Nervous immunity: A study on the role of complement system in neuronal degeneration and regeneration
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Chapter 6:

Functional recovery after transection-resuture injury of the sciatic nerve is not influenced by the terminal complement pathway in rats

Abstract

Complement activation and deposition of the membrane attack complex (MAC) are essential for rapid degeneration of peripheral nerves after crush injury. Deficiency for complement component 6 (C6−/−), which is essential to form MAC, slows down degeneration, but facilitates regeneration in a rat sciatic nerve crush model. Inhibiting MAC might therefore be used as a therapy to improve nerve regeneration after peripheral nerve trauma.

In this study we determined the effect of C6 deficiency on degeneration and regeneration in sciatic nerve transection-resuture injury in a rat model. This lesion differs from crush injury, the integrity of the nerve and blood nerve barrier is abolished, which eases influx of hematogenous factors. Even though the area of initial damage is smaller, in terms of micrometers compared to millimeters in the nerve crush model, recovery is much slower and incomplete. This is probably due to the fact that the contact with the distal nerve is completely lost, whereas in the crush injury model a part of the connection between proximal and distal part of the nerve is still intact, facilitating outgrowth of axons through the damaged area.

In this study we compared degeneration and regeneration of the nerve in WT and C6−/− animals and observed a similar degeneration and regeneration process. Low levels of complement activation were observed in WT and C6−/− animals after injury. A massive influx of phagocytes and cells was found in the transection area at 3 days post injury (d PI). Clearance of myelin and axonal debris was observed mainly in the transection area. Regeneration was slow in both animal groups and never reached full recovery. Signs of axonal regeneration and remyelination were observed at 8 weeks post injury (wk PI) in the distal tibial nerves. Morphologically no differences were observed between WT and C6−/− animals. Functional tests showed that the regeneration process started earlier in WT animals, but towards the end the difference between the groups diminished.

Interpretation: transection injury results in low levels of complement activation, since the initial area of damage is small. The massive influx of cells rapidly clears the remaining activation epitopes. Due to the very low level of complement activation levels in the WT animals the previously observed beneficial effect of complement inhibition on nerve regeneration was not present.

Conclusion: the type of nerve injury and committant complement activation should be taken into account before complement inhibition as a therapeutic target in nerve regeneration is considered.
Introduction

Injured peripheral nerves undergo a process called Wallerian Degeneration (WD), during which the nerve degenerates from the first node of Ranvier proximal of the damaged site all the way to its target (Waller, 1850). Myelin and axons degenerate and are removed from the nerve by phagocytes (Friede and Bruck, 1993; Omura et al., 2005). After WD the nerve can regenerate by new axons sprouting from the proximal nerve stumps guided by extracellular matrix proteins secreted by dedifferentiated Schwann cells (Chen et al., 2005; Tomita et al., 2007). Finally, the Schwann cells will engage in a one to one relationship with the newly grown axons and start remyelination. This results in a regenerated functional nerve.

However, this regeneration process is slow. Growing axon sprouts have a maximum speed of 2.5 mm a day (Sunderland, 1947; Wood et al., 2011). In an adult human, the distance the nerve has to span from the spinal cord to the foot is over 750 mm. Thus, if a nerve is damaged close to the spinal cord, regrowing at maximum speed recovery will take 300 days.

Not only is the process slow, often the regenerated nerve does not function properly, being either slower in nerve conduction or less sensitive than the undamaged nerve (Cragg and Thomas, 1964; Walbeehm et al., 2003). This is partly due to the smaller distance between the nodes of Ranvier in new grown nerves, but more often a result of improper remyelination.

During WD the complement system is activated, a scheme of the complement system is shown in chapter 1, Fig. 1. The classical pathway is involved in activation since C1q binding is seen on damaged cell products. This results in conversion of C3, results in C3 convertase and C5 cleaving. C5b will bind to the damaged cell membrane and serves as an anchor for MAC. Upon binding of C5b to the target membrane, C6, C7, C8 and C9 are recruited and together compose a barrel-shape structure that forms a pore in the target membrane, MAC. The alternative pathway serves as an enhancer of complement activation (for detailed review of the complement system see (Ricklin et al., 2010 or Alexander et al., 2008).

Previous studies showed that MAC formation after crush injury is important for WD, which was thought to be a prerequisite for regeneration (Ramaglia et al., 2007). However, regeneration of the sensory and motor function was more efficient in complement C6 deficient animals. Both functional and neuropathological examinations showed that the nerves of complement deficient animals recovered faster. Reconstitution of C6 deficient animals with C6, restoring complement function of the complement system, resulted in a delay in regeneration after crush injury, i.e. a shift towards the WT phenotype (Ramaglia et al., 2009). The results obtained from these studies indicate that the inhibition of MAC after peripheral nerve trauma can speed up nerve regeneration and ultimately improve regeneration outcome.

To determine whether inhibition of MAC formation after peripheral nerve trauma always improves recovery we studied a different type of injury, the transection-resuture injury. This type of injury is very severe; the regeneration of the nerves is often incomplete and slow. Furthermore, the regenerated nerve fibers are often misdirected and reinnervate a “wrong” target (Evans et al., 1991; Tomita et al., 2007).

In this study we analyzed the effect of complement C6 deficiency a transection-resuture injury. We compared WT and C6-/- animals at 3 d PI, 8 and 12 wk PI to determine how the inability to form MAC influenced degeneration and regeneration. In the nerve transection model the sciatic nerve was cut with micro scissors at the sciatic notch, immediately after transection...
the nerve was re-aligned and the epineurium resutured, with three filaments carefully placed in the epineurium. Resuturing of the nerve after transection will improve axonal guidance, without not fully restoring it. The differences between transection and crush injury are: the area of initial damage is smaller in transection injury compared to the crush injury. Another difference is that the integrity of the epineurium is disrupted in a transection injury, which severs axonal guidance and opens the blood-nerve-barrier. To study degeneration, we examined nerve morphology at 3 d PI. Activated macrophages are responsible for rapid clearance of debris from the degenerated nerves (unpublished data M. Sta et al. (chapter 2)), and (Bruck et al., 1996; Friede and Bruck, 1993). Presence and localization of phagocytes were determined with quantitative immunohistochemistry (Brald and Linington, 1996; Ramaglia et al., 2009). To study regeneration we performed in vivo functional tests. Sensory recovery was measured by the foot flick test and motor function recovery was determined using an alternative cat-walk test to determine a sciatic function index (SFI). Additionally, morphology of the regenerated tibial nerves was studied in semi-thick sections at two heights in the tibial nerves at 8 and 12 wk PI.

Materials and methods

Animals

Care and use of the animals in this study were approved by the Animal Care and Use Committee of the Academic Medical Center (AMC) and conform the protocols described 100763-101 en 100763-105 part of reference protocol DNL 1000/100663, which is conform national guidelines. For these experiments WT Piebald Virol Glaxo (PVG) (HSD/ola Harlan, UK) (www.harlan.com, 2013) and C6⁻/⁻ PVG (ARIA, AMC, the Netherlands) 12 weeks old ± 250 g male rats were studied. Animals were allowed a one week acclimatization period, were housed together in standard pertex cages under standard conditions and received food and water ad libitum. During the experiment animals were weighed and checked daily.

Genotyping of PVG C6⁻/⁻ rats

Breeding couples were genotyped to check if all animals had the homozygous C6 deficient genotype. C6⁻/⁻ animals carry a 31 basepair (bp) deletion in the C6 gene. Rat genomic DNA was prepared by proteinase K degradation of ear biopsies. Genotyping was performed by PCR (Ramaglia et al., 2007).

Surgery

Animals were anesthetized using 2.5% Vol isoflurane, 1L/min O₂ and temgesic 0.1 mg/kg. An incision was made at the height of the sciatic notch, gluteal muscle was split and the sciatic nerve exposed. The nerve was cut with Vannas scissors # 9600, 5 mm x 0.5 mm with straight, pointed blades (Moria, France), making sure there was as little compression of the nerve as possible. The nerve stumps were immediately realigned and reconnected with 3-4 sutures using Ethicon 0-10 wire. Muscles were massaged back into place and the wound was closed with surgical clips. Animals received 0.1 mg/kg temgesic for three days after surgery.

Degeneration

Animals were kept for 3 days (7 WT, 7 C6⁻/⁻) after nerve transection. At 3 d PI animals were terminated and both sciatic nerves, dorsal root ganglions (DRGs) L4 and L5 of transected
nerve and L5 of control nerve and the spinal cord 2 cm from the last rib down were harvested. Tissues were post fixed in 4% paraformaldehyde solution and further processed for analysis.

**In vivo functional tests**

Animals were kept for 8 or 12 weeks (8 wk: 8 WT, 8 C6/−; 12 wk: 8 WT, 8 C6/−). Functional tests were done weekly. Sensory recovery was assessed in vivo with a foot flick device. An electrical stimulus, with a variable stimulus intensity ranging between 20 and 50 µA, is given on a fixed position on the rat’s foot (de Koning et al., 1986). The stimulus intensity was gradually increased in 12 steps and the minimum current necessary to let the rat retract its paw after the stimulus threshold had been recorded. Intensity at which the foot was retracted was used as read out and normalized and plotted as mean ± SEM. The same foot flick assay was applied to the control leg, whose response was set to 100%. Before measurement, a check was done to determine proper function of the machine and responsiveness of the animal. Additionally, to avoid learned responses, animals were tested with and without current as well as the response to the sound of the switch (van der Hoop et al., 1988). Motor recovery was monitored from week 3 onward, by a footprint test. Rats had to walk on an ink pad and then on a sheet of paper. Parameters of the footprint were measured, print length (PL), toe spread (1st-5th toe) (TS) and intermediary toe spread (2nd-4th toe) (IT) for both the uninjured “normal” leg (N) and the injured “experimental” leg (E). SFI was calculated using the following formula:

\[-38.8 \times \left(\frac{EPL}{NPL}\right) + 109.5 \times \left(\frac{ETS}{NTS}\right) + 13.3 \times \left(\frac{EIT}{NIT}\right)\]

Normal SFI is acquired by analyzing the SFI before afflicting the injury and values were -9%.

Due to the development of neuropathic pain, some rats showed autotomy of the toes, these rats were immediately excluded from the experiment. Autotomy was prevented using Byte-X on the toes daily from week 8 onward.

**Tissue processing**

Animals sacrificed by exsanguination under deep isoflurane anesthesia, subsequently animals were perfused with 2% paraformaldehyde solution. Harvested tissues were post-fixed in 4% methanol buffered paraformaldehyde solution for immunohistochemistry and immunofluorescence, and processed for paraffin embedding. Nerves were embedded for transverse or longitudinal sectioning. 6µm sections were used. Cross sections were obtained at the transection site and at 5 mm distal of it.

For semi-thin sections, nerves were post fixed in MacDowell’s fixative (2% paraformaldehyde solution in sodium cacodylate buffer, with glutaraldehyde) and processed for EM by rinsing in sodium cacodylate buffer, washing in water and incubating in osmium tetroxide for 1 hour. Nerves were dehydrated in increasing % of ethanol, 70%, 80%, 90%, 96% and 100%. After dehydration nerves were transferred to polypropylene oxide, and polypropylene oxide and epoxy (1:1) and pure epoxy overnight. The nerves were cut in two, embedding the far distal and the middle parts of the tibial nerve for sectioning; epoxy was hardened for 72 hours at 60°C.
Semi-thin (1µm) sections were made and stained with thionine and examined light microscopically.

**Immunohistochemistry**

Sections were stained using a three step immunoperoxidase method at room temperature (RT). After deparaffination and rehydration the endogenous peroxidase activity was blocked with 1% H₂O₂ in methanol for 20 minutes. Antigen retrieval in the microwave was performed on all sections, for 3 minutes at 800W and 10 minutes at 440W in 10mM Tris/1mM EDTA (pH 6.5). Aspecific binding of antibodies was blocked using 10% normal serum in Tris buffered saline (TBS) of the secondary antibody host for 30 minutes (Dako Cytomation). Primary antibodies were diluted in 1% bovine serum albumin (BSA) and incubated for 90 minutes. Sections were incubated with biotinylated goat anti mouse or goat anti rabbit 1:200 in 1% BSA for 30 minutes and 30 minutes in horseradish peroxidase labelled streptavidin, 1:400 in 1% BSA. Detection was done by incubating the slides with 0.05% 3-amino-9-ethylcarbazole in acetate buffer (pH 5) for 5 minutes, followed by a counterstain with hematoxylin for 30 seconds. Slides were mounted in gelatin.

For immunofluorescence, no blocking of endogenous peroxidase activity was done. Primary antibodies were detected with directly conjugated goat anti-rabbit-FITC (green, 488nm) or sheep anti-mouse-Cy3 (red, 568 nm) (Sigma-Aldrich, Saint Louis, MI, USA). As a nuclear counterstain 4,6-diamidine-2-phenylindole, dihydrochloride (DAPI) (blue, 350 nm) was used. Sections were mounted in vectashield (VECTOR, Burlingame, CA, USA). For all stainings, a slide on which only the secondary antibodies were used was employed as a control.

**Quantification of cells**

Five non-consecutive 6µm sections were used for analysis. Between the sections an inter-space of 30µm was used. Per section, positive cells containing nuclear staining were counted in the entire nerve area by two individual researchers. The surface of the nerve area was calculated with ImagePro 7.0 software (Media Cybernetics, The Netherlands) using the area measurement tool in mm². Positive cells were divided between the area to calculate the amount of positive cells per mm².

**Results**

**Degeneration**

**Morphology**

We studied morphology of the transected nerves at 3 d PI, in transversal and longitudinal sections of injured nerves, stained for myelin basic protein (MBP, green) and neurofilament (NF, red). In the transected nerves, 3 d PI, damage of myelin and axons is found directly in and around the transection area (Fig.1D for localization in a longitudinal section of the transected-resutured nerve). A massive influx of cells, swelling of the axons and collapsed myelin sheaths (arrows) are observed in the distal area adjacent to the transection area of WT (Fig.1B) and C6⁻/⁻ animals (Fig.1C). The C6⁻/⁻ animals seem to show more remaining myelin debris. In figure 1 A we show an uninjured control nerve, in these nerves myelin surrounds the neurofilament (asterisk). WT and C6⁻/⁻ transected nerves, both retained some axons which displayed normal morphology (asterisks in Fig. 1 B and C). Analysis of the longitudinally
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Sectioned injured nerve (Fig. 1D) showed that most axonal and myelin damage was found in the transection site, with some spreading into the distal stump.

**Figure 1: Morphology of the transected nerves at 3 d PI.** Cross and longitudinal sections stained for neurofilament (Smi31; red) and myelin (MBP; green). Representative photomicrographs of uninjured (A), transected WT (B) and C6\(^{-/-}\) (C) nerves (D) longitudinal section of the transection site. Normal myelin and axons are found in the uninjured nerve, shown in A, myelin surrounds the axon (*). Only the nodes of Ranvier are unmyelinated (insert a). B and C cross sections in the transection site. Transected nerves (B, C) show collapsed myelin (arrows) and axonal swelling. DAPI staining for nuclei showed a massive amount of nuclei present in the transection area. No difference in the amount of axonal and myelin damage can be found between WT (B) and C6\(^{-/-}\) (C) animals, although it looks like the C6\(^{-/-}\) animals have more debris inside the nerve. Scale bar (A) equals 50µm. The longitudinal sections shows a massive influx of nuclei in the transection area (T) and also shows the changes in axonal and myelin morphology just distal and proximal of the transection site. It can be seen that most of the visible damage is around the transection site. Some changes in morphology distally suggest the damage has spread.

The mild decrease in axonal and myelin staining indicates that, in contrast to the situation in a crush injury, the damage in the transected nerves spreads only slowly. At 3 d PI the damage did not reach the point of 5 mm distal of the injury site (not shown), 5 mm distal of the lesion is the area where in crush injury most damage was visible.

**Complement activation**

The complement system (Fig. 2) was activated in both the WT and C6\(^{-/-}\) nerves, although only moderately. The amount of C1q deposition in the C6\(^{-/-}\) nerves (Fig. 2B) was similar to the levels found in the WT (Fig. 2A) animals (Fig. 2C). No C1q immunoreactivity was observed in uninjured nerves (Insert a in Fig. 2A) and at 5 mm distal of the transection area in the injured nerves (not shown).

C9 deposition, as a marker for the presence of MAC, was also analyzed by immunohistochemistry. We found some C9 staining in WT animals (Fig. 2D), and as expected very little in C6\(^{-/-}\) animals (Fig. 2E). Analysis of cross sections at different positions in the transected nerves showed that C9 was present close to the transection site (Fig. 2D) and
reduced in the C6 deficient animals (Fig. 2E). There was no C9 deposition detectable at 5 mm distal of the transection site (not shown). Differences in C9 deposition near the transection site between WT and C6\(^{-/-}\) animals were significant (p < 0.001) (Fig. 2F).

**Figure 2:** C1q and C9 positivity in transected nerves 3 d PI. Representative photomicrographs of cross sections of WT (A, E) and C6\(^{-/-}\) (B, F) transected nerves, stained for C1q (A, B) and C9 (E, F), scale bar in A equals 100µm. Quantification of the amount of positivity per area for complement factors C1q (C) and C9 (G). Error bar shows SD. Statistical analysis is done using one-way ANOVA and Bonferroni correction, significance is indicated with asterisks. In all nerves activation and deposition of complement components is relatively low. The WT nerve shows positivity for C1q (A), as well as deposition of C9 (E). The C6\(^{-/-}\) nerve shows positivity for C1q (B), but not for C9 (F). No difference was found in the amount of C1q deposition (C) between WT and C6\(^{-/-}\), both are significantly higher than C1q levels in the uninjured nerve. The C9 deposition (G) was significantly higher in WT animals compared to control and C6\(^{-/-}\). There was no difference between the C9 deposition in C6\(^{-/-}\) nerves compared to the control nerves.

**Phagocytes**

Although, not much complement activation is found and damage progression distally is little, there is a massive influx of cells at the injured site (Fig. 3). The distal spread of the influx is quite limited: only a few cells were found within the nerve at 5 mm distal of the injury. A large proportion of these invaded cells were CD68 positive. Quantification of the amount of CD68 positive cells showed no differences between the WT (Fig. 3A) and C6\(^{-/-}\) (Fig. 3B) animals in macrophage numbers in the transection area (quantification graphs Fig. 3C).

**Regeneration**

**In vivo functional tests**

We studied the regeneration process in vivo with two functional tests. For sensory recovery we used foot flick analysis (Fig. 4A) to calculate the percentage of sensory recovery where the uninjured nerve response is set to 100%, and for motor function recovery we used a print test to calculate the SFI (Fig. 4B). The foot flick analysis showed that the regeneration process starts a bit earlier in WT, which was significant between 6-8 weeks after injury (asterisks, Fig. 4A).
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There were no significant differences between the WT and the C6−/− animals in terms of speed and degree of functional recovery. For both genotypes, the in vivo regeneration tests showed overall poor recovery. The animals never attained normal values. For motor function, as measured by SFI, WT and C6−/− animals recovered to only 30% of normal function, shown by the dotted line at -9 (Fig. 4B). A major problem in this test was the development of neuropathic pain, due to which animals avoided using their paw. This occurred at week 8, 9 and 10. At these time points we also observed autotomy of the toes in some rats, which were immediately excluded from the tests.

Figure 3: Macrophages (CD68; ED1; red) and myelin (MBP; green) positivity at 3 d PI. Representative photomicrographs of transection area of WT (A) and C6−/− (B) nerves, stained for CD68 (red) and MBP (green), nuclei are blue (DAPI), scale bar equals 400µm. Quantification of the amount of CD68+ cells in the transection area (C). Error bar shows SD. Statistical analysis used a one-way ANOVA with Bonferroni correction, significance is indicated by asterisks. In both WT and C6−/− there are CD68+ cells present in the transection site, some of which progressed further inside the nerve area. The WT nerve seems to be more positive for CD68, compared to the C6−/− nerve (B). In the inlays in both A and B there is a 100 times magnification of the CD68+ cells. As can be seen there is no evidence for phagocytosis of myelin by these CD68+ cells, since there is no colocalization of CD68 with MBP. Furthermore, there is a massive influx of mononuclear cells inside the transection area; however most of these cells are not positive for CD68+. Quantification of the amount of CD68+ cells (C) showed that both WT and C6−/− animals have higher numbers of CD68+ cells than in the controls, no difference between WT and C6−/− was found.
Morphology of the regenerated nerves

The tibial nerve was studied at two positions to analyze the morphology of the regenerated nerves. The proximal part (supplementary Fig. 1) located in the middle of the tibial nerve, halfway of the calf muscle, and the distal part, located at the ankle of the animal (Fig. 5 and supplementary Fig. 1), were analyzed at 8 and 12 wk PI to follow the regeneration process. For comparison, the uninjured distal portions of the nerves are also shown in figure 5 (A, B). The proximal parts of the tibial nerve (supplementary Fig. 1B, D) at 8 wk PI showed signs of regeneration: new axons, some of which were already thinly myelinated. Inter-axonal space was quite large and a lot of cell nuclei could still be seen. There were no differences in the amounts of myelinated and unmyelinated regenerated axons. The distal portion of the tibial nerves (Fig. 5 B, D) also showed myelinated axons like those found in the proximal part of the nerve, only with a smaller diameter.
At 12 wk PI we found that the nerve fibers in the proximal part of the tibial nerve (supplementary Fig. 1 C,E) were larger in diameter, more regenerated myelinated fibres were found and the inter axonal space was smaller than at 8 wk PI. Furthermore, there were fewer visible nuclei between the axons. Also in the distal part of the tibial nerves at 12 wk PI (5 C, E), more regenerated myelinated fibres were found, although the diameter of the axons was slightly smaller than of those found in the proximal tibial nerve, the inter axonal space was also smaller. There were no morphological differences between the regenerated nerves of WT and C6−/− animals at either 8 and 12 wk PI.

Discussion

There are two major findings in this study. First there is no significant difference in neuronal degeneration and regeneration in WT and complement deficient rats after transection-resuture injury. Second; the induction of complement activation is only minor. These findings differ from findings we observed in prior crush injury studies of the sciatic nerve, where strong complement activation was seen (Ramaglia et al., 2009).

These findings can be explained by the fact that there are differences between transection and crush injuries. In a transected nerve, the integrity of axons, myelin, epineurium and blood-nerve-barrier is disrupted, exposing the axon to a different environment and the physical separation of the two nerve ends will have a severe impact on regeneration. The regenerating axonal growth cone must pass this barrier. In a crush injury the epineurium remains intact, providing a barrier between blood and nerve and a tube in which new axon sprouts can grow in the right direction.

Another striking difference between crush injured and transected nerve is the initial damage inflicted on axons and myelin. The damaged area in a transection, although it is a severe injury, is only small. The distance over which the axons and myelin are damaged, is in terms of micrometers versus the millimeters damaged in a crush injury. This reduces the amount of exposed molecules that can induce complement activation. In this way the level of complement activation and cellular infiltration will be much less than in a crush injury. In the crush model most damage and complement positivity was observed at 5 mm distal of the crush area (Ramaglia et al., 2007), whilst in the transection-resuture model most positivity was observed at and distal, next to the transection site at 3 d PI.

Even in the transection area, the amount of complement activation is low. In contrast to crush injured nerves, WT and C6−/− transected nerves show low levels of C1q deposition and the amount of C9 or MAC deposition was also low in WT animals at 3 d PI.

In a crush injury model, the complement system is likely an important trigger for the attraction and activation of endoneurial and hematogenous cells. In the transection injury model we do observe a massive influx of these cells, even when the level of complement activation is low. The disruption of the blood-nerve barrier might explain why there is such a massive influx of blood derived cells in the nerve transection area, whilst there is only little complement activation observed in the nerves. In addition, the nerve is exposed to cellular and humoral components straight from the bloodstream.

The low levels of complement activation observed in the transection-resuture model might explain why the effect of C6 deficiency and the inability to form MAC have no beneficial
effect on the degeneration and regeneration of these nerves, an effect previously observed in a small surface crush injury model (Chapter 4).

We observed an earlier onset of sensory recovery in the WT animals, although the differences between WT and C6⁻⁻ animals were lost over time. The restoration of motor function did not differ between WT and C6⁻⁻ animals. In contrast to the observations in the crush injury model (Ramaglia et al., 2009), animals did not regain full functional recovery at 12 weeks after transection injury. The sensory recovery seems to show a small dip after week 10, this might be explained by the development of neuropathic pain. Animals showed contraction of the muscles and avoided use of the injured paw. We observed autotomy of the toes in some animals as well around week 10; these animals were excluded from the trial.

**Figure 5: Morphology of the regenerated transected nerves.** Representative photomicrographs of semithin cross sections of the distal tibial nerve of the transected nerves at 8 (A, B, D) and 12 (C, E) weeks PI (wk PI). The scale bar in A equals 20µm. Uninjured nerve (A) show a thick myelin sheath surrounding large axonal fibres. The inter-axonal space is small and few cells are found inside the nerve area. The regenerated WT nerves (B, C) show at 8 wk PI (B) some regenerated fibres, although these are small, most of them are myelinated. Inter-axonal space is large and many nuclei are visible, at 12 wk PI (C) the number of axons, the diameter and the myelin layer are increased compared to 8 wk PI. The inter-axonal space is smaller. In the C6⁻⁻ nerves at 8 wk PI (D) we found similar changes than in the WT nerves. Same is true for the nerves at 12 wk PI (E).

It is interesting how the differences in complement activation between crush-injured and transected nerves could result in such diverse outcomes. Maybe the difference in damaged surface area explains why complement activation is so low in transected nerves. We previously showed (chapter 4) that the amount of complement activation is related to the amount of initial damage to the nerve. In the transection-resuture model the area of initial damage is small, in the range of micrometers instead of millimeters indicating that the amount of exposed complement activation molecules is also small. Additionally, massive influx of phagocytes and other mononuclear cells in the transection area was observed, which might be responsible for rapid clearance of these molecules (Hirata and Kawabuchi, 2002; Stoll et al., 1989). This rapid loss of complement activation molecules may be the reason why complement activation levels remain low during degeneration.
We can conclude that in case of low levels of initial complement activation no beneficial effect of complement C6 deficiency on the regeneration of peripheral nerves is observed.

The data from the transection-resuture model also suggest that a little complement activation might even be beneficial, as shown by the slightly earlier start of sensory regeneration in the WT animals. That initial complement activation is beneficial for proper WD and regeneration of the nerves has also been shown by other studies (Camara-Lemarroy et al., 2010; Kosins et al., 2012), where the depletion of C3 resulted in poor regeneration of injured peripheral nerves. Addition of a botulinum-derived C3 peptide promoted functional motor recovery in crush-injured and transected peripheral nerves (Huelsenbeck et al., 2012).

Taken together these data and the knowledge obtained from crush injury models (de Jonge et al., 2004; Maurer et al., 2002; Ramaglia et al., 2007; Ramaglia et al., 2008; Ramaglia et al., 2009), leads us to pose that, complement inhibition can have a positive effect on nerve regeneration, but only if there is enough damage. One should therefore take the type of injury into account before using complement inhibition as an intervention for enhancing post traumatic nerve regeneration.

Reference List


Part I: chapter 6


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Supplementary figure 1: Morphology of the regenerated transected nerves, proximal and distal tibial nerves at 8 and 12 wk PI (Fig. 5 extended). Representative photomicrographs of semithin cross sections of the proximal (left) and distal (right) tibial nerve of the transected nerves at 8 and 12 wk PI. The scale bar in A equals 20µm. A: uninjured nerve of WT. In proximal and distal uninjured tibial nerves (A) thick myelin sheath surrounding large axonal fibres are found. The inter-axonal space is small and few cells are found inside the nerve area. B, C: show regenerated WT nerves at 8 wk PI (B) and 12 wk PI (C). At 8 wk PI regenerated fibres are found in the proximal part of the tibial nerve (left), although some are large, most are small. Most fibres are myelinated. In the distal portion of the nerve a similar situation is found, however the axon diameter appears to be smaller. Inter-axonal space is large and many nuclei are visible. At 12 wk PI (C) the number of axons, the diameter and the myelin layer are increased compared to 8 wk PI in both proximal (left) and distal (right) portions of the tibial nerve. The inter-axonal space is smaller. D, E: show regenerated C6−/− nerves at 8 wk PI (D) and 12 wk PI (E). In the C6−/− nerves at 8 wk PI (D) we found similar changes than in the WT nerve. Same is true for the nerves at 12 wk PI (E).