross-linking and aligned collagen [80].

Plastic compressed collagen

More recently, a versatile new technique for the manufacture of fibrillar collagen scaffolds with improved mechanical properties and matrix density has been developed. This process known as plastic compression (PC) uses fibrillar collagen type I hydrogels as a start point [73]. The PC process involves rapid and controlled expulsion of much of the fluid trapped in

the hydrogel by unconfined compression. This results in fibrillar collagen sheets with functional mechanical properties [71, 73]. This process is versatile in terms of the volumes, densities and shapes that can be produced, with the 40 μm thick compressed sheets spiralled into 3 D multilayered conduits. Not only can these be easily layered to create a wide range of structures but resident cells survive the compression process to produce cell-populated scaffolds. The speed and control of typical PC fabrication offers the potential for tissue engineered implants which are made "at the bedside" for surgical repair[73] .

Fibrous scaffolds

These scaffolds rely on extracting insoluble collagen fibres from native matrix, then reassembling the fibres into scaffolds with the desired properties. A simple example is the nerve repair conduit made from a bundle of approximately 4000 type I collagen filaments [81]. Such constructs, prepared from decellularized collagen-rich and cross-linked bovine skin are now available commercially (Koken Co, Ltd, Japan) [81].

Nerve guide material made from fibronectin.

Fibronectin: structure, properties

Fibronectin (Fn) is a disulfide – linked dimeric glycoprotein prominent in many extracellular matrices and present at about 300 μg/ml in plasma. Its interactions with collagen, heparin, fibrin and cell surface receptors of the integrin family are involved in many processes including cell adhesion, morphology, migration, thrombosis and embryonic differentiation.

Fibronectin is composed of tandem repeats of three distinct types (I, II and III) of individually folded modules (Fig. 1). Fibronectin type III modules (FNIII) contain binding sites for several membrane receptors and ECM components that play a role in the assembly of the ECM. For example, it is now well established that some of the critical interactions are the binding of ^{10}FNIII to integrins; the binding of ^{1}FNIII to other fibronectin molecules and the binding of $^{12\text{-}14}$FNIII to heparin/heparan – a common proteoglycan component. The ^{10}FNIII region contains the integrin binding site defined by the RGD sequence. Deformation of this module by a mechanical force is predicted to affect the binding affinity for integrins [82].

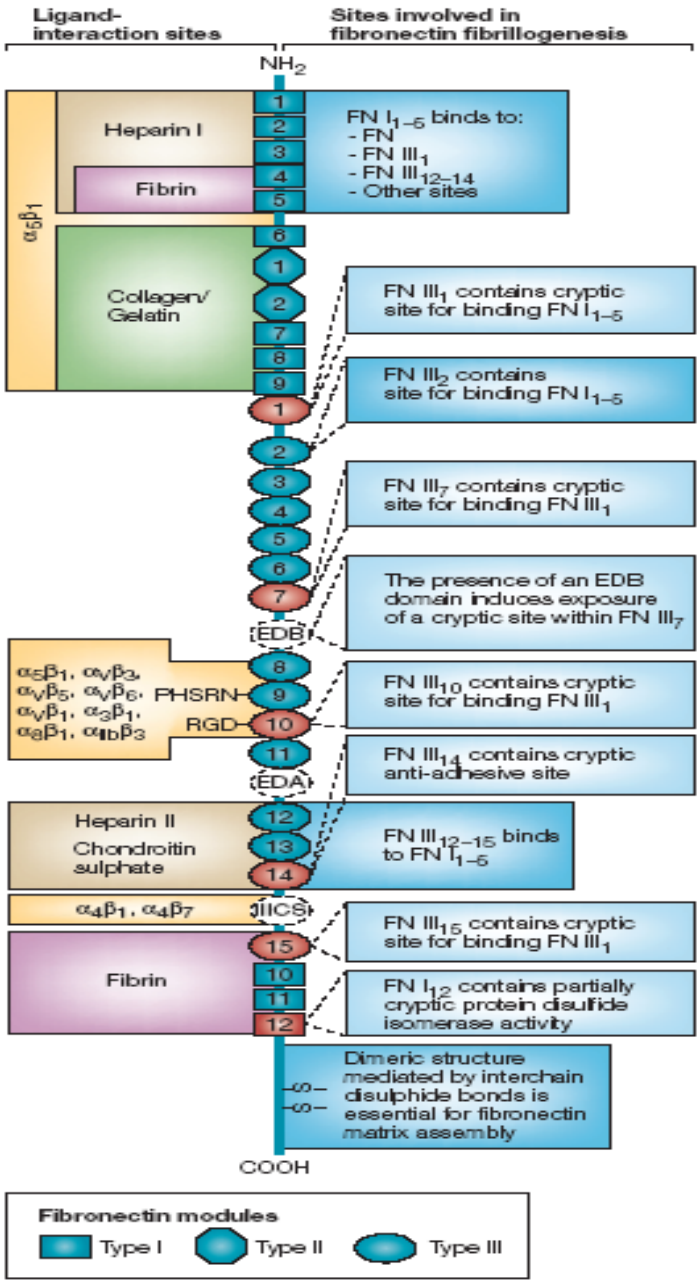

Fig. 1. Ligand-binding regions and interaction sites of fibronectin. Fibronectin (Fn) is composed of three repeating units, type I (rectangles 1-12), type II (octagons 1-2) and type III (ovals 1-15). Dotted ovals indicate units in which alternative splicing of messenger RNA insert type III modules termed ED-A (EDA) and ED-B (EDB), or portions of the variable IIICS region. The labels along the right side of the figure indicate exposed or cryptic self interaction sites involved in fibronectin fibrillogenesis. Modules reported to contain cryptic fibronectin-fibronectin interaction sites are coloured red. The labels at the left indicate regions involved in binding interactions with different members of the integrin family or other ECM molecules. The primary adhesive recognition (RGD) and synergy (PHSRN) sequences within the central cell-binding region of fibronectin molecules are also indicated. (Reproduced with kind permission from Macmillan Publishers Ltd: Nature Reviews. Molecular Cell Biology. (2001) V 2, 793-805 [83]).

The precise mechanism by which fibronectin fibrillogenesis occurs *in vivo* is not fully understood, but it involves cell contraction and the exposure of self-recognition sites [82, 84]. The repeat domains of fibronectin reversibly unfold in a distinctive sequence under applied tension [85, 86]. Cryptic sites may be revealed under certain mechanical conditions as cellular activity alters the tension applied to fibronectin molecules [87]. These cryptic sites include additional sequences which bind integrin receptors on cells, other matrix molecules and other fibronectin molecules, as well as anti-adhesive sites [88, 89]. Thus, exposure of cryptic sites by mechanical unfolding may act as a trigger for a variety of cell- matrix interactions.

Fibronectin materials for nerve regeneration

A new contact guidance material was developed made from plasma FN which is aggregated under unidirectional shear force in our Lab in the early 1990s [8, 9, 90, 91]. The shear aggregation process gives a predominant fibre orientation, which is able to orientate cells including fibroblasts [91], neurones [92], macrophages [93] and Schwann cells [8, 94]. Functional fibrous fibronectin biomaterials take the form of mats or cables [91]-[95], which have been developed for use in the repair of peripheral nerves [10] or injured spinal cord [9, 11-14, 96]. In addition to providing guidance substrates to support nerve repair, fibronectin materials have been used as a depot for the supply of soluble factors such as NGF [10], NT3 [15] and antibodies [12] by slow release to sites of nervous system repair.

Assessment of in vitro properties of fibronectin materials

When human dermal fibroblasts and Schwann cells (SCs) were cultured for three weeks on FN-cables attached to plastic culture dishes, cells that were attached to the dish appeared to migrate onto the ends of the cables [8]. Cells also lined up alongside the fibrils and became oriented parallel with the axis of the fibre, apparently by contact guidance. Transverse sections of unattached (free-floating) cables after three weeks in culture revealed a similar picture.

Stabilisation of cables of fibronectin with micromolar concentrations of copper or zinc

Zn^{2+} and Cu^{2+} treatment of FN-cables significantly increased their stability in a standard protein release assay (sterile incubation in PBS at 37°C, 21 days). Assessment of protein dissolution after incorporation of zinc showed a slightly lower level of stabilisation of FN materials, however, treatment of the mats with an equimolar concentrations of copper and zinc ions caused a greater stabilization of FN materials than copper or zinc alone [97]. In cables stabilised with low concentrations of Zn^{2+} or Cu^{2+}, cross-linking appeared to occur between adjacent fibres such that the whole structure became more compact [98].

Schwann cells, but apparently not fibroblasts, were highly sensitive to Cu^{2+}, such that higher doses of Cu^{2+}, (> 10 μM), were toxic to Schwann cells, but not fibroblasts [98]. Schwann cells were able to grow on FN treated with up to 10 μM zinc or copper/zinc [97]. This response suggests a means of focally manipulating materials in order to achieve, e.g. cell segregation, in an engineered construct *in vivo*. Copper, zinc or copper/zinc impregnated cables were able to support three-fold greater Schwann cell accumulation than untreated cables, suggesting a potentially beneficial effect of trace copper ion incorporation in improving repair [97, 98].

Fibronectin mats in spinal repair

FN forms insoluble fibrous aggregates at the rapidly moving interface in an ultrafiltration cell [91, 99], by extrusion of viscous precipitate from a syringe, or by shear aggregation in a viscous gel [9, 99]. This effect was hypothesised to be due to protein extension and elongation under mechanical force (for example, due to fluid shear at a liquid/solid interface in these systems), leading to lateral packing and protein aggregation. SEM revealed that mechanical shear caused the microscopic structure of the surface of the material after dehydration to change from an amorphous to an oriented fibrous structure [9] . Using FN as a substrate in an *in vivo* model of spinal cord repair has shown that the growth of neurites within the material is accompanied by migration of Schwann cells into the graft and the presence of reactive astrocytes at its surface [96]. Within the first two weeks of implantation, a number of cells and cellular elements replaced the FN mat as it dissolved. The first cells to infiltrate FN mats were macrophages (Fig. 2). It was shown that α4/β1, α5/β1 integrin receptors mediate adhesion and stimulate migration along FN mats [100], these receptors being present on macrophages [101], suggesting that FN mats directly stimulate migration of

macrophages. The presence of integrin receptors on Schwann cells suggests a similar mechanism may be responsible for the extensive infiltration of Schwann cells. The other major element that replaced the FN mat within the first month following implantation was laminin, expressed predominantly around blood vessels within FN mats (Fig. 2). The close spatial correspondence between laminin tubules and Schwann cells suggests that these were deposited by the Schwann cells.

At later time points (i.e. 3-4 months) when laminin had largely disappeared from the implant site Schwann cells remained in their myelinating phenotype (Fig. 2). In addition, laminin expression in FN mats appeared as discrete, well oriented laminin tubules, which contrasts with the generally random orientation of laminin expression following spinal cord injury. This suggests that FN mats may organise laminin deposition by Schwann cells in such a manner as to provide an endogenous matrix for axonal guidance. The last cellular elements to infiltrate FN mats were astrocytes (Fig. 2). Glial scarring, which is considered to be one of the primary barriers to CNS axonal regeneration, was present around the grafts but not within the material, indicating that the FN substrate provided a protected environment for neurite extension. By four weeks a glial scar similar to that described in numerous studies of CNS injury [13, 36] had formed around the implant and at later time points (two months post-implantation and later) the astrocytic area had almost completely contracted into the implant site. At this later time point elements such as macrophages and laminin had also largely disappeared. The remaining area was mainly composed of astrocytes intermingled with axons.

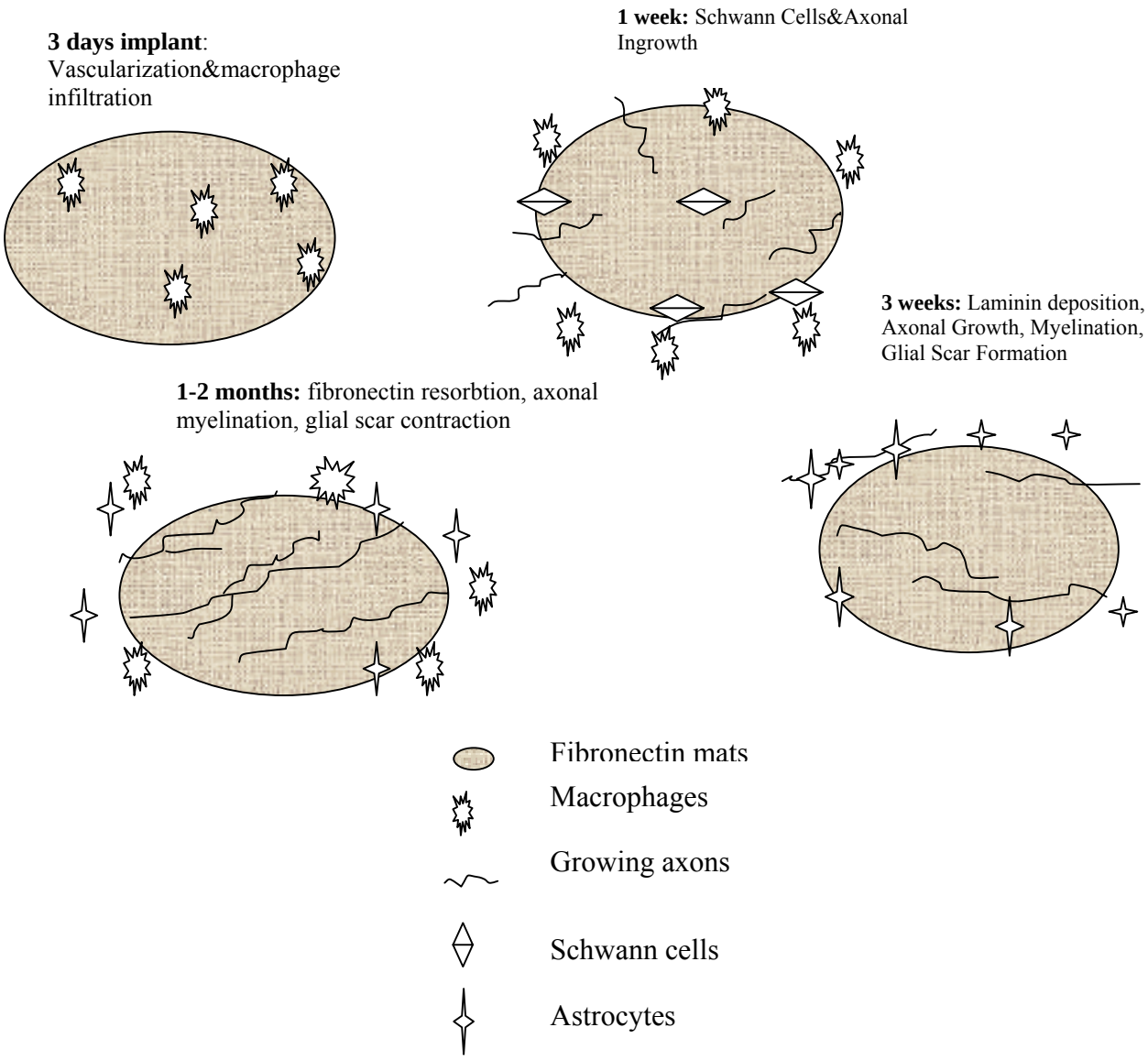

Fig. 2. The time course of cellular and non-cellular infiltrates into FN mats implanted into the spinal cord. By 3 days post-implantation, extremely dense macrophage infiltration as well as beginnings of angiogenesis are seen. By 1 week post-surgery, there are fewer macrophages in the implant site (although still present in large numbers) and extensive ingrowth of axons and non-myelinating Schwann cells. By 4 weeks post-surgery, while there are very few macrophages remaining within the implant site, the degree of axonal and Schwann cell infiltration has increased, with most axons and Schwann cells now being associated with laminin tubules that are now present. In addition, a dense glial scar has formed around the implant. By 3-4 months post surgery, axons remain in the implant site and are myelinated by Schwann cells, although many of the laminin-positive tubules with which they were associated at 4 weeks post-surgery have degraded. In addition, astrocytes have migrated into the implant site.

Fibronectin in spinal repair: Shear aggregated viscous fibronectin

Fibronectin exists in soluble and insoluble forms, the latter as an ECM component. FN matrices can provide migratory tracks during development and facilitate fibroblast migration and proliferation following tissue damage. Soluble forms, perhaps, accumulate in wounds,

opsonize macroaggregate debris for phagocytosis and promote revascularization. It has been shown that FN is secreted and assembled by wound fibroblasts [102], wound astrocytes [103] and endothelial cells [104, 105] into lesion sites after spinal cord injury.

The precise mechanisms by which FN is assembled into fibrillar matrix by cells has yet to be elucidated, but subjecting the molecule to tensile forces both through mechanical shearing at a moving plastic-liquid interface [90, 91] and by attachment to a lipid interface [106] has been shown to result in FN self-assembly. Basic shear mechanics suggest then shear dependent aggregation would be promoted when the viscosity of the solution is increased, enhancing aggregation of the FN monomers into stable aggregates. Such aggregates, used after drying/stabilization, are a good substrate for axonal growth when implanted into the lesioned spinal cord [9, 11] without apparent adverse immune reaction. Extruded viscous shear aggregated fibronectin has been used recently as injectable scaffolds to promote focal regeneration in a model of injured spinal cord. At one week post-injury the injectable fibronectin scaffold had supported strong neuron ingrowth into the lesion site and largely prevented cavity formation (Fig. 3). Immunostaining showed substantial infiltration of axons, Schwann cells, macrophages and blood vessels, and deposition of laminin. By four weeks post-injury, cavity formation, infiltration of axons, Schwann cells and blood vessels into the lesion were substantial. Whilst injectable FN scaffold can have little direct effect in terms of fibre alignment during regeneration, it does support vigorous ingrowth and may reduce post-injury neurite death [77], though direct evidence is not yet available.

Fibrin, fibronectin/fibrin matrices.

Fibrin matrices

Fibrinogen (Fg) has a monomeric molecular weight of 340 kDa and aggregates to form fibrin polymer following thrombin cleavage. Fibrin plays an important role in healing and tissue repair in adults, but less so during embryonic development. As a result, relatively few morphogenetic signals involved in development (adhesion factors or growth factors) interact specifically with fibrin, though they are most active when immobilized in a 3D matrix [104]. A great deal of effort has been placed in understanding the molecular and cell biology of morphogenetic growth factors, but understanding remains limited, particularly in terms of producing 3D scaffolds as effective therapeutics for repair/ regeneration.

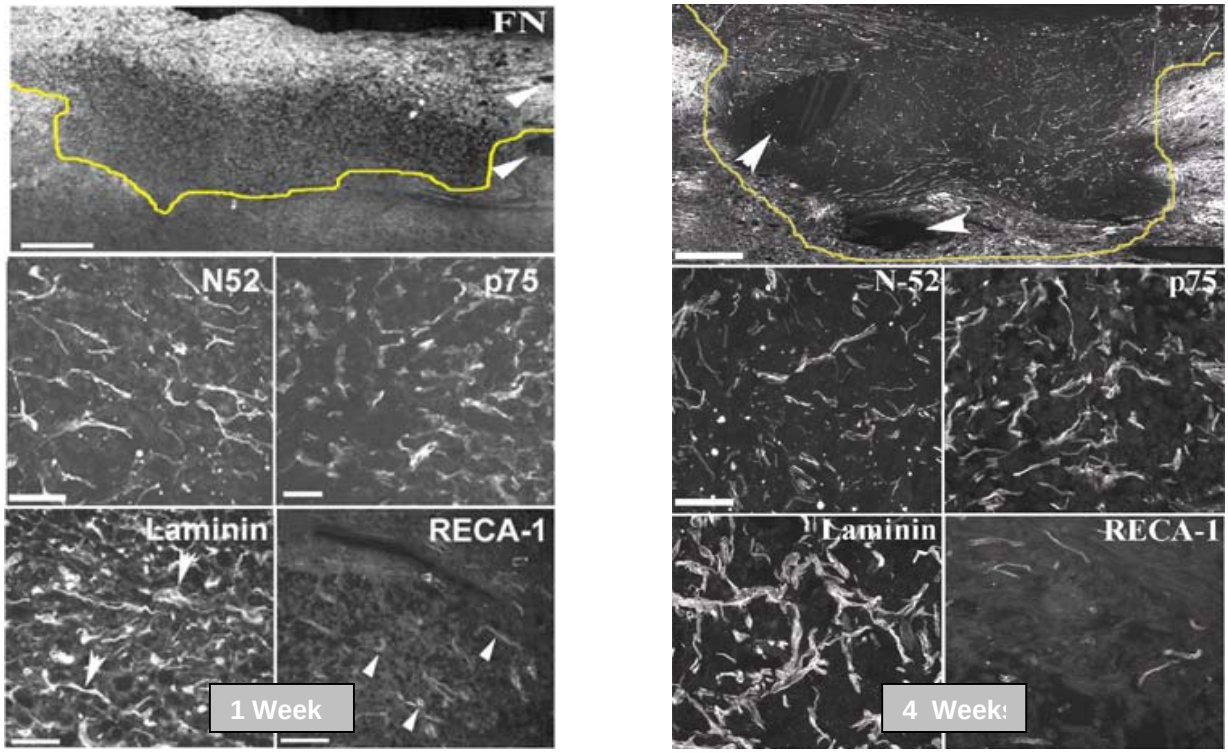

Fig. 3. Implantation of viscous fibronectin into rat spinal cord. Characterization of infiltrates at 1 and 4 weeks post-operations. PGP – 9.5, N52, p75, laminin, RECA – 1 labelling of horizontal sections through implants of 1 and 4 weeks after implantation; *Left panel:* Viscous FN implant (FN) at 1 week post-operation; *Right panel:* Viscous FN implant at 4 weeks post-operation.

Fibrin has been used as a scaffold protein for the immobilisation of adhesion and growth factors [107]. For example, heparin binding growth factors have been incorporated to fibrin scaffolds, bound to heparin, which is itself immobilised via a heparin-binding peptide enzymatically incorporated into the fibrin matrix (Fig. 4) [107]. As an alternative, mutant forms of the adhesion factors and growth factors have been developed, containing a novel protein domain for enzymatic coupling to fibrin and a second domain which is cleft by the enzymes typically produced during cell migration [107]. When mixed with fibrin precursors, these engineered adhesion and growth factors are covalently incorporated into the fibrin matrix by the enzymes involved in coagulation. In either strategy the morphogenetic factors remain tethered to the matrix during cell migration until locally released by cell action, mimicking normal biological mechanisms.

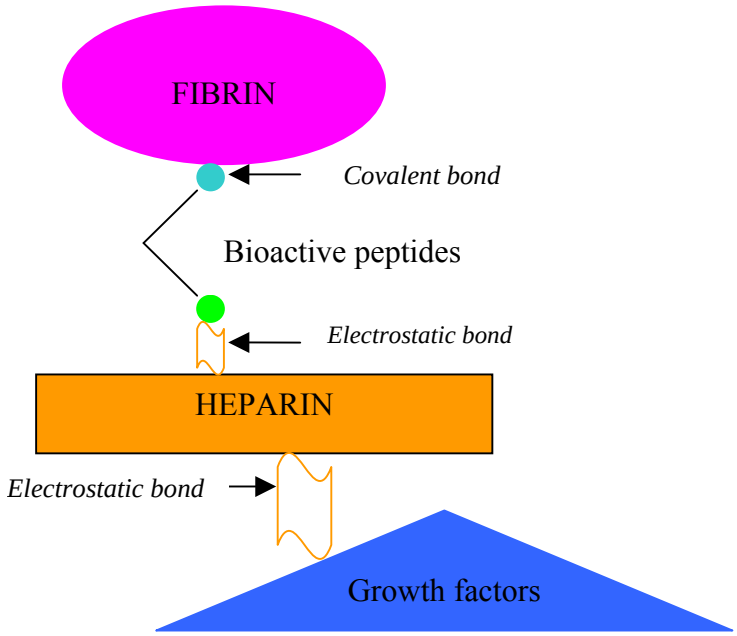

Fig. 4. Fibrin model for immobilization of heparin binding factors.

Fibrin matrices for tissue engineering

Fibrin, unlike collagen or fibronectin, is not associated with mature tissue structure, but rather as a temporary repair stage (early) ECM component. This fibrous matrix has been used in the form of "fibrin glue" as a tool for surgeons. Various kits are commercially available comprising fibrinogen and thrombin which are mixed at the point of use to produce fibrin glue [108]. Most fibrin glue formulations contain significant amounts of fibronectin which may inadvertently enhance cell migration within the fibrin matrix [108]. Fibrin has been used clinically as a scaffold matrix for the delivery of keratinocytes [109, 110], delivering mesenchymal stem cells transfected with growth factors [111], for spinal cord repair [112, 113] and repair of peripheral nerves [114].

Comparison of fibrin matrices and collagen hydrogels *in vitro* showed that after seeding with amnion cells collagen gels contracted substantially whereas fibrin scaffolds did not [115]. In general, matrices of fibrin tend to have greater intrinsic strength and support for cell ingrowth from surrounding tissues than other protein scaffolds [116]. The malleability and easy application of fibrin, coupled with its availability in a clinical form may be driving current research into its application rather than more rational design requirements. Indeed, there have been relatively few attempts to construct implantable fibrin scaffolds with controlled 3D architecture. This is perhaps surprising given the ability to adjust fibrin matrix

micromorphology by varying the fibrin or calcium concentration, or alignment of fibrin fibrils which can guide neuronal growth *in vitro* [78, 117].

Fibronectin/fibrinogen composite cables

Biomaterials containing mixtures of FN and Fg have been made as contact guidance substrates, particularly for repair of peripheral nerve lesions [118]. The composition of these biomaterials can be varied in terms of the ratio of FN to Fg in order to alter the resulting cell support properties [119]. All protein cables significantly increased cell migration *in vitro* compared to cells grown on plain glass substrata. Schwann cells and fibroblasts migrated faster on increasing concentrations of Fg with a peak at ratio of 50:50, Schwann cells migrating 40% faster on 50:50 FN:Fg - cables than on pure FN-cables. This represented an optimum migration substrate since increasing the Fg concentration beyond this point reduced cell velocity. Since the greatest reduction in cell velocity was seen for Schwann cells, it is suggested that Schwann cell attachment and subsequent migration is more sensitive to blocking by Fg. Fg has been shown to induce adhesion and spreading of human endothelial cells *in vitro* [120] and human dermal fibroblasts [121]. Maximum cell migration velocity has been mathematically predicted to occur at an intermediate adhesion balance, at which the cells can form new attachments at the cell leading edge but are still able to break attachment at the tailing edge rear [119].

FN/Fg composition has been due to prompt infiltration of neuronal cells, blood vessels, synapse formation and helps to reduce neural death in the rat spinal cord lesion model. At one week post-injury the FN/Fg gel supported strong regeneration into the lesion site, with little cavitations in contrast to gels of fibronectin alone and collagen [77]. Macrophage infiltration in the surrounding intact tissue was no more than that seen following injury alone. The results indicate that injectable FN/Fg gels provide a useful material for regeneration of the spinal cord and supported better neural structure regeneration than injectable collagen or fibronectin alone.

Discussion and conclusions

At the present time it is clear that different scaffolds made from native ECM proteins (collagen, fibronectin and fibrin) have the potential to improve the survival and repair of

damaged nerves and to some extent to replace lost structure. Implantation of such biomaterials provides a permissive environment that supports the growth of neurons in models of peripheral nerve and spinal cord repair. The ability of different polymer constructs to fill spinal cord lesions (e.g. cavities following crush injuries) or to bridge peripheral nerve gaps at injury sites depends in part on their mechanical properties. Thus, fibronectin based materials are more effective in models of brain and spinal cord injury, whereas, collagen has proved to be more effective in peripheral nerve injury models.

Successful reparation of the injured spinal cord should result in re-innervation of target regions but this is inhibited by non permissive molecules (e.g. CSPGs, Nogo, tenanscin and other) represented by the glial scar that grows around a spinal lesion site. Implants based on ECM are able to create a permissive environment in the injury site so that axons can enter the implant, but it is a major problem to encourage robust growth of axons beyond this permissive environment and produce reintegration into the host CNS. Implantation of FN/Fg blend biomaterials: suppresses cavitations, increases the opportunity for regenerating axons to re-enter the intact spinal cord by increasing implant to intact tissue interface, limits the extent of reactive astrocytosis and demonstrates neuroprotective properties in models of brain and spinal cord injury. These represent considerable benefits for repair particularly in relatively intact crush injury sites. Several strategies are currently used to overcome the inhibitory environment in models of CNS and PNS injures. These strategies include neutralization by myelin inhibitors and therapeutic degradation of inhibitory components of the glial scar such as CSPGs and Nogo. Axon regeneration after SCI has also been demonstrated following the administration of growth-promoting molecules, which make the lesion environment more permissive to growth. This includes the provision of exogenous neurotrophic factors, or the manipulation of pro-regenerative neuronal signalling pathways. Cellular transplantation strategies such as genetically modified fibroblasts, Schwann cell bridges, OECs and stem cells show great potentially by direct cell action in the repair process or indirectly by provision of physiological levels of growth and neurotrophic factors over a prolonged period. Regenerations of peripheral nerve across short and long gaps can be enhanced by introducing multilayer construct consisting of collagen, fibronectin, fibrin and other proteins in spatially distinct zones or layers. Such combination therapies aimed at tackling different needs at different stages or sites of the injury have also been shown to act synergistically to enhance the extent of whole nerve regeneration.

We conclude that, successful nerve regeneration is likely to be achieved where tissue engineered scaffolds provide not only mechanical support (mimicking connective tissue

elements) and directional guidance for growing neurites, but prevent ingrowth of scar tissue and provide biological signals (neurotrophic factors or cells) to speed up and direct axonal growth. In other words, the next generation of neural regeneration guides will need to incorporate a number of "packets" of control information to act on repair cells at distinct points of space (micro zones) and time after injury. Importantly, many of the properties of protein based scaffolds elements, in combination have the basic properties to achieve these subtle central events.

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