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Running title: Extracted nerve allografts and xenografts

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Abstract

When not enough conventional autologous nerve grafts are available, alternatives are needed to bridge nerve defects. Our aim was to study regeneration of nerves in chemically-extracted acellular nerve grafts from frogs, mice, humans (fresh and stored sural nerve), pigs and rats when defects in rat sciatic nerves were bridged. Secondly, we compared two different extraction procedures (techniques described by Sondell et al and Hudson et al) with respect to how efficiently they supported axonal outgrowth, and remaining laminin and myelin basic protein (MBP), after extraction. Isografts (rat) and xenografts (mouse) were transplanted into defects in rat sciatic nerves. Acellular nerve allografts from rats, extracted by the Sondell et al's technique, had a appreciably longer axonal outgrowt, based on immunohistochemical staining of neurofilaments, than acellular nerve xenografts except those from the pig. Among acellular xenografts there was considerably longer axonal outgrowth in the grafts from pigs compared with those from humans (fresh), but there were no other differences among the xenografts with respect to axonal outgrowth. Axonal outgrowth in acellular nerve xenografts from mice, extracted by the method designed by Sondell et al was longer than in those extracted by Hudson et al's method, while there was no difference in outgrowth between extracted nerve isografts from rats. Electrophoretic analysis of extracted acellular nerve grafts showed remaining laminin, but not MBP, after both extraction procedures. These preserved laminin and removed MBP in acellular nerve grafts. Such grafts can be used to reconstruct short defects in nerves irrespective of their origin. However, selecting and matching a suitable combination of graft and host species may improve axonal outgrowth.

Introduction

Transected peripheral nerve trunks should be treated by direct repair when possible to coapt the nerve endings. When the trunk is lacerated there may be a defect between the two nerve ends after the wound has been cleaned and necrotic tissue removed. Such a gap is usually bridged by an autologous nerve graft [1]. However, sometimes, there may be a shortage of graft material to bridge the defect, such as in lesions of the brachial plexus. The surgeon's reluctance to involve another extremity in the procedure, i.e. harvesting a sural nerve biopsy from the leg, requires alternatives to autologous nerve grafts. A short defect in an injured digital nerve may be appropriate to bridge with one of the alternatives to nerve grafts, which include various nerve conduits [2], longitudinal sutures [3], tendon autografts [4] or various matrices filled with Schwann cells [5]. The risk of complications from the donor nerve area may therefore be abolished. When alternative methods are not applicable, allografts (from another human) or xenografts (from another species) may be considered. However, these grafts induce rejection that requirres lifelong immunosuppressant treatment, with sometimes severe side effects [6,7]. The treatment would in many cases be worse than the ill it was meant to cure.

To overcome this problem an acellular nerve graft is an appealing alternative [8,9], as they are thought not to induce rejection [10]. Acellular nerve grafts retain the nerve's internal structure with preserved extracellular matrix components, such as laminin [8,9]. However, there is a risk that an immune reaction may develop, such as autoantibodies against myelin basic protein (MBP) that have been implicated in multiple sclerosis [11].

Different acellular nerve allografts, with an additional treatment with chondroitinase ABC, have been studied experimentally [12], and have been applied clinically [8,9,13,14].

However, acellular nerve grafts from different species (xenografts) can also be considered. The definitions of various nerve grafts are described in Table I.

Our aims were to study axonal outgrowth into extracted nerve grafts from different species transplanted into rat using the extraction method described by Sondell et al [8]. We also compared two different methods of extracting nerves [8,9] with respect to their ability to extract nerve components and their ability to sustain the regeneration process. We report that the extraction procedures preserve laminin and remove myelin basic protein in acellular nerve grafts. Irrespective of origin, acellular nerve grafts can be used to reconstruct short defects, but selection and matching of the most appropriate combination of graft and host species improve regeneration.

Material and methods

Experimental design

Axonal outgrowth in acellular nerve grafts (extraction method described by Sondell et al)
Sciatic nerve grafts (see below) from 5 NMRI mice (female, 20-22 g), 10 Wistar rats (female, 180 g), and 4 adult frogs (Rana temporaria) were retrieved for the extraction using the method described by Sondell et al (see below). In addition, tibial nerve segments from 5 pigs (Suidae Yorkshire) and segments from sural nerve fascicles (5 fresh and 5 stored) from humans were also obtained. A total of 34 female Sprague-Dawley rats (180 g) were used as hosts.

Comparison of the extraction methods described by Hudson et al and Sondell et al
Ten NMRI mice (female, 20-22 g) and 35 (5 for electrophoresis, 10 for grafts, and 20 used as hosts) Sprague-Dawley rats (female, 180 g) were used (Taconic, Denmark). Five fresh human sural nerve fascicles were used for electrophoresis.

The experimental protocol was reviewed and approved by the Human and Animal Ethical Committees at Lund University.

Nerve graft preparation

Rat and mouse

In the first set of experiments the animals were killed by an overdose of pentobarbital, and at least 10 mm of the left sciatic nerve was excised within two minutes of the first incision. The grafts were immediately placed in distilled water. In the second set of experiments the procedure was the same, but the grafts were randomly assigned to extraction either by the method described by Sondell et al (5 mice and 5 rats) or Hudson et al (5 mice and 5 rats) [8,9].

Frog

Frogs were anaesthetised with tricaine mesylate MS 222 and killed by decapitation. A segment of at least 10 mm of the tibial nerve was harvested and placed in distilled water. The nerves were then extracted using the method described by Sondell et al.

Pigs and humans

Segments of the tibial nerve fascicles from pigs obtained from a slaughterhouse were freshly dissected and placed in distilled water. Small segments of human sural nerve were obtained from remaining parts of the nerve that had been used for nerve reconstruction (freshly used or extracted after storage up to three weeks before use in PBS containing 0.05% azide to prevent bacterial growth). All nerve segments were cleaned of external debris and the stored nerve segments were washed extensively in PBS. For the segments of human sural and pig tibial

nerves an appropriate number of fascicles were dissected to obtain nerve segments with a diameter of about 1.3 mm, which matched a sciatic nerve in Sprague-Dawley rats.

Extraction methods

All segments of nerve were treated with chemical detergents to make them acellular.

The extraction method described by Sondell et al.

The nerve segments were immersed in distilled water, which was replaced several times during a seven-hour period at room temperature. The segments were then exposed to 3% Triton X-100 (Packard, USA) in distilled water overnight at room temperature and thereafter followed by a 24-hour agitation period at room temperature in a solution of 4% sodiumdeoxycholate (Sigma Chemical, USA) in distilled water. The extraction procedure was then repeated after the nerves had been finally washed in water. The nerves were stored in phosphate buffered saline (PBS, pH 7.2) at 4°C until use.

The extraction method described by Hudson et al.

The nerve segments were placed in Roswell Park Memorial Institute (RPMI)1640 solution at 4°C, and then immersed in deionised distilled water at 25°C for seven hours. The water was then aspirated and replaced by a 10 mmol/l phosphate buffered, 50 mmol/l sodium solution containing 125 mmol/l sulphobetaine-10 (SB-10). The nerves were agitated for 15 hours, and then rinsed for 15 minutes in 50 mmol/l phosphate buffered and 100 mmol/l sodium solution. This solution was replaced by a 10 mmol/l phosphate buffered and 50 mmol/l sodium solution containing 0.14% Triton X-200, and 0.6 mmol/l sulphobetaine-16 (SB-16). After another 24 hours of agitation the segments were rinsed with the washing solution. The nerve segments were again agitated in the SB-10 solution for 7 hours, washed once, and agitated in the SB-

16/Triton X-200 solution for 15 hours. After three more washes, each of 15 minutes, in a 10 mmol/l phosphate buffered and 50 mmol/l sodium solution, the segments were stored in the same solution at 4°C [13].

Electrophoresis and Western blotting

Nerve segments extracted from Sprague-Dawley rats and humans, which had been extracted by the technique described by Sondell et al (n=5) and by Hudson et al (n=5), were homogenised by sonication in 376 µl sample buffer (0.0625 M Tris-hydrochloric acid, pH 6.8; 2% SDS; 10% glycerol). β-mercaptoethanol and bromphenol blue were added to a final concentration of 5% and 4% (v/v), respectively. After the samples had been boiled for three minutes the vials were centrifuged at 10000 g for 10 minutes. Samples of the supernatants and standard (Magic Mark XP, Invitrogen Corp. Carlsbad, California) were applied on to pre-cast NuPAGE Novex 10% Bis-Tris gels and the proteins were separated at 150V/400 mA for 1.5 hours. The proteins were then transferred to a Hybond enhanced chemoluminescence (ECL) nitrocellulose membrane for 1 hour at 30 V constant using the XCell IIÔ Blot Module in NuPAGE transfer buffer. Transfer efficiency (not shown) was checked by fixing and staining the acrylamide gel with Coomassie® Brilliant Blue R-250 (Merck, Germany) 0.25% (w/v) after blotting. The membranes were then prepared for enhanced chemiluminescent (ECL) staining of laminin and myelin basic protein. The membranes were thereafter incubated with the primary antibodies diluted in PBS (rabbit antilaminin; Sigma L9393; 1:1000 and rabbit antiMBP; Chemicon Int; AB 980; 1/1000, respectively) overnight at 4 C. After washing the membranes with Tris-buffered saline (TBS), they were incubated with horse radish peroxidate (HRP)-conjugated Pig Antirabbit IgG secondary antibodies (DakoCytomation, Glostrup, Denmark) diluted 1/10000 in TBS for 1 hour at room temperature. After extensive washing in TBS the membranes were developed using an ECL kit (Amersham ECL Advance™ Western

Blotting Detecting Kit) according to the manufacturer's instructions. The gel was documented in a BioRad Gel Doc XR System. The gel images were further analysed using the ImageJ software (www.nih.gov).

Nerve grafting

The 53 remaining Sprague-Dawley rats were used as hosts for the extracted nerve grafts. After anaesthesia had been induced (pentobarbital 60 mg/ml, sterile saline, and diazepam 5 mg/ml in a 1/1/2 proportion; 0.25-0.30 ml intraperitoneally) the left sciatic nerve was exposed. A 7 mm segment of the sciatic nerve was excised at the midthigh level. The 10 mm long grafts were then sutured (cut to 10 mm length) with two epineurial sutures at each end (using 9/0 for rat, pig, human, and frog grafts and 10/0 for mouse grafts). The wound was then closed.

Retrieval of the grafts

After 10 days the rats were anaesthetised again using the solution described. The graft was exposed and excised together with at least 3 mm host sciatic nerve at both ends. The specimen was then fixed in Stefanini's solution overnight and then washed and placed in PBS containing 20% sucrose. The animals were killed by an overdose of pentobarbital.

Evaluation of length of axonal outgrowth

Immunocytochemical staining of neurofilaments was used to measure the axonal outgrowth in the grafts [15]. After being washed in PBS, the nerve grafts were cryoprotected in 20% buffered sucrose solution until they were sectioned. The nerve grafts with adjacent proximal and distal nerve segments were mounted in Tissue Tec® (Miles) and longitudinal sections 10 µm thick were cut in a Cryostat. The sections were collected on slides coated with poly-L-

lysine and air-dried for 30 minutes. Neurofilaments were visualised using techniques previously described [15] using monoclonal antibodies against 68 kDa neurofilaments (MO726, DAKO, Glostrup, Denmark) overnight at 4^oC. Control sections were incubated without either the primary or secondary antibody. The lengths of axonal outgrowth were measured in an Olympus AX70 microscope using a scale connected to the microscope. The length was estimated as the distance between the proximal suture line and the furthest point that neurofilament-stained fibres had reached (>5 continuously growing axons) and measured in at least three sections from each nerve graft. The longest distance measured was used.

Statistical analysis

The values are presented as median (interquartile range; IQR). The Mann Whitney test was used to detect differences between the two different extraction procedures. The Kruskal Wallis test, with subsequent Bonferroni test as a post hoc test, was used to detect any significant difference between the various nerve isografts, allografts and xenografts. Probabilities of less than 0.05 were accepted as significant.

Results

Axonal outgrowth in extracted nerve grafts (technique described by Sondell et al.) from different species used to bridge sciatic nerve defects in rat

The lengths of axonal outgrowth, where extracted nerve grafts from different species (acellular nerve xenografts and allografts) were used to bridge a 10 mm long nerve defect in rats and evaluated 10 days later, are shown in Table II. The longest axonal outgrowth was seen in extracted acellular nerve allografts from rats, which were different from the distances in the various acellular extracted xenografts except from those obtained from pigs (p=0.001). The outgrowth of axons in extracted nerve xenografts from the other species (frog, human,

stored human, mouse, and pig) showed no significant differences, except for a longer distance in pig compared with fresh human nerve xenografts (Table II).

Comparison of axonal outgrowth in nerve grafts made acellular by the two extraction techniques

The lengths of outgrowing axons (based on neurofilament staining) in the extracted nerve grafts that were harvested from mice (xenografts) and rats (isografts), made acellular by the two extraction techniques and evaluated in rat sciatic nerve defects 10 days after reconstruction, are shown in Table III. Axonal outgrowth in the 10 mm extracted nerve xenografts from mice was longer in the grafts made acellular by the technique described by Sondell et al. than in the grafts extracted by the technique described by Hudson et al. (p=0.04). By contrast, the length of axonal outgrowth in extracted nerve isografts from rats did not differ between the two extraction procedures (p=0.4).

Electrophoretic analysis and Western blot analysis of extracted rat and human nerve grafts

In the second set of experiments electrophoresis showed that laminin was preserved after both extraction methods at about the same intensity (measured using Image J) in both rat (Figure 1a) and human grafts. However, the intensity of the laminin bands was reduced compared with control (unextracted rat and human). The main laminin band in rat was located at 135 kD. Both extraction methods seemed to remove MBP completely (analysed with Image J) detected by electrophoresis in both rat and human nerves (Figure 1b).

Discussion

In the first part of this study we investigated axonal outgrowth in various types of allografts and xenografts made acellular by detergent extraction as described by Sondell et al. [8]. The

longest axonal outgrowth occurred when extracted nerve grafts from the same species (such as rat allografts) were used. Outgrowth was shorter in extracted xenografts (frog, mouse, and fresh and stored sural nerve fascicles from human), but not compared with pigs. Interestingly, the extracted nerve xenografts from pigs were more efficient than an extracted fresh human xenograft. We found no other differences with respect to species among the xenografts. Our results showed that the acellular allografts were superior to the acellular xenografts, but there was no simple relation between the origin of the graft and the consanguinity of the animals. Whether acellular allografting is also superior in humans is an open question. Until this has been studied, the clinical implication is that extracted grafts should preferably be created from the same species in which the nerve grafting should be done.

The present results show that regeneration occurs into acellular xenografts, and even in xenografts made acellular after storage (human) for a period of time. Interestingly, cold storage before transplantation of allografts may modify the axonal outgrowth and the immune response, most likely as a result of the reduced number and viability of Schwann cells during storage [16]. However, it was reported that cold storage does not modify the basal lamina and laminin distribution of Schwann cells [16] as predegeneration of an autologous nerve graft may do [17].

In the second part of the study we compared two extraction procedures: one developed in our laboratory (by Sondell et al. [8]) and the other by Hudson et al. [9]. The latter method has been claimed to be optimised over previous protocols [14]. We found that the axonal outgrowth in mice, but not in rats (isografts), was significantly longer after 10 days in grafts extracted by our method [8]. However, commercially the technique of Hudson et al. is used in conjunction with treatment with chondroitinase ABC to produce acellular nerve allografts

[12,14], which may increase the effective length of axonal outgrowth [12]. Other means or conditions by which regeneration can be improved into acellular grafts include an inflammatory response that is induced after implantation of the graft. Such a response involves migration of macrophages into acellular nerve and muscle grafts [18] or seeding the acellular nerve grafts with growth factors, basal lamina components, or cells [19-21].

The two extraction procedures did not differ with respect to the removal of laminin and MBP. Laminin is an important part of the extracellular matrix that interacts with axons and Schwann cells [22]. The basal lamina contains laminin subtypes 2 and 8, which are likely to be produced by Schwann cells [23]. Axons, and their growth cones, and Schwann cells bind to laminin through integrin receptors. After nerve injury, mRNA for laminin and integrins are upregulated in both the proximal and distal nerve stumps [23]. Knockout mice for a subchain in the laminin-8 molecule show no impaired regeneration after a nerve has been crushed, but do display neuropathy (morphological and behavioural). This impairment is probably mediated through lack of/faulty interaction between laminin and Schwann cells [24]. A nerve extraction, therefore, should not destroy or remove too much laminin, which is true also for other components of basal lamina, including fibronectin. The importance of these extracellular matriceal proteins is highlighted in reports of impaired regeneration in diabetes as a result of glycation of these proteins [25]. However, in this study we chose laminin, and did not study subtypes, as representative of the extracellular matrix.

Autoantibodies against MBP have been implicated in the pathogenesis of multiple sclerosis [11], so it may be important to remove MBP from extracted grafts. The two extraction techniques managed to extract MBP, which confirms the previous study by Hudson

et al. [9]. We cannot completely rule out that small amounts of MBP, or other substances, may remain and in the future trigger an immune reaction.

Taken together our results show that pieces of extracted nerve allografts and xenografts can be used to bridge defects, but there is no simple relation between the origin of the graft and the extent to which it supports axonal outgrowth. Both extraction procedures preserve laminin and remove myelin basic protein in acellular nerve grafts.

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Table I. Grafts used in the present study

- Autograft – a graft from the patient

- Isograft – a graft from another person with an identical genome

- Allograft – a graft from another member of the same species; in inbread species this is equivalent to an isograft

- Xenograft – a graft from another species

Table II. Axonal outgrowth in acellular nerve allografts and different acellular nerve xenografts made acellular by the extraction procedure described by Sondell et al. [8] used to bridge a 10 mm sciatic nerve defect in Sprague-Dawley rat. Axonal outgrowth lengths are expressed in mm [median interquartile range (IQR)].

Type of graft	No.	Axonal outgrowth (mm)
Acellular nerve allografts		
Rat	10	4.7 (4.0)
Acellular nerve xenografts		
Frog	4	2.4 (0.8) [a]
Mouse	5	2.6 (1.3) [a]
Pig	4	3.6 (1.6) [b]
Human (freshly extracted)	5	1.8 (1.1) [a]
Human (stored before extraction)	5	2.5 (0.3) [a]

$p=0.001$ between groups (Kruskal Wallis). [a]Significantly different from acellular nerve allografts from rats, and [b]significantly different from (fresh) xenografts from humans.
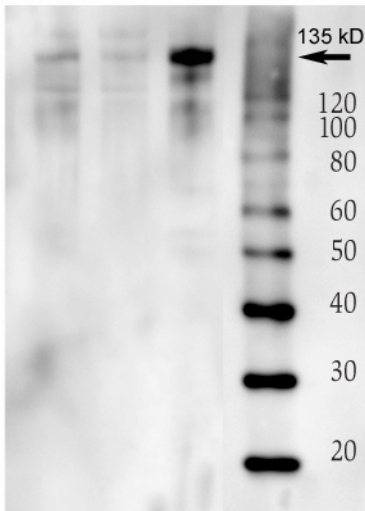
Table III. Axonal outgrowth in acellular nerve isografts and xenografts made acellular by two different extraction procedures [8,9,13] used to bridge a 10 mm rat (Sprague-Dawley) sciatic nerve defect. Axonal outgrowth lengths are expressed in mm [median and interquartile range (IQR); n=5 in each group].

Type of graft	Axonal outgrowth (mm)	
	Sondell et al.*	Hudson et al.*
Acellular nerve xenograft		
Mouse	3.7 (0.7)	3.1 (0.6)
Acellular nerve isograft		
Rat	2.9 (0.9)	3.1 (0.5)

[a]Significantly different from Hudson's technique (p=0.04; Mann Whitney). *Technique used.

Figure legends

Figure 1. (a) Enhanced chemoluminescence (ECL) for laminin, in nerve pieces extracted, according to Hudson et al's (Hu) or Sondell et al's (So) protocols. A fresh unextracted nerve (Sprague-Dawley) was used as control (C). The main laminin band had a molecular weight of about 135 kD (arrow). Both extraction methods had a lower intensity of the laminin bands than the control nerve; there were no differences between them. (b) Enhanced chemoluminescence (ECL) of rat (Sprague-Dawley) and fresh human nerves stained for myelin basic protein (MBP). The nerves were extracted, either by Hudson et al's (Hu) or Sondell et al's (So) protocols. Fresh unextracted nerves were used as controls (C). No myelin basic protein (MBP)-positive bands could be detected (using Image J) in the lanes of the extracted nerves. In the control lanes, bands were detected in both human (about 22.5 KD) and rat (about 15.5, 20.5 and 28.5 kD). Standard to the right in Figure 1a and b is Magic Mark (Invitrogen).

				135 kD
Hu	So	C		120
				100
				80
				60
				50
				40
				30
				20

Hu	So	C		Hu	So	C
	Rat				Human	