NEURONAL APOPTOSIS AND INTERVENTION STRATEGIES

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(Adunanza del 23 ottobre 2014)

SUNTO. - A seguito di una lesione distale di un nervo periferico, si riscontra la morte di una proporzione delle cellule neuronali sensoriali nei gangli dorsali. Se il trauma è più prossimale, per esempio una lesione del plesso brachiale, anche una proporzione dei corrispondenti neuroni motori muore. Questo fenomeno è stato poco studiato, ma è importante perchè le cellule neuronali perse non possono essere sostituite per la mancata potenzialità di divisione cellulare, con conseguente rigenerazione sub-optimale e deficit di recupero sensoriale del paziente. Ultimamente è stato accertato che la morte neuronale è dovuta al processo di apoptosi, come rivelato da studi di geni specifici che regolano i segnali apoptotici. Un intervento di riparazione chirurgica immediata del nervo conferisce una minima neuro-protezione, ma non abolisce completamente la morte cellulare dei neuroni, soprattutto questo approccio non è sempre possibile. I nostri studi hanno dimostrato che un intervento farmacologico usando sia acetil-L-carnitina (ALCAR) che N-acetil-cisteina (NAC) produce una protezione totale dei neuroni a seguito di lesioni dei nervi. Entrambi ALCAR e NAC sono già in uso clinico per altre applicazioni, ed i nostri studi hanno stabilito la miglior dose e la durata di somministrazione. L'efficacia della neuroprotezione di ALCAR e NAC può essere monitorata tramite risonanza magnetica del volume dei gangli dorsali, come dimostrato sia sperimentalmente che clinicamente. L'applicazione farmacologica sui pazienti richiede ulteriori studi, ma i risultati ottenuti finora dimostrano che questo approccio potrà determinare la sopravvivenza dei neuroni ed un miglioramento della rigenerazione dei nervi.

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ABSTRACT. - Following distal nerve injury significant sensory neuronal cell death occurs in the dorsal root ganglia, while after a more proximal injury, such as brachial plexus injury, a sizeable proportion of spinal motoneurons also undergo cell death. This phenomenon has been undervalued for a long time, but it has a significant role in the lack of functional recuperation, as neuronal cells cannot divide and be replaced, hence the resulting nerve regeneration is usually suboptimal. It is now accepted that this cell death is due to apoptosis, as indicated by analysis of specific genes involved in the apoptotic signalling cascade. Immediate nerve repair, either by direct suturing or nerve grafting, gives a degree of neuroprotection, but this approach does not fully prevent neuronal cell death and importantly it is not always possible. Our work has shown that pharmacological intervention using either acetyl-L-carnitine (ALCAR) or N-acetyl-cysteine (NAC) give complete neuroprotection in different types of peripheral nerve injury. Both compounds are clinically safe and experimental work has defined the best dose, timing after injury and duration of administration. The efficacy of neuroprotection of ALCAR and NAC can be monitored non-invasively using MRI, as demonstrated experimentally and more recently by clinical studies of the volume of dorsal root ganglia. Translation to patients of this pharmacological intervention requires further work, but the available results indicate that this approach will help to secure a better functional outcome following peripheral nerve injury and repair.

1. PERIPHERAL NERVE INJURY AND NEURONAL CELL DEATH

Peripheral nerve injury triggers a number of cellular and molecular events which can be fundamental for successful nerve regeneration following nerve repair. Distal to the injury, the process known as Wallerian degeneration is responsible for the dying out of the distal axons and the de-differentiation of Schwann cells. These proliferate and secrete a number of growth factors which have tropic and trophic effects on the regrowing axons [1, 2, 3]. However, a more important and often neglected aspect is the effect of the injury on the neuronal cells. It is now accepted that various factors influence the survival of neuronal cells following peripheral nerve injury, with sensory neurons in dorsal root ganglia more susceptible to cell death following distal nerve injury [3, 4, 5] while spinal motoneurons die only after proximal injury [1, 3, 6].

1.1 Sensory neurons cell death

Following distal nerve injury and nerve repair, limited motor function restoration, due to survival of motoneurons, can compensate

for poor axonal regeneration of sensory motoneurons, but sensory recovery is affected more substantially as the innervation density of target organs is critical for restoration of sensory function [7, 8, 9]. There is no doubt that that neuronal cell death contributes to poor functional recovery following nerve repair, in particular when surgical repair is delayed post-injury. Sensory neuronal cell death starts soon after nerve axotomy. Experimentally it has been shown that a statistically significant cell loss is already evident in the dorsal root ganglia at one week post-injury if repair is not carried out [10, 11, 12]. Loss of sensory neurons increases with time reaching a value of 35-40% of the total neuronal cell population in the dorsal root ganglia at 2 months post-injury [11]. Sensory cell loss varies considerably according to the level of injury along the nerve, with more proximal injury provoking a wider neuronal cell death [13].

These experimental results have been validated in one human post-mortem study [14], where the magnitude of neuronal cell loss appears to correspond to the loss of functional sensory recovery after nerve repair [15]. Although motoneurons survive a distal nerve injury, approximately 30% of motoneurons die after spinal nerve injury, and 50% or more after experimental ventral rhizotomy or root avulsion [5, 6, 16]. As neuronal cells cannot be replaced after death, it is inevitable that regeneration will be poor, with significant loss of sensory and motor functions [7, 9, 17, 18].

Interestingly, cutaneous sensory afferent neurons appear to be more susceptible to cell death than sensory motor afferents [4, 19, 20]. In particular small diameter cutaneous afferent neurons undergo cell death more easily than large diameter cutaneous afferent ones, although there are no clear links to quality of sensation after nerve regeneration. This difference is more likely to be due to the dependence of the small sensory neurons on neurotrophins, such as NGF, BDNF, NT-3 and NT-4, as following injury there is a loss of neutrophic support derived from the target organs [1, 3, 21]. The regenerative potential of both cutaneous and muscular afferent neurons is dependent on the activation of peripherin and ATF3 gene expression, while those neurons lacking regeneration have a significant lower expression of these genes [22]. After injury there is activation of both regenerative and cell death signalling in any type of neuron, and it is the balance between the two that determines whether the neuronal cell will survive and regenerate or die [23].

1.2 Signalling pathways of neuronal cell death

This equilibrium is probably due to various intrinsic and extrinsic cellular signalling pathways, and our experiments have indicated that the balance between intracellular pro-apoptotic and pro-survival signalling determines the fate of the cell. Neuronal cell death is regulated by an apoptosis pathway, and regulating this pathways are the pro-survival mediator Bcl-2 and the pro-apoptotic Bax, which interact at the mitochondrial level determining their membrane permeabilization [24, 25, 26]. The predominance of Bax signalling leads to the formation of pores in the mitochondrial membrane, allowing the release of cytochrome C and other apoptotic molecules that cause activation of the caspase cascade [27], and in particular of caspase-3 which exerts a death-inducing action on cells [28, 29]. Following differential nerve axotomy of medial gastrocnemius nerve or sural nerve, gene analysis was carried out on the corresponding sensory neurons, which have been previously shown respectively to survive or die following nerve injury [20]. Interestingly, the survival of the muscle afferent sensory neurons of the gastrocnemius nerve corresponded to a significant upregulation of the pro-survival Bcl-2 gene and downregulation of Bax and caspase-3, while in the cutaneous sensory afferent neurons there is an upregulation of the pro-apoptotic Bax and caspase-3 gene expression, which is the likely cause of their death [30].

It is evident from all these studies that neuronal cell death is a significant and common occurrence after nerve injury, which if not addressed would results in deficient axonal regeneration and loss of functional recuperation. Neuronal survival is essential for regeneration [1], and immediate nerve repair after injury reduces loss of both sensory and motor neurons [11, 31]. Although early repair is recommended where appropriate, it is recognized that this surgical strategy is not always applicable [32], and a pharmacological approach to neuroprotection is therefore essential.

2. Pharmacological neuroprotective strategies

Neuroprotective intervention has been studied widely for the central nervous system [33, 34], but very little has been reported for the peripheral nervous system, and it is generally related to the administra-

tion of neurotrophic factors [3, 35]. However, this latter approach has limited clinical relevance as different neuronal sub-populations respond specifically to the administration of different neurotrophic factors, hence the need to use different mixtures of growth factors for each type of nerve. Furthermore, side effects of such treatments have discouraged their clinical application [36, 37]. More recently, two pharmacological agents, acetyl-L-carnitine (ALCAR) and N-acetyl-cysteine (NAC), have been shown to have the requisite neuroprotective potential, possibly due to their antioxidant effect, and both are established as safe clinical pharmaceutical agents [38].

2.1 ALCAR

Systemic administration of ALCAR after experimental sciatic nerve injury without repair reduces significantly neuronal cell loss in dorsal root ganglia, and this neuroprotective effect is maintained up to 2 months post-injury [39]. A dose response study has established an optimal dose of 50mg/kg/day [40], and a delayed administration of 24 hours post-injury still has efficacy in preventing neuronal cell loss [41], another important point for the future clinical translation of this treatment. Interestingly, ALCAR administration promotes nerve regeneration separately from its neuroprotective effect [42] with increased target organ reinnervation [43]. Unfortunately, ALCAR does not have a neuroprotective effect on motoneurons, which would limit its applicability only to distal nerve injury.

2.2 NAC

A neuroprotective effect has also been demonstrated for NAC in both sensory [44] and motor neurons [16]. Systemic administration of 150mg/kg/day was optimal for sensory neuronal survival after experimental sciatic nerve injury [45], and a treatment duration of 4 weeks reduces the cell loss to only 5%, with total neuroprotection if the NAC is administered for 8 weeks post-injury (unpublished). A higher dose of 750mg/kg/day was more effective for motoneuron survival after ventral rhizotomy [16], but importantly there was no loss of neuroprotective effect if the administration of NAC was delayed for 1 week after root avulsion [16]. The anti-apoptotic effect of NAC administration was demonstrated by the preservation of the mitochondria structure in sen-

sory neurons following nerve injury [44], which is consistent with the marked downregulation of the pro-apoptotic genes Bax and caspase-3 in cutaneous sensory afferent neurons following sural nerve injury and NAC administration [30], confirming the neuroprotective properties of this compound.

2.3 Clinical application

The problem faced by the clinician in applying these neuroprotective strategies is how to define the success of the treatment. In the experimental studies described above the dorsal root ganglia and spinal cord are harvested from the animal at the end of the experiments, and neuronal counts are carried out on with stereological techniques [46]. However, it was shown that the neuronal loss observed after nerve transection is correlated with the volume of the axotomized dorsal root ganglia [11]. In the past, magnetic resonance imaging (MRI) has provided a non-invasive assessment of rodent peripheral nervous system structures [47, 48], and in our experience it has been useful to determine the volume of dorsal root ganglia as a proxy measure of neuronal cells death or survival. Following rat sciatic nerve axotomy, we have compared morphological measurement of sensory ganglia volume and neuronal cells death with calculation of volume carried out on MRI images. There is a strong correlation between the morphological and MRI results, with the MRI volume of the dorsal root ganglia being a representative measure of the number of neurons in the ganglia [49].

Hence, it appears that MRI volumetric quantification of dorsal root ganglia can be developed clinically as a non-invasive measure of sensory neuronal survival after ALCAR or NAC treatment. As a precursor to such a clinical trial, we have carried out morphological assessment of numbers of sensory neurons and ganglia volume on cadavers and compared them with the ganglia volume of healthy volunteers, and of patients that have suffered arm amputations and median/ulnar nerve injuries [50]. Using cadaver ganglia, we have shown that there was low inter-individual variability in DRG neuronal counts and volume, and strong correlation between counts and MRI volume measurements. MRI also gives good resolution for volumetric analysis in patients, confirming the validity of proxy measurement of sensory neurons in human, but further clinical studies are necessary to determine the sensitivity for the MRI technique used following different nerve injuries at different times.

3. Conclusions

The functional outcome of peripheral nerve injury remains inadequate despite the technical advances of microsurgical nerve repair. The understanding of the cellular and molecular mechanisms underlying the neuronal response to nerve injury have indicated that a combined surgical and neuroprotective approach could give the best results. The work presented here shows that pharmacological intervention can be used to prevent neuronal cells death, and that the efficacy of the treatment can be assessed non-invasively.

Although initially these drugs should not be given outside the framework of an appropriate clinical trial, with a specific and sensitive outcome measure, there is little doubt that in future such applications will result in an improved functional outcome for the patients allowing them to return to a better work and social lives.

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