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Nanovector-Mediated Drug Delivery in Spinal Cord Injury: A Multitarget Approach

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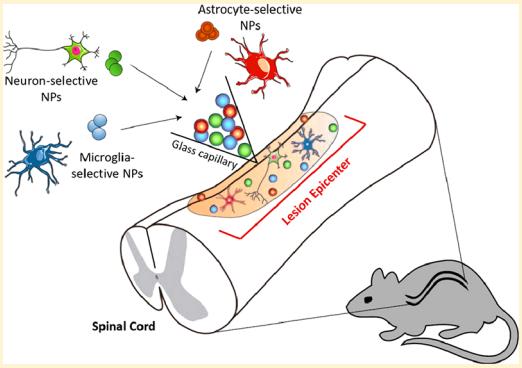
ABSTRACT: Many preclinical studies seek cures for spinal cord injury (SCI), but when the results are translated to clinical trials they give scant efficacy. One possible reason is that most strategies use treatments directed toward a single pathological mechanism, while a multitherapeutic approach needs to be tested to significantly improve outcomes after SCI. Most of the preclinical reports gave better outcomes when a combination of different compounds was used instead of a single drug. This promising approach, however, must still be improved because it raises some criticism: (i) the blood−spinal cord barrier limits drug distribution, (ii) it is hard to understand the interactions among the pharmacological components after systemic administration, and (iii) the timing of treatments is crucial: the spread of the lesion is a process finely regulated over time, so therapies must be scheduled at precise times during the postinjury course. Nanomedicine could be useful to overcome these limitations. Nanotools allow finely regulated drug administration in terms of cell selectivity and release kinetics. We believe that excellent therapeutic results could be obtained by exploiting this tool in multitherapy. Combining nanoparticles loaded with different compounds that act on the main pathological pathways could overcome the restrictions of traditional drug delivery routes, a major limit for the clinical application of multitherapy. This review digs into these topics, discussing the critical aspects of multitherapies now proposed and suggesting new points of view.



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INTRODUCTION

Spinal cord injury (SCI) is a debilitating condition caused by damage to the spinal cord. It is the most frequent disabling spinal injury: an estimated 2.5 million people worldwide live with SCI, and more than 130 000 new injuries are reported every year[1] (http://www.wingsforlife.com).

Persisting SCI has a physical, emotional, and economic impact on patients and places a heavy burden on society in terms of healthcare costs, primary care, and loss of income.[2,3] Spinal cord trauma is the result of two phases. First, a *primary injury* to the spinal cord occurs, causing tissue compression, transection, contusion, or laceration. The most frequent causes of *primary* mechanical trauma are auto or motorcycle or bicycle accidents, falls, gunshot wounds, falling objects, medical and surgical complications, person-to-person contact, and pedestrian injuries. SCI can also result from no traumatic causes such as infection, insufficient blood flow, and tumors. This review will focus on traumatic SCI. From *primary injury* arises a multifactorial *secondary injury*, involving a complex pathological mechanism that starts after *primary* SCI and can last months.[4] These events include, but are not limited to, neuronal injury and death, neuroinflammation, breakdown of the blood−spinal cord barrier (BScB), and oxidative stress. This results in autonomic, somatosensory, and/or motor dysfunction below the lesion, with the progression of chronic pain syndromes.

Up until now, the usual procedure in the case of SCI is surgical stabilization and decompression of the spinal cord, combined with high-dose methylprednisolone.[5] This approach is still controversial since it gives only limited improvements in outcome, often with severe side effects,[6] so effective therapy for SCI remains a great challenge.

Our understanding of the pathophysiology of SCI has greatly increased in recent decades as a result of fruitful preclinical research. In particular, there have been remarkable advances in our understanding of the *secondary injury* events, and several different *secondary injury* processes have been identified.[7,8] It is now clear that the main source of the complexity of *secondary injury* is the different starting time of the various pathological processes involved. Inflammation characterizes the acute phase after SCI (seconds to minutes), when microglia begin to remove debris from the injured area.[9] This leads to astrocyte activation, marking the subacute phase (minutes to weeks), with the stabilization of a glial barrier to the axonal regeneration.[10] Finally, weeks, months, or even

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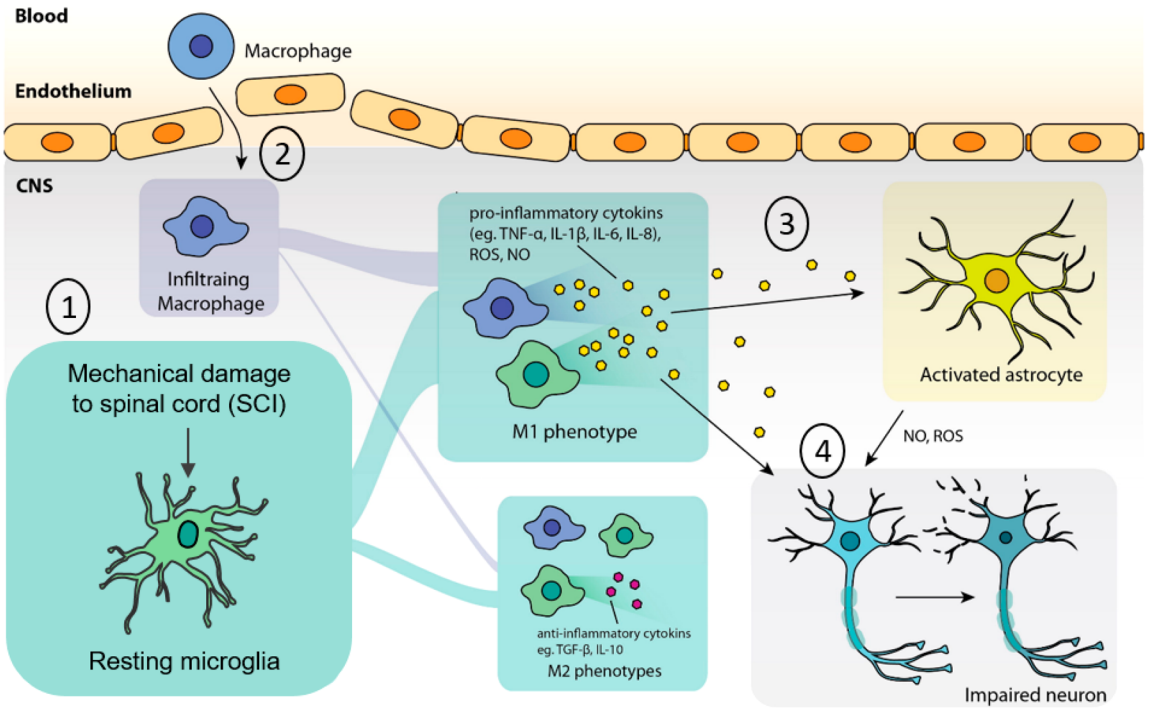

Figure 1. Schematic representation of *secondary injury*. (1) Following mechanical damage to the spinal cord (*primary injury*), resting microglia became activated and release anti- and pro-inflammatory cytokines. (2) Peripheral macrophages are recruited to the injury site and tend to polarize to the M1 phenotype. (3) Some pro-inflammatory cytokines can activate astrocyte, which exacerbate the injury process and create a glial scar. (4) Impaired neurons are unable to regenerate and under the influence of the toxic environment undergo apoptosis or necrosis (Adapted with permission from Zhang, F., Lin, Y.-A., Kannan, S. and Kannan, R. M. Targeting specific cells in the brain with nanomedicines for CNS therapies. *J. Control. Release Off. J. Control. Release Soc. 240*, 212−226 (2016) Copyright 2016 Elsevier.

years after the injury (chronic phase), oligodendrocytes continue to undergo apoptosis, leading to axon demyelination and neuropathic pain as well as further neuronal degeneration[9] (for a detailed table of the sequence of pathophysiological events during SCI, see ref 8). The clarification of this scenario has offered various potential therapeutic opportunities to counteract the spreading of *secondary injury*.

A large number of treatments (molecular, cellular, and pharmacological) have been proposed as potential therapeutic approaches, but few of them could be usefully translated to humans because of poor efficacy. The most probable cause of these unsatisfactory results lies in SCI progression itself. As mentioned above, *secondary injury* involves different times for degenerative pathways, with the contribution of different cellular components, and a single-target therapy is probably insufficient to counteract its spread. Now, however, is increasingly shared the expectation that a combined therapy, targeting multiple pathological mechanisms at the same time, is a promising approach.

Many studies have focused on multitherapeutic strategies, and over half of them show protective effects in preclinical settings.[11] However, combined therapies have to be carefully planned since different drugs may require different administration times to follow the time course of *secondary injury* and may not work synergistically, because of the complexity of the different phases. Fine-regulated therapy aimed at multiple cellular targets remains essential for successful treatment. Nanoparticles are a promising tool that can be easily adapted with regards to administration time and biodistribution (different cell targets affected at different times after SCI). The emerging research field of nanomedicine offers a variety of nanodelivery tools to load drugs and gain therapeutic efficacy by selective time-controlled cell treatment, depending on the composition and degradation kinetics.[12−14]

This review offers an overview of the main pathological events in *secondary injury*, looking at the pathological role of CNS cells in each process and the time course of their contribution after SCI. We will focus on pathological processes that turn out to be the target of the most promising therapies proposed in the literature. We assess the most recent single and multitherapeutic strategies proposed, to highlight the validity of the multidrug approach, and discuss some critical aspects. Considering the current limits for the transferability to the clinic of a multitherapeutic approach, in the final part of the review we will discuss an alternative route of administration that we believe to be the most promising for the overcoming of the current therapeutic restrictions. We discuss the advantages of combining different neuroprotective treatments administered with nanotools to improve the pharmacokinetics, biodistribution, and efficacy of free drugs through a cell-specific treatment.

SECONDARY INJURY: A MULTIFACTORIAL PROCESS

Secondary injury is due to a complex balance of cellular responses and contributions to a dynamic SCI microenvironment, which has a profound impact on the global pathophysiology of the spinal cord. This complex cellular and extracellular cascade has protective and reparative roles but also damage exacerbation.[15] We can try to simplify it by defining three steps: (1) Inflammation: resident microglia activate, proliferate, and mediate the recruitment of non-

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resident immune cells and fibroblasts to the injury site. (2) Gliosis: astrocyte activation orchestrates the composition of a glial scar composed of fibroblasts, immune cells, and extracellular matrix proteins that surround the lesion epicenter, forming a barrier that prevents axonal regrowth through the lesion but also helps sequester toxic substances and rescues penumbral tissues. (3) Axonal degeneration and demyelination: cells within the scar produce chemorepellants, chemoattractants, and trophic factors that influence the microenvironment, causing a failure in neuronal regeneration. Meanwhile, activated immune cells, including endogenous microglia and peripheral neutrophils and macrophages, adopt a spectrum of phenotypes with a variety of roles. These include debris clearance and toxic and trophic factor release that further influence the cellular and extracellular microenvironments[16] (Figure 1).

The most promising therapeutic strategies can be divided over three broad targets: (i) inflammation and oxidative stress, for the promotion of a pro-regenerative environment; (ii) destruction of an inhibitory glial scar with, at the same time, (iii) enhancement of neuronal regeneration. This classification highlights the important contribution to the progression of the *secondary injury* of the pathological processes that underlies these mechanisms. To recapitulate the main features of these pathological pathways, we give an updated summary of preclinical therapeutic options to define the key cellular therapeutic targets.

INFLAMMATORY RESPONSE TO INJURY

In physiological conditions, the blood−brain barrier (BBB) limits the entry into the CNS of patrolling bone marrow (BM)-derived immune cells, while resident microglia provide physiological surveillance. In response to mechanical *primary injury* to the spinal cord, microglia become rapidly activated, undergoing morphological and molecular changes often associated with neurotoxicity and initiating the inflammatory cascade that mainly characterizes the acute phase of the *secondary injury*. The composition and the potential effects of the cellular and molecular inflammatory cascade change in relation to the time and distance from the epicenter of the lesion.

The time course of the inflammatory reaction has been arbitrarily divided into three stages by Shin et al.:[18] (1) an early inflammatory stage, from the injury to the first 3 days, (2) a cleaning stage, from approximately 4 days to about 2 weeks postinjury, and (3) reactive gliosis starting 2 weeks postinjury.[18] This timeline has been defined for rodent models, but it has been demonstrated by previous studies that, despite variations in SCI lesions, the main features of neuropathological changes following SCI are similar between rodent models and human patients.[19]

The first stage involves hemorrhage, destruction of myelin, cell death, edema, and infiltration of inflammatory cells due to the disruption at the BBB. As residents of the spinal cord, microglia are the first cells to respond to tissue damage.[20] These cells are important for re-establishing tissue homeostasis.[21] They release chemokines and cytokines, both pro-inflammatory (tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, interferon (IFN)-γ), and anti-inflammatory (IL-10 and transforming growth factor (TGF)-β1) and other vasoactive substances, such as reactive oxygen species (ROS), kinins, histamines, nitric oxide, and elastase, that enhance recruitment of peripheral leukocytes.[22,23] Recruited macrophages persist in SCI lesions for as long as any study has examined, years and months, respectively, for humans and rodents.[24,25]

During the cleaning stage, edema is reduced and there is massive proliferation of macrophages/activated microglia in the site of the lesion.[26,27] In the lesion's core, macrophages remove cells and myelin debris, reducing edema and inducing the formation of a cavity.[28]

The third phase is characterized by astrogliosis and/or shrinkage of the spinal cord volume. Astrocytes become the predominant cell type[29] and form a "scarlike" barrier between the fluid-filled cavitation and normal tissue.[30] We shall discuss this phase further on. Recently, a later phase of cellular inflammation has been observed, persisting up to 180 days post injury (DPI), with a peak in the macrophage/microglial response at 60 DPI, double the site of the earlier peak. This later peak did not coincide with any change in locomotor function, suggesting that the second phase is not enough to affect locomotor recovery.[31]

The role of neuroinflammation is still controversial, being both beneficial and detrimental for recovery after the trauma. The immune response may facilitate the recovery from injury by reducing the size of the lesion, facilitating wound repair, and stimulating axonal regeneration.[32,33] In particular, further discussion surrounds microglia/macrophage phenotypes, including classically activated M1 and alternatively activated M2 macrophages. Although the M1 phenotype is usually associated with a neurotoxic effect,[34] it is also involved in axonal regeneration,[35] while M2 microglia, with neuroprotective effects, lack an agreeable environment to maintain their phenotype.[36] The current view is that polarization into M1 and M2 microglia phenotypes are the two ends of the same spectrum. In between, there is a continuum of intermediate states defined by unique molecular cell signatures.[37] Each cellular substate takes its cue from the changing environment and responds accordingly by adapting its functional phenotype.

For all these aspects, the inflammatory response after injury could be considered a "double-edged sword", having both neuroprotective and neurotoxic properties, and the detrimental phases of inflammation are interspersed with neuroprotective events.[8] This reflects the microglial diversity and functional plasticity, including its immunoregulatory roles after SCI. In order to exploit this diversity, a thorough understanding of regulatory mechanisms is required for the best therapeutic strategy, particularly in regards to neurorepair and neuroregeneration. From this perspective, accurate definition of the therapeutic window of intervention acquires great importance for any worthwhile anti-inflammatory treatment, because it should limit the neurotoxic potential and enhance the reparative mechanisms, depending on the different phases of inflammatory reaction during the progression of SCI.

Because the innate immune inflammation comes early, the inflammatory reaction offers an attractive option as a first therapeutic target in SCI. Nonsteroidal anti-inflammatory drugs (NSAIDs) have become the focus of various experimental SCI models as their potent anti-inflammatory effects may be expected to reduce inflammation in secondary damage.[38] Experimentally, they display neuro-protective and apoptotic effects by suppressing axonal regrowth, thus inhibiting the RhoA pathway, which leads to apoptotic cell death, in addition to the recovery of motor functions and some histological improvement. However, histological improvement

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is not always significantly associated with any gain of motor function.[7]

Rapamycin and minocycline are CNS-penetrating antibiotics that also inhibit microglial activation and have different anti-inflammatory properties.[39,40] Minocycline has been examined in different SCI preclinical models,[7,41] and the promising results led to a clinical trial by researchers at the University of Calgary (ClinicalTrials.gov Identifier: NCT01828203) that was completed in June 2018. Despite the benefits of this treatment, its effect was often not restricted only to immune system cells, and oligodendrocytes, astrocytes, and neurons could also be affected.[42] It therefore appears that systemic administration of a general immunosuppressant may be undesirable in a pathological condition such as SCI.[43]

Finally, Maresin-1, a recently isolated highly conserved specialized proresolving mediator[44] was identified as an interventional candidate to attenuate dysregulated inflammation and restore functional recovery after SCI.[45]

Although all these cited treatments show promising results, there remain questions about their time window of applicability and efficacy and their biodistribution in the injured cord. Further preclinical work is needed to refine and optimize the treatment paradigms for human study.

GLIOSIS

Several days after SCI, astrocytes are activated. This is linked to resident microglial activation[46] and causes synapse phagocytosis, clearance of debris and dead cells, and the formation of a glial scar (gliosis), which can restrict the spread of additional cytotoxic inflammation while, at the same time, limiting the axonal sprouting.[47] Astrocytes surrounding the injury site begin to show hypertrophy with morphological and molecular changes. They accumulate, extend, and overlap their processes, forming tight junctions that constitute the scar around the injury site.[48,49] Although the mechanism underlying the reactive response of astrocytes is not yet fully understood, it has recently been shown that immune cells play an important role in the induction and formation of the glial scar.[50] After the lesion, microglia are activated first and responsible for the transformation of the astrocytic phenotype.[47] Moreover, macrophages migrate to the lesion site and produce factors that induce astrocyte proliferation and their reactive phenotype.[51] In addition, recent evidence show that pericytes, perivascular cells located on microvessels, react to the lesion and participate in scar formation.

In particular, following the lesion, a specific subset of pericytes (pericitis of type A) begins to proliferate, leaves the walls of the blood vessels, and differs in cells similar to scarring fibroblasts that contribute to forming the nucleus of the scar.[52]

The glial scar has a role in re-establishing the physical and chemical integrity of the CNS and closing the BBB to reduce infiltration of non-CNS tissue, minimizing infection and the spread of cellular damage.[53,54] On the other hand, the glial scar produces chemical signals inhibiting axonal sprouting, as discussed below.

As amply explained in a review by Liddelow and Barres,[46] gene profiling can be useful to classify the reactive astrocytes and, most importantly, to define their function. Similar to the microglia classification, they identified two astrocyte phenotypes, A1 and A2. A1 reactive astrocytes turn out to be responsible for the lack of repair after different CNS pathologies and are considered "harmful". On the other hand, A2 astrocytes upregulate many genes that promote survival and growth of neurons, with a "helpful" function. For instance, when the formation of A1 astrocytes is inhibited, the death of axotomized CNS neurons in vivo is prevented.[46] On the other side, the removal of proliferative scar-forming astrocytes by STAT3-mediated ablation worsens the SCI outcome, with extensive axon dieback.[55] These "helpful" proliferative astrocytes might well be the A2 phenotype, becoming important therapeutic mediators.

FAILURE IN AXONAL REGENERATION

Axonal regeneration is the therapeutic goal mainly pursued for post-SCI therapies. Several factors are implicated in the failure of CNS neurons to regenerate their axons after injury. They can be divided into two main groups: deficit in intrinsic regenerative pathways of the axotomized CNS neuron, which lacks an appropriate cell body response in terms of activating proper pro-survival gene expression (regenerative associated genes, RAGs),[56] or inhibition by the toxic environment, i.e., related to molecules and/or physical barriers that inhibit axonal growth.[57] We are still far from an effective clinical therapy based on neutralization of inhibitory factors or enhancement of RAGs.

The extracellular inhibitors regulate axon outgrowth by acting on receptors located on the growth cones and can be divided in those associated with myelin (e.g., Nogo, myelin-associated glycoprotein, oligodendrocyte myelin glycoprotein) and those related to the glial scar, acting as a chemical (e.g., chondroitin sulfate proteoglycans, CSPGs) or physical barrier. After SCI, CSPGs are upregulated around the injury site,[58,59] and this upregulation can inhibit neurite outgrowth in vitro[60,61] and in vivo.[62] Different studies utilized the bacterial enzyme chondroitinase ABC (ChABC) to digest chondroitin sulfate glycosaminoglycans (CS-GAGs) of CSPGs. There is robust preclinical evidence replicated in a number of different injury models of the beneficial effects of ChABC after SCI, such as enhancement of axon regeneration.[63−65]

Among RAGs, Growth Associated Protein 43 (GAP-43) is required for neurite growth since it regulates the actin cytoskeleton.[66] However, increasing the expression of only one RAG, such as GAP-43, seems not to be sufficient to boost the neuronal regeneration.[67,68] Probably the simultaneous expression of other RAGs is necessary.

Different strategies have been proposed to stimulate the functioning of the regenerative machine or inhibit the molecules that counteract axonal regeneration. The different approaches can be divided into biologic and pharmacological treatments.

Biologic Therapy. Among the strategies exploited to stimulate neuroregeneration after SCI, neurotrophins are widely used. Neurotrophins are a family of proteins that regulate synaptic function, neuronal survival, and neurotransmitter release and elicit the plasticity and growth of axons within the adult CNS. The most widely used neurotrophins are nerve growth factor (NGF), basic fibroblast growth factor (bFGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3).[69] Generally, the majority of studies had significant success when neurotrophic factors are applied in or close to the lesion site during the acute or subacute phase after SCI. Fewer studies have tested neurotrophic factors to directly target injured neurons. The administration route differs among acute injection of recombinant proteins, subacute or chronic delivery using a variety of strategies including osmotic minipumps, cell-

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Table 1. Neuroprotective Strategies Undergoing Clinical Evaluation for Spinal Cord Injury[a]

treatment	therapeutic action	clinical trials
riluzole	Blocking tetrodotoxin-sodium channels associated with injured neurons. Inhibition of presynaptic glutamate release and increased reuptake to modulate excitotoxicity	Phase II/III randomized controlled trial (NCT01597518) (Estimated completion date: December 2018)
magnesium	*N*-methyl-D-aspartate receptor antagonist; antiexcytotoxic and antiapoptotic properties	Phase I/II placebo−controlled (Interrupted)
glibenclamide	Blocking nonspecific cation channels of the capillary endothelium to avoid capillary fragmentation and hemorrhage	Phase I/II clinical trial NCT02524379 (Estimated completion date: 2020)

[a]These compounds are mainly injected systemically.

mediated delivery, delivery using polymer release vehicles or supporting bridges of some sort, or gene therapy to modify neurons.

Adeno-associated viral (AAV) vectors have been widely tested to introduce neurotrophic factors into neurons. Different AAV serotypes displayed different transduction efficiencies and regional specificities (AAV5 for neurons in reticular formation, AAV1 for raphe neurons). Nonviral vectors are an attractive alternative: they can be targeted to specific neuronal subpopulations, offering better safety profiles than viruses and lower production costs. However, nonviral vectors have lower transfection efficiency than viruses, severely limiting their utility in neuron-targeted delivery applications.[70] Though gene therapy has been successful in treatment of SCI in several animal models, it is still not available for clinical practice,[71] and other options are required for a selective administration of neurotrophins.

The neutralization of myelin-mediated inhibition of neurite outgrowth with anti-Nogo (IN-1) antibodies or other Nogo-related approaches and the inhibition of Rho activation (e.g., with BA-210, Cethrin) are two other biological strategies that have received considerable attention in the last 2 decades and are in various stages of clinical translation to promote endogenous neuroregenerative repair after SCI. Their use in preclinical models of SCI is amply discussed in a dedicated review by Kwon and collaborators[7] or by Ahuja and Fehlings.[41] As the authors explain, it is often difficult to define the real benefits of these approaches, mainly because of differences in experimental paradigms and some difficulty in replicating positive results. These approaches have already been tested in clinical trials, but there is some concern in the translation of these therapies in regards to their biodistribution in the injured spinal cord. The signaling glycoprotein G-CSF (granulocyte colony-stimulating factor) can enhance the survival of ischemic CNS cells, protect against glutamate-induced apoptosis, and reduce TNF-α and IL-1β expression in vivo.[72]

Pharmacologic Agents. Among the various neuroprotective pharmacological treatments reported, we have selected those with high potential for clinical translation (because they are already used in other clinical applications). They are discussed in detail in up-to-date reviews by Ahuja and collaborators[73,41] and Ulndreaj and collaborators[74] and comprise different approaches, recapitulated in Table 1.

Another strategy recently pursued is based on microtubule stabilization. Some compounds such as epothilone D or paclitaxel, which promote microtubule stabilization, show neuroprotective properties after systemic injection in contused rats by activating the intrinsic axonal growth machinery and reducing the inhibitory fibrotic lesion scar.[75,76]

COMBINATORIAL THERAPIES FOR A SYNERGISTIC EFFECT

The primary injury sets off a variety of secondary events, which result in an expanded lesion area. Ultimately the tissue fails to regenerate so management of this secondary cascade is an important first step in achieving recovery of normal function. Single therapies have given only limited effects. This is quite likely due to a combination of factors responsible for the staggered development times of different biochemical pathways of degeneration, which overcome other toxic or inhibitory factors. To boost the small protective effect seen after a single neuroprotective treatment, the most promising strategy is probably simultaneous treatment of different pathological processes. This should give better effects than single target therapy. *In vivo* combination studies require great effort because the introduction of an additional treatment triples the number of experimental groups.[77] However, different studies show that a multitherapy approach is more effective than single treatments.[11] In this direction, recent research has focused on multitherapeutic compounds to target the multiple mechanisms involved in the *secondary injury*.

An extensive literature analysis show that several studies use combinatorial approach in preclinical models. Most of them are exposed and summarized in specific reviews. To offer a rapid overview on this topic, we can divide the combination therapies tested in SCI in four groups: (1) combinations of stem cells transplantation and neurotrophic factors (e.g., NT3, BDNF); (2) combinations of different growth factors (e.g., bFGF, NGF) that enhance neuronal survival, act on glial phenotype, and/or promote plasticity and axonal regrowth;[78] (3) combinations of different drugs;[79] or (4) combinations of cells/trophic factors and biomaterial scaffolds, which could serve as proper substrates for cell transplantation,[80] bridging the cavity, guiding axonal regeneration, as a cell delivery tool, and a reservoir for sustained drug delivery. Other studies have used in addition ChABC to counteract scar formation and increase axonal growth into the scaffold, further improving behavioral outcome.[81]

Reviews by Silva and Harvey provide exhaustive summary tables of, respectively, combinatorial therapies and combinatorial use of neurotrophic factors for SCI regeneration.[11,78] It is worth mentioning that recent evidence indicates the importance of motor rehabilitation combined with pharmacological treatments. In general, an early rehabilitation and a greater intensity of training seem to be beneficial to favoring maximum functional recovery. This is demonstrated by different studies on rodent models: pharmacological treatment combined with rehabilitation lead to re-establishment of gait in completely transected rats.[82] It has also been shown that

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combining treatment with ChABC and rehabilitation promotes functional recovery in acute spinal cord injury.[83] Musieko et al. showed that combining epidural and pharmacological stimulation to manipulate serotonergic, dopaminergic, and noradrenergic pathways with rehabilitation restored posterior limb locomotion in rats.[84] Even if also recent clinical trials demonstrate a beneficial effect of rehabilitation,[85] the underlying neuronal mechanisms leading to improvements are not yet fully understood.[82]

From overall observation and analysis of these combinatorial studies, we can offer some considerations. The compound (trophic or growth factors, drugs) is often given systemically, so its biodistribution is limited by the BScB. Indeed, the BScB strictly regulate diffusion transport of molecules to the spinal cord parenchyma, allowing only molecules with MW < 400 Da to cross the endothelium.[86] Even if there is a partial rupture of the BScB following SCI, it is difficult to assess the extent of this gap and then evaluate how much drug actually manages to reach the site of injury, meaning higher doses may be needed to give a protective outcome. Another important critical point is the lack of selectivity of these treatments after systemic administration. For example, combined treatments using different growth/trophic factors to promote axonal sprouting or neuron regeneration may affect different axonal populations in different ways and this may result in adverse outcomes such as enhanced plasticity and/or altered responsiveness of the nociceptive spinal circuitry.[87−89]

Finally, when used as single doses, neurotrophic factors gave limited improvements, due to the impossibility of maintaining a constant biological effect in vivo. Ideally, the biological effect should be maintained until the regenerative process finishes, requiring multiple doses or invasive therapies.[90] These results suggest that the multitherapeutic approach is promising but it still needs to be improved.

TARGETED DRUG DELIVERY IN SCI

Treatments for SCI can be designed in an increasingly rational manner, ultimately improving their potential for translation to the clinic. Although multitherapeutic options provide evident benefits in SCI preclinical models,[11] there is a critical need for novel methods to treat the injured spinal cord, to improve biodistribution across the BScB after systemic administration, to avoid side effects due to the different treatments or their antagonism when administered simultaneously, and most of all to boost the selectivity of treatments.

A promising solution to improve the multitherapy approach is targeted therapy, where single compounds are directed toward their cellular targets, avoiding the interactions with nontarget cells of systemic distribution. Various approaches have been proposed to obtain a selective cellular response. Nanomedicine is the most achievable and an increasingly used option in the field of SCI target therapy. Nanomedicine offers great potential for improving the efficacy of therapeutic drugs in clinical settings for many CNS disorders. The uptake of nanoparticles by target cells and so the interaction with cell membranes and receptors is strongly associated with the ability of nanoparticles to form protein corona associating biomolecules from the cellular microenvironment and body fluids.[91] Indeed nanoparticles do not interact directly with the cells, but the protein coronas of nanoparticles play a key role in the interaction with lipids or protein receptors of the cell membrane.[92] The nanoparticle surface and its specific chemical compounds resulting from the engineering processes (postpolymer functionalization), the methods used for dispersion, and experimental preparation determine the selective cell uptake though the activation of specific signaling pathways.[93]

A few studies have proposed new smart nanostructured biomaterials to deliver therapeutic compounds in situ, demonstrating that these tools are safe and adjustable for a multitherapeutic approach.[12] Engineered polymeric particles can offer advantages in many aspects of therapeutic delivery: while drug entry to the spinal cord is tightly restricted by the physical limitation of the BScB, drug-carrying nanoparticles can give significantly better CNS pharmacokinetics and biodistribution than the free drugs.[94] Nanoparticles can improve the solubility of hydrophobic compounds in aqueous environments, prolong the half-life of therapeutics in the blood, provide an assortment of controlled release profiles, improve the bioavailability of drugs, and have fewer of the adverse side effects of delivering therapeutics locally.[95−97] A very important feature of this tool is that the rate of release of therapeutics from polymeric particles can be tailored by several methods: particle properties (polymer composition or porosity), polymer molecular weight and arrangement of the polymer chains, particle size and shape, as well as the amount and type of therapeutics loaded.[12] The use of nanoparticles for drug delivery is not only widely reported in preclinical studies but is now also being implemented for some clinical applications. Caron and collaborators and Ordikhani and collaborators provide ample overviews of the use of polymeric nanoparticles to deliver neurotrophic and growth factors, drugs, and other therapeutic molecules to treat SCI.[12,14]

NANOMEDICINE TO TARGET DIFFERENT CELLS SIMULTANEOUSLY

As discussed above, nanomedicine is becoming increasingly popular in the field of SCI thanks to its versatility. Nanoparticles can be prepared following different routes that depend on the polymeric material and are reviewed in refs 98 and 99. Self-assembly, driven by different affinity, of preformed polymers is the most common method used to synthesize nanoparticles. Among the different strategies used, two of them are the most widely used: (i) emulsion-solvent evaporation and (ii) nanoprecipitation or solvent diffusion. The emulsion-solvent evaporation is an emulsification, using ultrasounds or microfluidizers, of hydrophobic polymeric solution with an aqueous phase containing surfactants. The organic phase is then removed under pressure. The nanoprecipitation process needs two miscible solvents: the polymer soluble in the first solvent but insoluble in the second one (cosolvent). Nanoprecipitation takes place through a quick desolvation of the polymer when the solvent is added to the nonsolvent driven by complex phenomena of diffusion, flow, and variation of surface tension. The key difference between these two methods is that in nanoprecipitation the presence of surfactants is not required. Nanoparticles can also be prepared starting from monomers using emulsion polymerization; strong attention should be paid to complete removal of catalysts and initiators.[100]

Different studies show that encapsulation of a drug in a nanoparticle delivery tool can improve the drug's pharmacological efficacy compared to its free administration. For example, because methylprednisolone is already used to treat SCI, several groups have attempted to improve its local delivery with the goal of avoiding side effects and improving its efficacy. The result showed a better outcome than with

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methylprednisolone alone in vivo with reduction of the lesion and prolonged drug release (4 and 14 days) giving a better locomotor score for the methylprednisolone-loaded nanoparticles than for methylprednisolone alone.[101,102] To further support the potential of this approach, our laboratory has developed and used a nanovector delivery tool (poly-ε-caprolactone-based nanoparticles, PCL) to selectively treat/target activated microglia. When administered acutely in SCI mice, PCL loaded with minocycline, a widely used anti-inflammatory drug, acted efficiently on the resident microglial cells reducing the proinflammatory response. We did not get the same result when minocycline was given alone, supporting the hypothesis that targeted delivery of the drug improves its efficacy.[20] Linking the advantages of nanoparticles as drug delivery vehicles with the multitherapy strategy, we suggested that a combined approach using smart drug delivery systems, able to provide multiple treatments with different release kinetics, could have synergistic action on treatment efficacy. The key point of this strategy is to obtain a temporally defined treatment to selectively influence cell activation at different stages of the *secondary injury*. Polymeric nanoparticles specifically functionalized that can be internalized by cells (microglia/macrophages or astrocytes) and then selectively release different active compounds could be used.

The rationale of this approach is to reduce inflammation, counteracting the M1/A1 phenotype in the first stage and then help neuroregeneration, by promoting the M2 and A2 pro-regenerative phenotypes. An early release of anti-inflammatory factors would be beneficial, as resident microglia are the first cells to activate after injury.[103] Factors promoting glial scar degradation would be more useful if embedded in nanoparticles with long-term release. Neuroregeneration, finally, could be achieved using nanoparticles functionalized to be neuron-selective, loaded specifically with neurotrophic factors. The further added value of the combined nanoparticle therapy is in the possibility of finely adjusting the release of the treatments over time, following the course of the *secondary* lesion. Understanding how different kinds of cells are able to internalize nanoparticles is essential to improve the selectivity of nanotools. Microglia and macrophages have an intrinsic ability to take up foreign bodies through their phagocytic activity. Clathrin-dependent endocytosis is the principal uptake mechanism, especially in activated microglia, which is the principal therapeutic target.[104] Different synthetic nanoparticles have been used to achieve astrocyte selectivity, though for now studies are limited to in vitro experiments.[105,101] Neuron-specific uptake is the major challenge because they are not phagocytic and are surrounded by glial cells in the CNS. Some authors suggest exploiting the ability of apolipoprotein E (ApoE) to deliver lipids into neurons and synthesize nanoparticles with a lipid nature, able to interact with apoE receptors. A review by Zhang and coauthors provides a summary of platforms reported in recent studies to target CNS cell populations.[17] Although the choice of nanoparticles is essential to achieve a higher degree of cellular selectivity and avoid broader treatment of different cell types with the same nanoparticle, the main problem remains the transition of the biomaterial into the CNS. The intrathecal route is usually used to treat different spinal cord insults,[106] but local delivery by intraparenchymal infusions has gained increasing favor for the treatment of neurodegenerative disorders.[107] According to our laboratory's experience, in situ drug administration can be done with a glass capillary to maximize the proportion of treated cells.[103,108] This technique has been used in different preclinical paradigms and recently has been proposed in clinical practice.[109] Nanoparticles administered by intraparenchymal infusion could be combined with vertebral decompression, already practiced on SCI patients, avoiding different surgical interventions.

FINAL REMARKS

SCI causes substantial physical and psychological damage to patients, including paralysis, neuropathic pain, and bladder dysfunction. To date, there are no valid therapeutic options. Clinical interventions such as surgery and methylprednisolone alleviate inflammation and pain and limit further damage but do not lead to functional recovery and present troublesome side effects. It is increasingly evident that a promising therapy should target more than one pathological event, so recent research has focused on setting up multitherapy, where different combinations of compounds are administered simultaneously. This approach turned out to be more effective than single treatments in several studies, but some limitations emerge, including (i) lack of selectivity, (ii) side effects due to aberrant interactions among the different drugs or to systemic treatment, and (iii) some difficulty in finding the best therapeutic window. To overcome these limitations, injectable nanoparticles can localize and sustain the release of molecular therapeutics to the lesion site in a minimally invasive manner. Such therapies will most probably be multimodal, where timed delivery of various constituents (i.e., drugs, biological compounds) could be used to modulate cell and tissue responses at the site of injury during the different stages of recovery.

The ability of nanoparticles to control and the sustain the release of drugs is widely studied and analyzed in several papers.[99,110] The mechanisms behind drug delivery are three: (i) diffusion, (ii) swelling, and (iii) degradation. In diffusion controlled systems, drug delivery is driven by the gradient of concentration existing between the inside and the outside of the device. In swelling controlled ones, the swelling step is the only release rate-controlling phenomenon. Upon contact with water, the polymer chains "relax", with a consequent volume increase. Obviously, the conditions for drug transport in these two states (nonswollen versus swollen) are fundamentally different and can be used to accurately control the release rate of the incorporated drug. In the degradation controlled system, the delivery of drug is promoted by the degradation of the polymeric structure that takes place in the body via hydrolysis and/or enzymatic degradation.

Analyzing the different stages of *secondary injury*, we can identify three main pathological pathways that call for action: inflammation, gliosis, and neurodegeneration. These processes start at different times after injury and are orchestrated by two principal cell populations: resident microglia and astrocytes, while neurons are mainly affected by a hostile environment and the need for regenerative stimuli. A combined therapy with different nanoparticles that can be selectively internalized by microglia, astrocytes, or neurons, loaded with specific drugs/molecules, could limit the spread of *secondary injury*. Although a considerable amount of work is still needed to characterize the use of nanoparticles in SCI properly before clinical trials are possible, we believe this is a promising therapeutic option. The versatility of this tool could allow a single in situ injection of nanoparticles to avoid systemic routes and to achieve a selective treatment instead of repeated dosing to cover the

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different phases after SCI. In addition, the development of new nanoparticles capable of overcoming the BScB could further improve the efficiency of treatment, reducing the risks associated with in situ administration. To conclude, this approach could possibly be adapted to other CNS diseases where many factors contribute to the worsening of the clinical outcome.

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Author Contributions

S.P. wrote the manuscript. F.R, I.V., G.F, and P.V. contributed to the final version of the manuscript. All authors provided critical feedback.

Notes

The authors declare no competing financial interest.

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