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Delayed nerve repair increases number of caspase 3 stained Schwann cells

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ABSTRACT

Caspase 3 staining in Schwann cells was investigated with immunohistochemistry, as a measure of Schwann cell apoptosis, after transection and immediate (day 0) or delayed rat sciatic nerve repair (30, 90 and 180 days post injury). Cleaved caspase 3 stained Schwann cells significantly increased at the site of lesion (SNL; median [IQR], 15.2 [7.0] %) and in the distal nerve segment (SND; 9.5 [3.6] %) 10 days after immediate repair. The number of cleaved caspase stained Schwann cells also increased significantly after delayed repair, irrespective of length of delay, at both locations (SNL: 22.0–27.1%; SND: 18.5–22.1%; $p < 0.05$). Some cleaved caspase 3 stained satellite cells were seen in dorsal root ganglia on the injured side, but no stained motor or sensory neurons were observed at any time-point. Delayed nerve repair is associated with more pronounced Schwann cell apoptosis which may explain impaired nerve regeneration after nerve injury and delayed repair.

Delay of the repair of nerve trunks results in impaired regeneration, particularly after brachial plexus injuries [14]. The basis of such impaired regeneration can be attributed to alterations in and the death of Schwann cells and neurons during the delay in response to the injury [6,9,15,18,20,23]. In the distal nerve segment, Schwann cells rapidly respond to nerve transection with proliferation [24], preceded by up-regulation of e.g. Erk1,2 [19], but Schwann cells and neurons may also die by caspase mediated apoptosis [3–5,17,22,27,29,30]. The cascade of caspases can be triggered by release of proapoptotic molecules from mitochondria, but also through cell surfaced molecules [2,26,28]. Our aim was to examine and quantify apoptosis in Schwann cells by immunostaining of cleaved caspase 3 after transection and immediate and delayed nerve repair in rat sciatic nerve.

Twenty female Wistar rats, body weight around 200 g, were anaesthetised after approval of the Local Animal Ethical Committee at Lund University. In addition, measures were taken to minimize pain and discomfort (e.g. Temgesic®) postoperatively. The sciatic nerve in the hind limb was exposed and transected. In five rats the transected sciatic nerve was immediately repaired with three epineurial sutures. In the remaining 15 rats the ends of the proximal and distal nerve segments were positioned so that growth of axons over the gap was prevented, i.e. turned away from each other. The skin was sutured and the animals were allowed to recover. After a delay period of 30 ($n = 5$), 90 ($n = 5$) and 180 ($n = 5$) days the transected sciatic nerve stumps were mobilised and retransected to allow coaptation. The sciatic nerve was repaired with three epineurial sutures. After an additional 10 days spinal cord, L4 and L5 dorsal root ganglia (DRG) and 30 mm (15 mm on either side of the site of lesion) of the sciatic nerve were harvested, fixed in Stefanini solution and then transferred to 20% sucrose in phosphate buffered saline (PBS) for cryoprotection (for details see Ref. [23]).

The 30 mm long sciatic nerve segment was transected at the site of lesion and the proximal and distal nerve segments were longitudinally sectioned (10 μm thick sections). The sections were rinsed in PBS for 3 × 5 min where after they were incubated with an antibody to cleaved caspase 3 at a dilution of 1:200 (In vitro Sweden AB, Stockholm, Sweden) in 0.25% bovine serum albumin (BSA) and 0.25% Triton-X in PBS at 4 °C overnight. After an additional rinse in PBS 3 × 5 min, the sections were incubated with anti-rabbit Alexa Fluor (488) at a dilution of 1:500 (Invitrogen, Lidingö, Sweden) for 1 h at room temperature. The sections were mounted after washing and counterstaining of nuclei with DAPI.

The total number of cleaved caspase 3 stained Schwann cells (elongated nucleus located within basal lamina) was counted and expressed as a percent of total number of DAPI stained cells [23]. The cleaved caspase 3 stained Schwann cells were counted in three randomly selected sections (100–200 μm apart) from the sciatic nerve. Pictures were taken (size 500 μm × 400 μm) and 100 μm × 100 μm large areas from the distal side of the site of lesion (SNL) and 15 mm distal to the site of the sutures were randomly selected for counting. The number of cleaved caspase 3 stained Schwann cells located in the endoneurial space was also examined 15 mm proximal to the site of nerve suture.

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To elucidate if caspase 3 stained cells were equivalent to Schwann cells double staining with caspase 3 and S-100 was performed. Sections were rinsed in PBS for 15 min and thereafter incubated with anti-cleaved fragment caspase 3 antibody (1:200) as described. After an additional rinse in PBS, sections were incubated with Alexa Fluor (488; 1:500), rinsed again in PBS three times and the anti-S-100 antibody (α/β chain, SC-58839, Santa Cruz Biotechnology, CA, USA; 1:300) in 0.25% BSA and 0.25% Triton-X in PBS were added overnight at 4 °C. After an additional rinse in PBS, sections were incubated with Rhodamine-conjugated anti-mouse goat IgG diluted 1/500 (Cappel; Aurora OI) at room temperature for 1 h. After a final rinse in PBS the sections were mounted in glycerol:PBS 1:1. All sections were examined with a fluorescence microscope with a digital camera system [23].

Sections from spinal cord and dorsal root ganglia were also sectioned (10 μm sections) and stained for cleaved caspase 3 as described above.

The number of cleaved caspase 3 stained Schwann cells (identified by shape and location [23]) per total number of DAPI stained cells (i.e. % cleaved caspase 3 stained cells) at the site of lesion and in the distal nerve segment were expressed as median [interquartile range, IQR]. Kruskal–Wallis analysis was performed with subsequent Bonferoni test [25] to observe differences between groups.

Cleaved caspase 3 stained Schwann cells were observed both at the site of lesion and in the distal nerve segment. Only single positive Schwann cells were seen 15 mm proximal to the site of lesion and in the contralateral uninjured sciatic nerve (Fig. 1). Double staining with cleaved caspase 3 and S-100 revealed that all cells with an elongated nucleus and located within the basal lamina (our definition of Schwann cells) also stained for S-100 indicating that all our selection criteria were correct and cells counted in the sciatic nerve were Schwann cells (Fig. 2). The percentage of cleaved caspase 3 stained Schwann cells at the site of lesion 10 days after repair was 15.2 [7.0] % and 9.5 [3.6] %, in the distal nerve segment when the sciatic nerve was transected and immediately repaired (Fig. 1). Delayed nerve repair showed statistically higher number of cleaved caspase 3 stained Schwann cells at 10 days both at the site of the lesion (SNL; Kruskal–Wallis $p = 0.02$) and in the distal nerve segment (SND $p = 0.01$; Fig. 1b–d). Subsequent Bonferoni test revealed significant difference between immediate nerve repair and repair with a delay of 30 (22.0 [4.6] %) and 180 (27.1 [9.0] %) days, but not after 90 days (24.5 [7.3] %), at the site of lesion (Fig. 1). There was also a significance difference between immediate nerve repair and the other groups (30 days: 18.7 [7.5] %; 90 days: 18.5 [4.1] %; 180 days: 22.1 [8.9] %) in the distal nerve segment (Fig. 1). Thus, an increased number of caspase 3 stained Schwann cells were observed at the site of lesion and in the distal nerve segment when the sciatic nerve was transected and repaired after a delay.

No motor or sensory neurons in the spinal cord and DRG, respectively, showed any staining for cleaved caspase 3 at any time-points on the injured or uninjured sides. However, some cleaved caspase 3 stained satellite cells surrounding sensory neurons were observed in DRG at the injured side, while only single stained satellite cells were seen on the contralateral side (Fig. 2). In addition, single Schwann cells could be observed in the dorsal root ganglia at the injured, but not on the uninjured side.

We showed that cleaved caspase 3 stained Schwann cells are present 10 days after immediate nerve repair at the site of the lesion and in the distal nerve segment. The numbers were significantly higher if nerve repair was delayed after transection. Satellite cells with cleaved caspase 3 staining, surrounding sensory neurons in DRG, were also more frequently visible after transection. In contrast, neither motor nor sensory neurons showed any staining for cleaved caspase 3 at any time point.

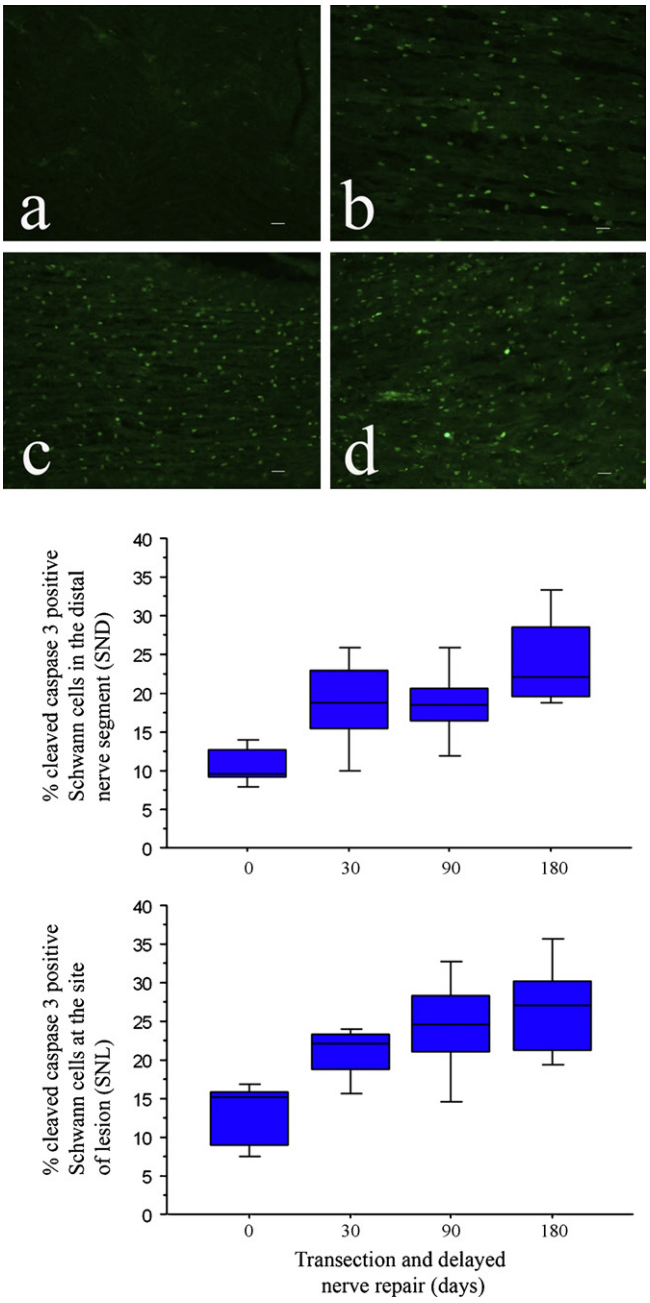

Fig. 1. Staining of cleaved caspase 3 in Schwann cells in rat sciatic nerve on the uninjured contralateral side (a) and in a transected nerve after immediate (b) and delayed (30 days, c; 180 days, d) nerve repair. The diagrams show the percent of cleaved caspase 3 stained Schwann cells after immediate and delayed nerve repair in the distal nerve segment (SND; top diagram) and at the site of lesion (SNL; bottom diagram). Delayed nerve repair resulted in significantly higher number of cleaved caspase 3 stained Schwann cells at both locations compared to immediate repair ($p = 0.02$ and 0.01). Length of bar = 50 μm.

Cleaved caspase 3 staining was used as a marker for apoptosis in the Schwann cells. Apoptosis of Schwann cells, as detected by presence of cleaved caspase 3 and TUNEL staining in mouse and rat, is induced in the sciatic nerve after nerve crush or transaction [4,30], after ischemia-reperfusion injury (>35% stained Schwann cells 14–42 days post-ischemia [13]) or nerve compression (only 1–2% TUNEL positive Schwann cells [7]). Interestingly, there seems to be a difference between rat and mouse in their sensitivity to Schwann cell apoptosis [30]. No previous studies have described cleaved caspase 3 staining in Schwann cells after delayed nerve repair.