Chapter 1

Understanding Molecular Pathology along Injured Spinal Cord Axis: Moving Frontiers toward Effective Neuroprotection and Regeneration

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Abstract

Spinal cord injury (SCI) is a severe, often life threatening, traumatic condition leading to serious neurological dysfunctions. The pathological hallmarks of SCI include inflammation, reactive gliosis, axonal demyelination, neuronal death, and cyst formation. Although much has been learned about the progression of SCI pathology affecting a large number of biochemical cascades and reactions, the roles of proteins involved in these processes are not well understood. Advances in proteomic technologies have made it possible to examine the spinal cord proteome from healthy and experimental animals and disclose a detailed overview on the spatial and temporal regionalization of these secondary processes. Data clearly demonstrated that neurotrophic molecules dominated in the segment above the central lesion, while the proteins associated with necrotic/apoptotic pathways abound the segment below the lesion. This knowledge is extremely important in finding optimal targets and pathways on which complementary neuroprotective and neuroregenerative approaches should be focused on. In terms of neuroprotection, several active substances and cell-based therapy together with biomaterials releasing bioactive substances showed partial improvement of spinal cord injury. However, one of the major challenges is to select specific therapies that can be combined safely and in the appropriate order to provide the maximum value of each individual treatment.

Keywords: spinal cord injury, secondary processes, proteome, biomaterials

1. Introduction

Intensive lifestyle brought about by the modern age of the twenty-first century often brings risks of trauma to the CNS. Both trauma of brain and spinal cord are considered not only as life-threatening conditions, but also as substantial, social, and economic problems that affect mainly the young population. The increased incidence of trauma may be related to popular sports such as ice hockey, American football, rugby, horse riding, and diving, but the most common causes include traffic accidents [1]. Spinal cord trauma accounts for 70% of the total number of CNS injuries.

Many spinal cord (SCI) patients remain permanently paralyzed with complete or partial loss of neurological functions below the site of injury [2]. The most common is paralysis of the body, usually affecting both lower limbs. At the same time, complications may arise when loss of sensitivity, urinary tract control, or the development of spasticity occur in the affected area [3]. Statistics shows that victims are twice as often men as women, with the highest occurrence of cases between the 19 and 40 years of age [4]. Care for patients with injured spinal cord is demanding and often requires lifelong financial costs [4].

The neurological outcomes depend on the range of damaged neuronal populations at the injury site, the level of disconnection of ascending and descending neuronal pathways, the secondary damage (edema, inflammation, and ischemia), and the age-dependent activation of regenerative processes (endogenous production of trophic factors and revascularization). Thus, patients with incomplete injury who retain some sensory or motor function below the lesion, undergo an extensive rehabilitation program to have a better chance of recovering some function. On the contrary, severe spinal cord injury causes a life-lasting disability for which currently no effective therapy is available. Another important factor is age; statistics shows that younger patients have better prognosis of recovery.

Therefore, the main objective of biomedical research is the development of new therapeutic procedures that would contribute to a more effective functional outcome and improvement of the quality of life.

In this chapter, we would like to highlight pathological consequences that could be evaluated by temporal and spatial proteomic analyses, leading to discrimination of the proteome within the entire spinal cord after acute injury. These data will be correlated with delivery of individual neuroprotective and combinatory neuroregenerative strategies for SCI treatment.

2. Pathology

Spinal cord trauma triggers a pathophysiological complex of cellular and molecular reactions leading to edema, hemorrhage, free radical formation, glutamate excitotoxicity, ischemia, macrophage phagocytic activation, glial scar formation, and apoptotic changes in the injured tissue [5]. These processes take place within a few minutes to weeks and years after the injury.

During this time, under the influence of secondary events, small primary damage will spread to the surrounding healthy area within the craniocaudal axis, causing partial or complete loss of physiological functions below the site of injury.

One of the key events of secondary processes is inflammation characterized by fluid accumulation (edema) and the recruitment of immune cells (neutrophils, T-cells, macrophages, and monocytes) [6]. In fact, spinal cord microglial cells normally function as a kind of reactive immune cells that begin to respond to signals after pathological stimuli (injury, infection, or tumors) [7] and are activated at the lesion epicenter [8]. It has been suggested that microglia/macrophages can be polarized into M1-neurotoxic or M2-neuroprotective states and produce a variety of cytokines, chemokines, and neurotrophic factors. However, the mechanisms regulating microglial polarity remain unclear [9].

In addition, not only stimulated microglia/macrophages but also astrocytes, meningeal cells, and fibroblasts together with the increased production of inhibitory chondroitin sulfate proteoglycans (CSPGs) are involved in the spinal cord pathogenesis [10]. Macrophages can alter their phenotypes and functions according to changes in the spinal cord microenvironment during subacute and chronic phases. Thus, SCI triggers an excessive inflammatory response mediated by the invasion of M1/M2 macrophages into and around the central lesion at subacute phase, but not at chronic phase when the formation of glial scar occurs.

2.1. Neuroinflammation

In the CNS, immune cells acquire diverse phenotypes depending on the pathophysiology of the microenvironment.

The inflammatory environment of injured spinal cord contains pro-inflammatory cytokines such as tumor necrosis factor α (TNFα), interleukins IL-1 and IL -6. Anti-inflammatory molecules, like transforming growth factor β1 (TGFβ) and IL-10, are released as well. Immune response in the CNS is mediated by resident microglia and astrocytes, which are innate immune cells without direct counterparts in the periphery.

Among glial cells, microglia are firstly activated and are able to play a bifunctional role. They secrete toxic factors and contribute to tissue damage, but at the same time also release neuroprotective and neurotrophic molecules to allow tissue repair [11]. Interestingly, microglia and astrocytes are able to cross-talk with CNS-infiltrating immune cells, such as neutrophils, T cells, and other components of the innate immune system, as well as with neurons.

Neutrophils are considered as the first inflammatory cells to arrive at the site of injury with a peak at 24 h after injury [12]. They are rapidly mobilized from the bone marrow in response to signals from pro-inflammatory CXC (CXCL8) family chemokines, IL- and cytokine-induced neutrophil chemoattractant 1 (CINC-1) to mediate pleiotropic functions in the immune-inflammatory response [13]. Neutrophils adhere to post-capillary venules 6–12 h post SCI and afterwards they migrate into the lesion site to phagocytose debris [14]. Neutrophils generate their own cytokines after stimulation by pro-inflammatory mediators and produce proteases

via the NF-kB translocation pathway. Phagocytic activity can induce NF-kB activation [15, 16], and other mediators such as matrix metalloproteases (MMPs), and cytokines TNFα, IL-1, IL-8, and TGF-β [17].

Microglia are a unique myeloid cell population, derived from the yolk sac during a narrow time window during development (before vascularization or definitive hematopoiesis) in the embryo. Microglia cells, present in the CNS parenchyma, are sustained by the proliferation of resident progenitors, independently of blood cells.

Their response following pathological stimuli is characterized by an accumulation at the lesion site and the release of various bioactive molecules. Two categories of molecules are released, some are cytotoxic or pro-inflammatory, and others may aid survival and regeneration. Resident monocytes are the first cell types to respond after injury within 1–2 h, which starts the initial acute inflammatory response accompanied by an expression of TNFα and IL-1 (M1 phenotype). This leads to the recruitment of other immune cells. M1 macrophages promote phagocytosis. Eight hours after injury, the production of pro-inflammatory cytokines is terminated, thus promoting the differentiation of macrophages into an anti-inflammatory M2 phenotype with the expression of arginase 1 and a mannose receptor (CD206). M2 macrophages promote angiogenesis and matrix remodeling, while suppressing destructive immunity [18]. The ratio M1/M2 varies in terms of the microenvironment.

These findings correlate with accumulating evidence pointing to a chronological time line expression of different degeneration- and regeneration-associated genes that are involved in the pathogenesis and endogenous repair or plasticity during days to months following SCI.

2.2. Neuro-glial interactions

Microglia activation may be beneficial, deleterious or neutral [8, 9]. Neurons express cell surface glycoproteins (CD22, CD47, CD200, and NCAM) to prevent microglia activation [10, 19]. A relationship between the nervous and immune system has been studied this past decade. Indeed, glial cells (microglia and astrocytes) not only perform supportive and nutritive roles for neurons, but also serve to defend the CNS. On the other hand, excessive and prolonged glial cell activation may result in more severe and chronic neuronal damage, leading to neuroinflammation and neurodegeneration [11, 13].

Neurons are able to control microglia with two types of signals: “On” or “Off” [20]. Off signals (TGF-β, CD22, CX3CL1, neurotransmitters, and CD20) are found in healthy conditions to maintain homeostasis and also restrict microglial activities under inflammatory conditions to prevent damage to healthy tissue. Conversely, “On” signals [CCL21, CXCL10, and MMP3 (from apoptotic neurons)] are produced by damaged and impaired neurons to activate microglia (pro- or anti-inflammatory) [21].

2.3. Glial scar

Glial scar is the accompanying pathological phenomenon of various CNS injuries. The site of injury is infiltrated by macrophages from the bloodstream, fibroblasts, astrocytes, microglia, and oligodendrocytes [8]. Later, precursors of oligodendrocytes and meningeal cells are activated.

Activated astrocytes proliferate and, together with other glial cells, produce a glial scar that encloses the lesion site and prevents the diffusion of ions, neurotransmitters, and other metabolites from damaged tissue into surrounding healthy tissue [22]. This protects undamaged tissue from inflammation and demyelinization, while at the same time, it also prevents regeneration of nerve fibers, which is a serious problem for the treatment of spinal cord injuries. Activated astrocytes reveal thicker projections that intersect with each other and are connected by tight joints. Astrogliosis is accompanied by increased expression of glial fibrillary acidic protein (GFAP), vimentin, and markers for neural precursor cells (Nestin) [23]. In reactive astrocytes, increased synthesis of extracellular matrix protein CSPGs has been reported, which are inhibitory to axon growth itself [23]. Similarly, oligodendroglia, together with meningeal cells migrating into the lesion, form a significant barrier for axonal growth by producing inhibitory molecules (NOGO) and other proteoglycans [24].

2.4. Inhibitory molecules

NOGO inhibitory protein [25, 26], myelin glycoprotein oligodendrocyte (OMGP) [27], myelin-associated glycoprotein (MAG) [28] together with secondary inhibitors, including the large group of chondroitin sulfate proteoglycans (CSPGs), are among the major inhibitory molecules that block axonal regeneration [24, 29]. While blocking the penetration of axons, they contribute to the formation of so-called blind clusters, unable to form functional connections with terminal neurons. These pathological formations often cause painful irritable syndrome [30].

Inhibitory CSPGs are synthesized by neurons and glial cells. They play an important role in the physiological development of the CNS, such as cell migration, maturation, differentiation, survival, and tissue homeostasis, but in the case of disruption of tissue homeostasis, increase their expression and consequently inhibit regeneration [31]. These molecules interact extensively with extracellular matrix components [32], for example, with laminin, fibronectin, tenascin, and collagen [33]. Additionally, they bind to growth factors, midkine, pleiotrophin, fibroblast growth factor [34], or inhibitory growth factors such as semaphorins [19] and contribute to the formation of a glial scar that inhibits regeneration of axons [35]. NG2 glycoprotein, which belongs to the most important inhibitors of the CSPGs group, is produced by oligodendrocytes precursor, meningeal cells and macrophages [36]. Accumulation of NG2 was seen at the site of injury, where it blocks regeneration of the axons [31]. Co-expression of NG2 and PDGF-α receptors in the same population of CNS cells confirmed its specific expression in oligodendrocyte precursors [37]. NG2-positive oligodendrocyte precursor cells are often the first cells to respond to injury. Unlike microglia, reactive oligodendrocyte changes are local and occur only in the immediate vicinity of the injury. Previous experiments confirm the initiation of spontaneous regeneration in SCI, as reflected by the incidence of GAP-43-positive axons. They were found in the segments above the lesion at first week [38]. In the central lesion, which forms a mechanical and chemical barrier, the inhibitory proteoglycan NG2 was significantly enhanced [39]. Immunohistochemical analyses using specific NG2/GAP-43 antibody confirmed that increased accumulation of NG2-positive cells at the central injury creates a barrier for successful diffusion and further ingrowth of GAP-43-labeled axonal fibers at acute phase [38]. Sequential administration of ChABC enzyme caused degradation of NG2 glycoprotein, which modified the extracellular matrix and created a tolerant environment for longer term recovery (2–3 weeks).

2.5. Neuropathological consequences based on proteomic analyses

Based on the recent analyses of SCI pathological processes, it seems that complex changes in gene and protein expression as well as in cellular interactions are taking place not only at the central lesion but also in adjacent segments. However, the exact mechanisms by which proteins involved during inflammation, recruitment and microglia activation, glial scaring, remyelination, or axonal growth function remain to be further explored [5, 10, 21, 35]. Therefore, understanding of the molecular cross-talk occurring between cells at the lesion site and in the adjacent segments needs to be further investigated [21]. In particular, studies that are able to take into account both spatial and temporal data may identify interesting molecular targets [40]. Such an investigation could be performed by a **proteomics approach**, which can be connected to cellular and physiological studies as well as to a global regeneration-activated gene (RAG) investigation. Mass spectrometry (MS) plays a central role among proteomics approaches. Several developments allow fast identification of lower abundance proteins such as cytokines and chemokines [41]. Furthermore, MS is highly used in neuroscience to discover biomarker candidates and also to study the differential expression of proteins at any given time in a proteome and they are then compared with the pattern of those from healthy ones.

Thus, to better understand the pathology based on secondary injury processes and plasticity, it is necessary to analyze entire spinal cord tissues in time, thus collecting tissues from the epicenter and both adjacent segments above (rostral) and below (caudal) the lesion firstly in acute, and afterwards in chronic SCI experimental models, expecting the release of different molecules. They will most likely reflect pathology *in situ*, at each specific segment, which may contribute to the final view of ascending or descending pathway disruption resulting in aggravation of clinical symptoms [41].

Nowadays, we can count on innovative proteomics technologies that can screen, identify image lipids and peptides in each spinal cord segment-derived conditioned medium (CM), or in the spinal cord tissue obtained *in vitro*, to better understand protein composition changes along the rostro-caudal axis after SCI with time in SCI.

Recently, application of shotgun proteomic analysis and label-free quantification to conditioned medium from the injured spinal cord (CM) identified chemokines (CXCL1; CXCL2; CXCL7, CCL2, CCL3, CCL22, CLCF1, and EMAP II) and neurotrophic factors (TGF, FGF-1, PDGF, and FGF1) in the lesion and rostral segments. These molecules are known to have immune-modulator and neurotrophic properties and ability to polarize macrophages/microglial cells into the M2 phenotype [10].

Chemokines are the most important molecules released immediately after SCI. Specific chemokines (CXCL1, CXCL2, CXCL3, CXCL5, CXCL7, CCL3, CCL20, and IL6) that are secreted by macrophages or epithelial cells after injury have the ability to attract neutrophils and lymphocytes, activate inflammation and stimulate extracellular matrix synthesis and tissue remodeling. Recent data showed that the cytokine profile changes in time between the segment above and below the lesion. This is in line with the hypothesis that immune cells that are attracted along the spinal cord upon injury insult are quite different between rostral (R1) and caudal (C1) segments in time. Recently, using proteomic analysis it has been documented that specific immune cells initially migrate toward R1 and then C1 segment [41]. In line with this,

IL6 and CCL20, which are known to attract T regulator lymphocytes through CCR6 binding, were expressed firstly in R1 at 3 days after SCI and secondly appeared in C1 at 7 days [40]. Furthermore, results from proteomic analysis were re-confirmed with cytokine/chemokine arrays and correlated with immunohistochemistry for neutrophils and Tregs. These experiments confirmed that neutrophils were abundantly detected in both R1 and C1 segments with a peak reached 3 days after SCI without any differences in terms of amount between each segment. However, their level decreased in time. In comparison, Tregs were present 3 days after SCI, in higher amounts in the rostral segment than in the caudal one. Their levels peak at 7 days for both segments and then decrease at 10 days [40]. These data are in line with the presence of CXCL1, CXCL3, CXCL5, CCL20, TIMP-1, and IL6 in R1 at 3 days, which are known to attract neutrophils and lymphocytes. In C1, a delay was observed in the recruitment of the Tregs, which were detected 7 days after SCI and correlated with the detection of CCL20 in C1 only at 7 days, whereas neutrophils and microglial cells were already present at 3 days [40]. Taken together, the results showed that C1 is clearly different from R1 in terms of cell types and molecular content in a time course manner, and is revealed to be a target segment for therapy.

The functionality of chemokine released from injured spinal cord tissue can be evaluated by chemotaxis assay, thus investigating the BV2 (microglial) cells activation, followed by Western blot, and M1/M2 polarization through CX3CR1 and CD206 expression.

In vitro chemotaxis assays confirmed that BV2 cells were highly responsive to the cytokine cocktail present in the CM from lesion and rostral sites, compared to CM from the caudal site after SCI. Interestingly, the BV2 migratory potency induced by CM derived from rostral and lesion segments was 37-fold higher compared to the ATP or LPS stimulations that increase their migration by close to 3-fold, due to the specific factors found in the complex CM [41, 42]. Furthermore, immunocytochemical studies prove that activated BV2 cells exposed to CM from the rostral segment overexpressed the CX3CR1 receptor, known to correspond with the M2 profile. This finding was strengthened by Western blot analysis and lack of labeling with C2KR, an M1 receptor [41]. These data together with *in vivo* CX3CR1 expression were in close coherence with published transcriptomic experiments showing that in the injured spinal cord, M2 gene expression is transiently expressed during 7 days after injury, while the M1 gene expression is maintained for up to 1 month [43].

Spatio-temporal proteomic analysis of spinal cord tissue between 3 and 10 days after injury provide clear evidence of regionalization between the rostral and caudal axes, with an expression of neurotrophic and immune modulatory factors in the rostral region, in contrast to inflammatory and apoptotic molecules in the caudal region.

Neutrophic factors were found at 3 and 7 days after injury and disappeared at 10 days. They were replaced by synaptogenesis factors reflecting the fact that a neurorepair process is taking place in the rostral segment after 10 days. In fact, more neurotrophic factors have been detected in the lesion and rostral parts, i.e., CTGF (connective tissue growth factor), NOV (Protein NOV homolog), PIGF (placenta growth factor), FGF-1 (fibroblast growth factor 1), BMP 2 or BMP3 (bone morphogenetic proteins (2 or 3), NGF, PGF, TGF beta (1–3) (transforming growth factor beta), periostin, GAP-43, neurotrimin, neurofascin, and hepatocyte growth factor-regulated tyrosine kinase substrate (HGS). In addition, molecules involved in neuronal development/differentiation/neuronal migration, i.e., CRIP1 (cysteine-rich protein 1), DRP-5 (dihydropyrimidinase-related

protein 5), Negr1 (neuronal growth regulator 1), NCAN (neurocan core protein), CD44, Wnt8, syndecan-4, nexin, and Bcl-2, were identified. Specific factors involved in immune cell chemotaxis or cellular adhesion, including complement factors (C1qb, C1qc, factor D, factor I, and CD59), tetraspanins (CD9 and CD82), and CD14 have also been characterized [40, 41].

In contrast, proteins produced in the caudal region were related to necrosis factors (BAX, BAD, Caspase 6, and neogenin), cytoskeleton proteins, synaptic vesicle exocytosis, chemoattractant factors, and neuronal postsynaptic density.

These data are in line with our previous *in vivo* results demonstrating that neurite outgrowth takes place from rostral to lesion but never in the opposite direction from caudal to the lesion. Furthermore, the presence of chemokines, lectins, and growth factors in the rostral but not in the caudal segment clearly document the immediate inflammatory response together with activity-dependent factors released by neurons and glia.

In order to investigate the neurotrophic role of CM derived from the injured tissue, studies testing neurite outgrowth in rat DRG explants have been undertaken. Data from these experiments confirmed that enhanced neurite sprouting of DRGs facilitated by CM from rostral and lesion segments were most likely mediated by the content of neurotrophic factors, i.e., FGF-1, NGF, PGF, BMP 2 or BMP3, GAP-43, neurotrimin, neurofascin, and other molecules involved in neuronal development/differentiation/migration. Although the principal role of NGF/TrkA pathways in sensory axon outgrowth has been widely demonstrated, other neurotrophic factors including the BMPs (members of the TGFβ superfamily) or GAP-43 have to be taken into account [41, 44].

In summary, it has been demonstrated that few days after SCI, a clear regionalization occurs between the rostral and caudal axes, with expression of neurotrophic and immunomodulatory factors in the rostral region, in contrast to inflammatory and apoptotic molecules in the caudal region. These data indicate the importance of stimulating neurite sprouting at segments below the lesion by inhibiting inflammation and turning polarization of M1 cells to the M2 state, which could have a clear impact on neurorepair. Therefore, these findings should be taken into account when planning new treatment strategies.

3. Neuroprotection in the CNS

Neuroprotection is defined as a curative strategy against harmful biochemical and molecular lesions that, if left untreated, lead to CNS damage [29]. The main purpose is to protect the damaged area by modifying the pathophysiological cascade with the limitation of harmful processes at secondary damage. In particular, the objective is to save those cell populations that are not directly affected by the injury, but due to secondary processes will underlay delayed apoptosis [45, 46]. In this regard, the primary goal is to suppress secondary inflammatory processes, edema and hemorrhage, and excitotoxicity that expand from the lesion center above and below the lesion site and acts destructively on healthy cells. Neuroprotection is among the specific therapies used in CNS injuries [47].

One of the important concepts that have recently resonated is the use of neuroprotective strategies that are applied to the spinal cord in conjunction with clinically proven operative methods

of decompression and reconstruction of the spine. This clearly indicates that early intervention on traumatic spinal cord injuries can significantly affect the prognosis of the disease [3]. Therefore, great attention has been paid to studies that deal with the optimal timing of surgical procedures for acute spinal cord injury [48]. Previous data suggest that patients undergoing surgical decompression within 24 h after spinal cord injury have a significantly better recovery prognosis [29]. Currently, a number of innovative neuroprotective strategies for acute spinal cord injuries are being tested and evaluated in randomized controlled trials. Experimental studies on animal models showing promising results, such as ChABC, minocycline, riluzole, granulocyte colony stimulating factor (G-CSF), are now being tested in clinical studies [2, 49]. Hypothermia induced by intravascular cooling infusion administered epidural or subcutaneously has achieved success during acute SCI treatment.

3.1. Pharmacotherapy

Pharmacotherapy is one of the most widespread forms of treating secondary damage that use a wide variety of different types of molecules to target specific secondary processes. These are comprised of anti-inflammatory or neurostimulating compounds such as, minocycline, neurotrophic factors (BDNF, GDNF, NGF, and erythropoietin), and molecules that alleviate regenerating axons from the inhibitory effects of extracellular matrix molecules.

In particular, chondroitinase ABC eliminate CSPG with the major component NG2 which inhibits the regeneration of damaged axons [50]. Nogo-neutralizing antibodies or blockers of the post-receptors components RhoA, are used to improve long-distance axon regeneration and sprouting [25]. Previous studies have identified Rho pathway as important to control the neuronal response after CNS injury. Therefore, a drug called Cethrin® that blocks activation of Rho is actually in phase I/IIa of clinical trials [48]. The most encouraging findings were observed in patients with cervical SCI, whereas patients with injuries at thoracic level received only modest neurological recovery. Although the patient numbers were small in this trial, the results obtained indicate some evidence of efficacy to enhance functional recovery and warrant further clinical trials [51].

3.2. Molecular therapies: chondroitinase ABC, minocycline, tacrolimus, riluzole

Chondroitinase ABC is a bacterial enzyme that reduces the inhibitory effect of CSPGs at the site of injury. In order to increase CNS regeneration, only chondroitinase ABC purified from Proteus vulgaris [52] should be delivered. The mechanism of action lies in removing GAG chains from the nuclear protein and converting them to unsaturated disaccharides [34]. These stimulate the release of growth factors and proteins attached to GAGs of CSPGs, thereby enabling their diffusion and interaction with neural cell receptors. ChABC has been shown to promote neuroprotection and neuroregeneration [53]. Experimental administration of ChABC after cervical SCI positively affects the branching of damaged and intact descending pathways around which increased accumulation of CSPGs and then inhibition of axonal growth occurs. The neuroprotective effect of ChABC has been described also for the hemisection of the spinal cord [50, 54], transection of dorsal columns [55], and after compression injury of the thoracic spinal cord and the peripheral nerve [56] or in adult rats with visual deficits [57].ChABC administration is often

combined with other therapeutic elements such as LiCl or Schwann cell transplantation [58] that can trigger regeneration. On the other hand, axonal plasticity supported by histological analyses did not correlate with motor function improvements of hind limbs. A similar conclusion was obtained by a group led by Cafferty [59]. There are several explanations for the negative correlation between the growths of axons without functional enhancements.

3.2.1. Orientation and quantity of functional synaptic connections

Functional recovery is dependent on correct orientation of axons and their functional links to the target structure. In some studies, linearly oriented as well as disordered nerve fibers that regrow through the lesion in different directions have been observed. Theoretically, they might increase the plasticity of tissues, because they cover a broader area. On the other hand, disorganized nerve fibers are often losing functional links with the target structure [60].

3.2.2. The time required for the maturation of functional linkages

Another possible negative factor that influenced clinical outcome may be the short-term survival of experimental animals required for functional contact formations. The intensive regeneration process in human patients progresses for months or years, and it is therefore necessary to prolong the length of survival in experimental animals from 3 to 6 months.

3.2.3. Method of ChABC administration

The important factors that affect the efficiency of ChABC therapy are: (i) method of local delivery, (ii) dose, (iii) timing of therapy, and (iv) efficacy of ChABC, since it is a bacterial enzyme which loses its activity *in vivo*. Therefore, to ensure its activity, repeated intrathecal delivery of ChABC or thermostable ChABC should be considered.

The undesirable effects of ChABC delivery have been observed only in rare cases. They are often related to the immune response against the enzyme or to neoepitopes (cleavage products) that this bacterial enzyme forms. Despite the rare negative effects, ChABC broadly reorganizes extracellular matrix, changes cell adhesion and tissue diffusion, and stimulates the functional recovery of damaged CNS [38].

In summary, the results confirmed that early reduction of NG2 allows extracellular matrix reorganization, creating a favorable environment for the initial neuroprotective processes to enable significant regrowth of injured axons in the epicenter of damage. Experimental studies also demonstrate that in order to achieve a better neurological outcome, ChABC needs to be combined with other therapeutic approaches. These may increase the plasticity of the injured tissue, create an environment for the axon outgrowth of fibers, and navigate these fibers to the right direction for the creation of fully functional synaptic connections.

Minocycline is a second-generation, semisynthetic tetracycline that has been commonly used in the treatment of acne vulgaris in children, because of its antibiotic properties against both gram-positive and gram-negative bacteria. However, it has been shown that minocycline can exert a variety of biological actions that are independent of their anti-microbial activity, including anti-inflammatory and anti-apoptotic activities, inhibition of proteolysis, angiogenesis, and tumor metastasis. Minocycline reveals high lipid solubility [61] and therefore easily

crosses the blood–brain barrier [62]. This drug has been shown to be beneficial in various experimental animal models of CNS diseases. Primary mechanisms of action lie on the inhibition of microglia activation, which would justify its potential effectiveness in the treatment of neuroinflammatory and/or neurodegenerative disorders [63]. Different *in vitro* studies have described minocycline's ability to block LPS-stimulated inflammatory cytokine secretion and Toll-like-receptor (TLR)-2 surface expression in the BV-2 cell line and on primary microglia isolated from the brains of adult mice. Minocycline also attenuated the mRNA expression of inflammatory genes, including IL-6, IL-1β, major histocompatibility complex (MHC) II, and TLR-2. In experimental models of SCI, minocycline delivery significantly improved the function and strength of both hindlimbs, reduced the gross lesion size in the spinal cord, and enhanced axonal sparing. Minocycline-treated rats showed decreased release of cytochrome c from the mitochondria, resulting in markedly enhanced long-term hindlimb locomotion [64]. In traumatic SCI, results [65] showed that both short and long-term treatment with minocycline had a neuroprotective effect on the spinal cord segments located rostral to the injury epicenter. Minocycline has also been shown to improve functional recovery after SCI through the inhibition of pro-nerve growth factor production by microglia, thereby reducing oligodendrocyte death and apoptosis after traumatic SCI. It has been shown to inhibit the expression of p75 neurotrophin receptor and the activation of the Ras homolog gene family, member A (RhoA) after SCI [61]. Furthermore, previous study reported that minocycline might also exert a neuroprotective effect in SCI by inhibiting caspase expression and matrix metalloproteinases [65]. Metalloproteinases belong to a group of proteases that are responsible for the degradation and remodeling of the individual components of the intracellular matrix in normal tissue, and their activity is regulated by endogenous inhibitors. However, many pathological CNS conditions are characterized by increased metalloproteinase activity due to the reduced activity of their tissue inhibitors. The imbalance between intracellular matrix metalloproteinases and their inhibitors may lead to destructive proteolytic damage to the CNS tissue [45]. Minocycline has shown beneficial effects in many experimental studies [65] and was therefore also approved for phase I and II clinical trials in patients with completely injured spinal cord. The overall results confirmed the safety of the drug, but did not show improved motor outcomes in patients treated with minocycline compared to placebo. However, in a subset of patients with incomplete spinal cord injuries, patients experienced significant improvement [66]. Based on this promising outcome, a Phase III clinical trial was initiated in patients with acute spinal cord injury. This is currently ongoing and will be completed in 2018 [67].

Another interesting formulation is **FK506** (tacrolimus) isolated from the bacterium *Streptomyces tsukubaensis*, which presents a potent immunosuppressive drug. Primarily, it is used to reduce allograft rejection in organ transplantation, but also offers neuroprotective properties for central nervous system trauma. FK506 blocks the activation of calcineurin through the formation of complexes with immunophilins. However, it binds to a different immunophilin than cyclosporine A (CsA) [68]. FK506 has been found to increase nerve regeneration and functional re-innervation after peripheral nerve injury, as well as prevent axonal damage in toxic neuropathies [69]. Several studies document that FK506 delivery protects tissue from secondary injury and showed a beneficial effect during an acute SCI [70]. However, long-term administration of FK506 after experimental spinal cord injury in rats has shown to be not as effective [71]. FK506 was also used as a potent inhibitor of activated T-cells that infiltrates the injured spinal cord. Thus, it can modulate inflammation and ameliorate neuroprotection through its immunosuppressive

action on immune cells [72]. Furthermore, the immunosuppressive action of FK506 was proven by the prevention of graft rejection following spinal cord ischemia and SCI [44].

Riluzole is commonly used in the treatment of amyotrophic lateral sclerosis (ALS) to protect against nerve cell degeneration. The possible mechanism of action of riluzole is blocking sodium channels as well as glutamate excitotoxicity. The deleterious effect of glutamate overproduction during CNS damage can be reduced by both reducing the synthesis and preventing its release into the synaptic cleft. In the case of riluzole, its mechanism of action is most likely thought to be the reduction of glutamate synthesis and thereby its release into the presynaptic region of the neuron. In a recent study, 155 patients were randomized to riluzole treatment (100 mg/day) or to placebo. The patients were monitored for 12–21 months [48]. Survival was significantly longer in the riluzole-treated group compared to placebo-treated patients. The median survival time was 17.7 months for riluzole compared to 14.9 months for placebo [4]. Additionally, there was a significant improvement in motor function in patients with cervical SCI receiving 50 mg riluzole twice a day for 14 days after injury, compared to the control group [73].

4. Regeneration

Regenerative medicine is a dynamically developing area of medicine whose mission is to restore damaged tissue. Although different tissues and organs have different recovery capabilities, there are diseases and CNS injuries that have limited regeneration and, unfortunately, they cannot be treated by conventional therapies. One of the innovative regenerative medicinal approaches is the use of stem cells and biomaterial-based treatments in order to replace damaged tissue or to supplement missing trophic factors in various CNS diseases [3].

The twenty-first century resonates with the rapid development of regenerative medicine, where methods of isolation and processing of stem cells and the use of highly compatible biodegradable materials and nanotechnologies directed to the treatment of SCI patients has been improved [74]. However, successful cell therapy is influenced by various factors such as: (i) selection and processing of stem cells (adult, induced pluripotent stem cells), (ii) delivery strategies (local, systemic), (iii) dosage (single, continuous), and (iv) appropriate timing of administration (acute, chronic phase of SCI). Selection of stem cells is important for their compatibility with host tissue. For this reason, in clinical studies, stem cells obtained from the tissues of the patient are preferred. Autologous stem cell transplantation obtained from the bone marrow and adipose tissue of a patient is used in the treatment of hematopoietic diseases, in the regeneration of bone tissue and cartilage, and possibly also in spinal trauma [74]. At present, an autologous transplantation of the so-called induced pluripotent stem cells (iPKB) derived from adult somatic cell patients has also been considered. By new procedures, we can reprogram a fully differentiated somatic cell (fibroblast) toward a cell with primitive pluripotent origin that is derived into the desired cell population [75]. In other cases, allogenic stem cells that meet the compatibility criteria (ABO, HLA) may still be used, but patients must still receive immunosuppressive therapy for a lifetime. In addition, stem cells are a major tool for gene therapy when they can produce some trophic factors and other molecules that are necessary for the regeneration of injured nerve tissue.

Among different **mono-therapies**, more **complex cellular therapy** has reached considerable attention due to targeting multiple aims, such as bridging the cavities or cysts, replacing dead cells, and creating a favorable environment allowing axonal regeneration [76].

4.1. Regenerative approaches toward biomaterials

SCI results in cysts or cavities at the site of the lesion, which gradually expand in the caudal direction. From this point of view, cell therapy alone for such a progressive pathological process as SCI is insufficient. Therefore, it is recommended to combine the administration of stem cells with biodegradable biomaterials that fill the cavities. The main objective is to optimize mechanical properties, cell adhesion, and biodegradability of synthetic or natural materials and develop new methods to deliver cells to the lesion site. One of the most important features for the successful integration of the implant into damaged tissue of the spinal cord is its optimal mechanical strength. If the biomaterial is too rigid, it can cause compression of regenerating axons and the formation of additional secondary cavities between the implant and surrounding spinal tissue. Therefore, it is preferable to use an injectable biomaterial that can properly adapt to the lesion [63, 77].The stem cells with which the implant should colonize also require the presence of growth factors that help them to survive in the unfavorable environment of the injured spinal cord. Chen and his scientific group compared the regenerative capabilities of several biodegradable multichannel biomaterials with different mechanical properties that were colonized by Schwann cells and implanted into the spinal cord after transection [63]. Compared to the poly-caprolactone fumarate material, which had significantly higher compression modulus values, biomaterials based on hydrogels showed significantly smaller cavities and promoted material vascularization and Schwann cell infiltration [63].

The biomaterial has to be biocompatible; this depends on the properties of the surface of the material and its interactions with cells or proteins [78]. However, we have to be aware of non-specific inflammatory responses of the recipient to the foreign biomaterial, and its extent determines the rate of implant biocompatibility. Interestingly, the acute response of the immune system that is mediated by macrophages or dendritic cells can be neuroprotective and can promote CNS regeneration. Modulation of the inflammatory response by the type of biomaterial surface can therefore be an auxiliary tool for repair mechanisms of the tissue. In principle, the physical properties of biomaterials should simulate the extracellular environment of the central nervous system and thereby ensure the diffusion of neurotrophic factors. Interactions between biomaterial surface and living tissue are usually mediated by a layer of proteins. Most biomaterials have an optimized surface with bioactive molecules or oligopeptide sequences [77]. This guarantees the adhesion of specific cells or their parts (e.g., axons).

Biodegradable materials are more desirable than non-degradable ones. Their degradation is most often mediated by hydrolysis and enzymatic cleavage. The rate of degradation can be controlled by various factors, such as molecular weight and polymer structure, crosslinking, and use of copolymers [79]. Of course, degradation products must not cause any immune response and the rate of breakdown of the material must be appropriate to the formation of new tissue. Biomaterials that are used to regenerate nerve tissue usually degrade for weeks or months, depending on the axonal and vascular material growth. Degradation can take place by gradual erosion of the surface of the material while maintaining the structural integrity of the material

or by the gradual breakdown of the material structures. The first method is more advantageous because the collapse of the material may stop the regeneration process.

Alginate materials are natural and have a significant role because most of them are biodegradable. This group of natural materials also includes collagen, methylcellulose, or hyaluronic acid-based materials. The disadvantage is their natural variability and the risk of immunogenicity. The implantation of lyophilized alginate into the cavity of newborn or young rats stimulated the growth of non-myelinated and myelinated fibers in the hydrogel [80], as well as the formation of functional neuronal connections that have been demonstrated. In another study, the optimal combination of EGF and bFGF was chosen routinely in conventional 2D cultures in order to obtain the desired amount of proliferating cells. The goal was to create a strong but reversible binding of both factors to alginate-sulfate [81], allowing their prolonged and sustained local presentation to neural progenitors in cell culture. This develops an active biomaterial that eliminates the need for external continuous growth factor substitution during cell culture. However, it is crucial to determine the optimal concentration of growth factors that could mimic similar concentrations of bFGF/EGF commonly used in the 2D system culture (10–20 ng/ml for each factor/3 days). In this case, the equilibrium binding constant of the selected factors on alginate-sulfate plays an important role. The initial concentration of both bFGF and EGF factors (200 ng) was shown to be sufficient for their continuous release over 21-day incubation [82]. The concentration of growth factors released within the first week *in vitro* initiated cell proliferation and the formation of typical 3D neurospheres. Consequently, there was a decline in the growth factor concentrations; the cells migrated from the neurosphere and differentiated to neurons, astrocytes and oligodendrocytes. These results confirmed that the 3D alginate biomaterial, which gradually released growth factors, creates optimal conditions for long-term survival and differentiation of neural progenitors *in vitro* [82].

The developed 3D biomaterial was implanted locally into SCI rats. The results confirmed that the optimal bioavailability of growth factors (EGF and bFGF) from the implant stimulated neuroregenerative processes. Enhanced sparing of spinal cord tissue and increased number of surviving neurons (ChAT-cholinacetyltransferase-positive neurons), corticospinal fibers (BDA-labeled), and blood vessels at the site of injury [83] occurred. Inflammatory processes were partially suppressed, but not astrogliosis. These partial results indicate the possible use of active alginate biomaterials enriched with bioactive molecules in the treatment of CNS trauma [83].

Although the biomaterials themselves can affect nerve tissue regeneration by creating a space for cell growth through the lesion, it is increasingly clear that combined therapy has a synergistic effect and leads to better results. Therefore, biomaterials are most often combined with different types of cells or enzymes digesting proteoglycans in glial scars, as known for chondroitinase ABC. The most commonly used cells are MSC, Schwann cells, and neural stem cells that can express Noggin, promoting neurogenesis and suppressing gliogenesis [84]. Biomaterials can also serve to release the biologically active substance, which can then create a gradient that promotes cell growth into the implant. Biologically active agents may be growth factors (EGF, FGF), cytokines, neurotrophins (NT3, NGF, BDNF, and GDNF), neurotransmitters, and anti-axon growth inhibitory antibodies [85].

In conclusion, it is necessary to combine these strategies to further enhance the final effect.

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