REMYELINATION IN EXPERIMENTALLY DEMYELINATED CONNEXIN 32 KNOCKOUT MICE

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Abstract – The aim of this study was to evaluate the role of connexin 32 (Cx 32) during remyelination of the peripheral nervous system, through a local injection of either 0,1% ethidium bromide solution or saline in the sciatic nerve of Cx 32 knockout mice. Euthanasia was performed ranging from 1, 2, 3, 7, 15, 21 to 30 days after injection. Histochemical, immunohistochemical, immunofluorescence and transmission electron microscopical techniques were used to analyze the development of the lesions. Within the sciatic nerves, Schwann cells initially showed signs of intoxication and rejected their sheaths; after seven days, some thin newly formed myelin sheaths with uneven compactness and redundant loops (tomacula) were conspicuous. We concluded that the regeneration of lost myelin sheaths within the PNS followed the pattern already reported for this model in other laboratory species. Therefore, these results suggest that absence of Cx 32 did not interfere with the normal pattern of remyelination in this model in young mice.

KEY WORDS: connexins, myelin, KnockOut mice.

Remielinização em camundongos KnockOut para conexina 32 desmielinizados experimentalmente

Resumo – Este estudo visou avaliar o papel da conexina 32 (Cx 32) durante a remielinização no sistema nervoso periférico. Uma injeção local de 0,1% de solução de brometo de etídio foi realizada no nervo ciático de camundongos deletados para a Cx 32, com eutanásia dos animais aos 1, 2, 3, 7, 15, 21 e 30 dias pós-injeção. Avaliações histoquímicas, imunoistoquímicas, por imunofluorescência e por microscopia eletrônica de transmissão foram utilizadas na análise do desenvolvimento das lesões. Nos nervos ciáticos, células de Schwann mostraram inicialmente sinais de intoxicação e rejeitaram suas bainhas. Após sete dias, observaram-se finas bainhas neoformadas, com compactação desigual e alças redundantes (tomácula). Conclui-se que a regeneração de bainhas de mielina perdidas no SNP seguiu o padrão já relatado deste modelo em outras espécies de laboratório. Portanto, estes resultados sugerem que a ausência da Cx 32 não interferiu com o padrão normal de remielinização em camundongos jovens neste modelo.

PALAVRAS-CHAVE: conexinas, mielina, camundongos KnockOut.

Myelination of peripheral axons and maintenance of the myelin sheaths are carried out by Schwann cells. After axonal segregation and proliferation of Schwann cells, myelination proceeds until all axons with a diameter bigger than 1 μm are myelinated. Smaller diameter axons which act as sensor fibers are ensheathed by Schwann cells but do not receive a myelin sheath[1]. Each Schwann cell produces one internode of myelin, very rarely two. The signal for Schwann cell differentiation does not come from the axon alone, rather than from the axon[2] and the extracellular matrix, which provides collagen as a third element for myelination in the peripheral nervous system (PNS). Collagen fibers are produced and secreted by Schwann cells themselves[3]. The relationship of the axon and the ensheathing

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Received 15 October 2008, received in final form 26 January 2009. Accepted 22 April 2009.

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cell is very close, and during development both structures grow together in such a way that, after segmental demyelination, the length of the Schwann cell goes back to the embryonary length with the production of shorter internodes[4]. Such an exquisite composition certainly requires complex communication between the neuron and the peripheral glial cells. Proper communication is provided by gap junctions, sites at the cell membrane with intercellular channels composed of twelve connexin proteins. Each cell contribute with one connexon formed by six connexins[5]. This kind of junction represents an efficient way of intercellular communication in most tissues, including the nervous tissue, and allows the passage of ions, secondary messengers and metabolites up to 1 kilodaltons between the cells. Gap junctions may be composed by twelve identical connexins (homotypic) or different on each cell connexon (heterotypic) or intercalated (heteromeric) [5]. Autologous communications between the cells may also take place[6].

Connexin 32 (Cx 32) is a protein found in both the PNS and the CNS, respectively in Schwann cells and oligodendrocytes and their myelin sheaths[7]. It occurs within non-compacted myelin sheath domains, paranodal loops and the Schmidt-Lantermann incisures, which create a radial pathway for molecule diffusion[6]. Blockage of that pathway is seen in knockout mice and in some spontaneous mutations in humans. This lack of Cx 32 induces changes such as increase of periaxonal collars and non-compactness of myelin[8]. In the PNS, autologous communication between myelin lamellae involve Cx 29 and Cx 32, located at the paranodal loops and Schmidt-Lantermann incisures of the myelin sheath[6]. Some pathological conditions are related with alteration in gap junction function[9]. Some human diseases are caused by connexin mutations[10], i.e. mutations on the Cx 32 gene induce a peripheral neuropathy named Charcot-Marie-Tooth disease, an X-linked change. The mechanisms altered by Cx 32 mutations in Charcot-Marie-Tooth disease interfere with nervous conduction and include altered protein traffic to the gap junctions, altered permeability within the channels and altered conformation of heterotypical channels[7]. Ethidium bromide is a well known gliotoxic chemical, extensively used to induce demyelination in the CNS[11-16] and the PNS[17].

The goal of this investigation was to evaluate the processes of demyelination and remyelination induced by ethidium bromide in the PNS of C57Bl-6 mice knockout for Cx 32, and therefore assess the role of connexin 32 in PNS remyelination.

METHOD

Animals

Cx 32 knockout mice were obtained from the International Agency for Research on Cancer (IARC, Lyon, France). The mice were produced originally in the C57BL/6 strain, and both Cx $32^{-/y}$ and wild type Cx $32^{+/y}$ mice were used in this study.

These mice were bred and housed at the laboratory animal facility of the Department of Pathology of the School of Veterinary Medicine and Animal Science of the University of São Paulo, São Paulo, Brazil. Animals were weaned at the age of 4 weeks and kept under controlled conditions (22ºC±2ºC; 65±15% relative humidity, air exchange rate 15 times/h, 12h-12h light-dark cycle), in filtered cages. Animals received a standard pelleted diet (Purina Lab Chow, Brazil) and tap water *ad libitum* during the study.

Two-month-old F1 (C57Bl/6 × ADCx 32) male mice (n=70) knockout for Cx 32 and 50 C57BL-6 wild-type were submitted to surgery at the Department of Pathology of the School of Veterinary Medicine and Animal Science, University of São Paulo, Brazil. Wild type C57BL-6 were obtained at the FEPPS/POA/RS and operated at the Veterinary Pathology Laboratory of the Santa Maria Federal University. The mice were divided into three groups: one group (60 mice) had a single injection of 1 µl of 0.1% ethidium bromide (EB) in 0.9% saline; the second group (55 mice) had a single 0.9% saline injection and the third group (5 mice) was not injected and was used for morphological studies (Table).

Surgical preparation

Injections were made at the middle third of the right sciatic nerve with a 10 µl Hamilton syringe. All procedures were conducted in agreement with the international ethical rules of the National Research Council.

Anesthetic induction was achieved through an association of xylazine and ketamine; the surgical procedure and the method of injection are described elsewhere[17]. The mice received morphine (10 mg/kg every 4h) for up to 48 hours.

Table. Distribution of the number of animals in each experimental group and time points for euthanasia after injection of EB.

Time / treatment		24 hrs		48 hrs		3 days		7 days		15 days		21 days		30 days		*	Total
Procedure		SS	BE	SS	BE	SS	BE	SS	BE	SS	BE	SS	BE	SS	BE	–	
Electron	Normal	3	3	3	3	2	2	3	3	3	3	3	3	3	3	–	40
microscopy	KO Cx32	2	2	2	2	2	2	2	2	2	2	2	2	2	2	–	28
Parafin	Normal	–	–	–	–	–	–	–	–	–	5	–	–	–	–	5	10
	KO Cx32	3	3	3	3	3	3	3	3	3	3	3	3	3	3	–	42
Total		8	8	8	8	7	7	8	8	8	13	8	8	8	8	5	120

*SS: saline solution; *morphologic control group.*

Sample collection

The mice were perfused through the heart at 1, 2, 3, 7, 15, 21, and 30 days post injection (p.i.) with 0.9% saline with EDTA; for the mice destined for electron microscopy analysis the perfusion was completed with 2.5% glutaraldehyde; in mice destined for histochemical, immunohistochemical and immunofluorescence analysis the perfusion was followed by immersion in methyl Carnoy's fixative for 24 hours. Following procedures, standard sample embedding in either epoxy resin or paraffin was conducted. From each mice, a sample of the tail was obtained for DNA profiling as described by Anzini et al.[18].

Coronal and longitudinal sciatic nerve samples were obtained; 5 μm sections were deparaffinized and stained with hematoxylin and eosin, luxol fast blue and toluidine blue methods, analyzed and photographed under an Olympus BX41 light microscope.

For immunohistochemical (IHQ) studies, 5 μm sections of the sciatic nerve were deparaffinized and treated with hydrogen peroxide prior to incubation with primary antibody. Incubation was performed in a humidity chamber at 37ºC for 2 hours with anti-S100 primary antibody (DakoCytomation, Z0311, 1:1000), followed by incubation with biotin conjugated antibody (DakoCytomation) and incubation with streptavidin for 30 minutes at room temperature. After exposure to the appropriate chromogen (DAB), slides were counterstained with Mayer's hematoxylin and mounted in synthetic resin. For IF studies, sections were deparaffinized and incubated in a humidity chamber at 4ºC for 18–22 hours with anti-Cx 32 primary antibody (Santa Cruz Biotechnology, sc-7258, 1:100), followed by incubation with a secondary antibody (DakoCytomation) and a fluorescent antibody (FITC – DakoCytomation) both for 2 hours at room temperature and mounted in aqueous medium (Vectashield mounting media, Vector Laboratories, USA). The slides were analyzed under an Olympus BX41 or a fluorescence Olympus BX41 microscope with a FITC filter, respectively.

Standard embedding procedures for electron microscopy studies were followed by preparation of 0.5 μm-thick sections stained with toluidine blue; from selected areas of these sections, 70-nm-thick ultrathin sections were cut using a diamond bladed Leica Reichert Supernova ultramicrotome. These sections were stained with uranyl acetate and lead citrate and analyzed with a Morgagni 268D transmission electron microscope equipped with an integrated image capture system (MegaView).

RESULTS

After complete recovery from the anesthesia, the experimental mice were observed daily and did not show any neurological changes: they moved, fed themselves and interacted normally.

PCR profiling confirmed the male $Cx^{Y/-}$ genotype; a single 414 pb band was visualized, which represents the genotype of homozygous KO mice for Cx 32. IF method confirmed the lack of expression of Cx 32 (image not shown).

Changes induced by the injection of 1 μl of 0.1% EB in the sciatic nerve were of variable extension and involved myelinated fibers, the perineurium and the epineurium. These changes consisted of segmental demyelination followed by remyelination, and of wallerian degeneration due to the injection trauma.

Histochemical and immunohistochemical results

Inflammatory infiltrate was found in all lesions, although it was more conspicuous in early lesions up to seven days p.i.. That infiltrate was more pronounced in EB induced lesions and occurred more markedly within the perineurium. An increase in cell numbers was observed mainly in EB induced lesions from 3 to 15 days. This cell increase was related to Büngner bands composed by packed reacting Schwann cells and to phagocytic cells within occasional digestion chambers. Mast cells were consistently detected within the lesions: some were endoneurial perivascular cells, whereas others were detected among the nerve fibers and within the perineurium.

S100 protein labelling was irregularly detected within the cytoplasm and nucleus of Schwann cells and also in axons. A wide range of labelling was observed independently of the treatment used.

Ultrastructural analysis

Ultrastructural changes were similar for normal and KO mice and therefore will be described simultaneously. In acute lesions intracellular and extracellular edema was the main change (Fig 1). After 24 hours, intoxicated Schwann cells rejected their myelin sheaths and some re-

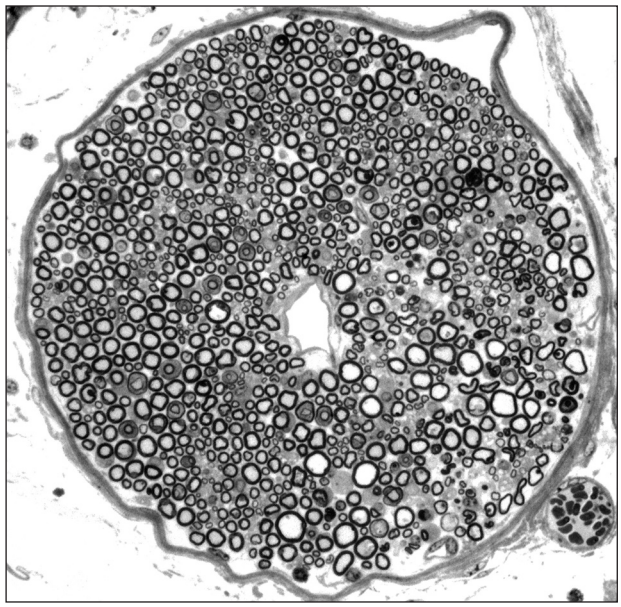

Fig 1. Coronal semi-thick section of a sciatic nerve fascicle of a Cx32 KO mouse. Edema at the right side of the section. 48 h p.i. Toluidine blue, Obj. 20.

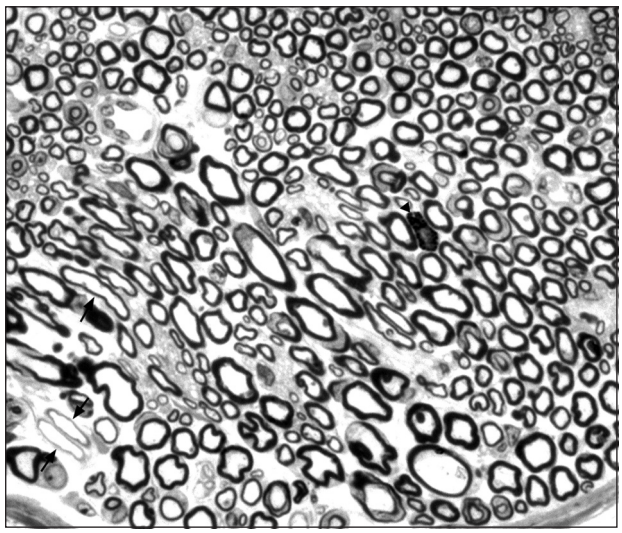

Fig 2. Thin myelin sheaths (remyelinated) are detected in this semithick section of a sciatic nerve fascicle of a Cx32 KO mouse (arrows). A mast cell is observed (arrowhead) among the fibers. 30 days lesion of a Cx32 KO mouse. Toluidine blue, Obj. 40

maining lamellae were observed within their cytoplasm. At three days p.i. the rejected sheaths were removed by macrophages. These cells were loaded with myelin in various stages of breakdown, from vesiculated myelin to neutral fat globules. From 7 days onwards, thin newly formed irregularly compacted thin myelin sheaths and shorter internodes were detected along remyelinated fibers, which laid among some normal fibers (Fig 2). Redundant myelin loops (tomacula) were conspicuous in 21 and 30 day-old lesions. Within the fascicles, Wallerian degeneration was detected in most lesions as well as Remak fibers. The latter were more frequent in KO mice (Fig 3).

DISCUSSION

Ethidium bromide intoxicates Schwann cells when injected within the sciatic nerve of rats[17]. The intoxicated cells reject their myelin internode but do not die; such a cell resistance is highlighted by Cavanagh[19] and it is considered independent of the etiological agent. The changes of the fibers after EB injection in mice, likewise in rats, are of segmental demyelination. It is common knowledge that the damaged axons communicate through gap junctions to induce regeneration[20]. Likewise, demyelinated axons may attract Schwann cells in order to induce remyelination[11].

KO mice for Cx 32 were used in this experiment because they are the experimental model to a form of Charcot-Marie-Tooth disease (CMT1X)[21]. EB-induced lesions in KO mice for Cx 32 are very similar to the EB-induced lesions in rats[17] and in normal C56BL-6 mice used in this investigation. After the degenerative phase and removal of debris by macrophages, myelin sheaths repaired by Schwann cells were found from 7 days onward. Myelin sheaths were thinner than normal for the diameter of the axon and internodes were shorter. Such dimensions are considered as indicative of remyelination as well as fiber regeneration[4].

During myelination, the committed Schwann cells form a sheath and grow with the axon, and a relationship between number of myelin lamellae, thickness of the sheath and length of the internode is established. After demyelination in adult individuals, the already sized axon demands for myelin and a profound alteration in the dimensions listed above takes place; the process is regulated by intrinsic axonal factors and induced by the ensheathing cell[22]. In this investigation the morphological development followed the same pattern in normal and KO mice, indicating that there are no alternatives for the

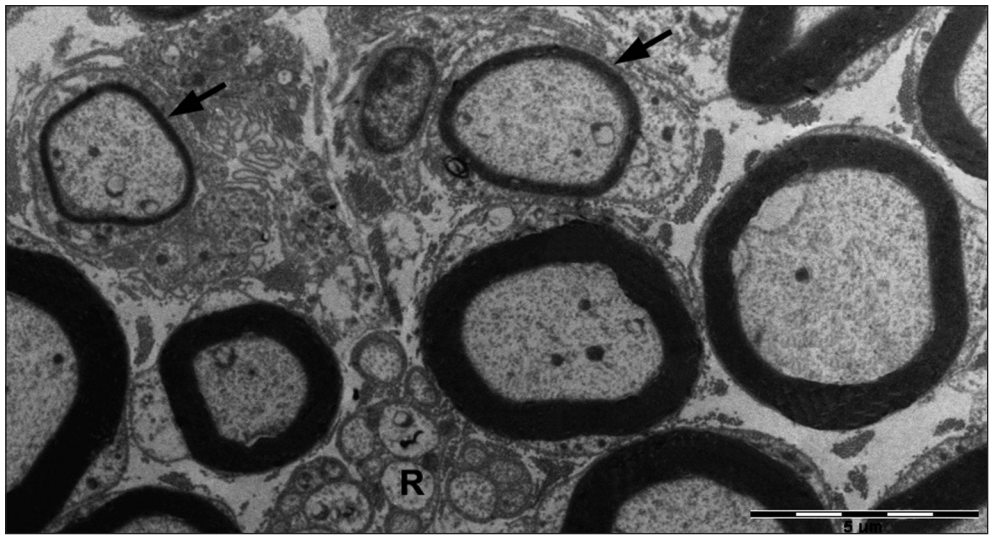

Fig 3. Ultraestructure of a 21 days lesion of the sciatic nerve of a Cx32 KO mouse. Two thinly repaired myelin sheaths of uneven shape (arrows) are detected among normal myelinated axons. One Remak fiber is also observed (R). Bar: 5 µm.

repair, at least in these young adult mice, before the age when they suffer spontaneous demyelination and are less able to repair their sheaths.

In all lesions the presence of mast cells was conspicuous. They were observed within the endoneurium, perineurium and epineurium, without a mandatory consistent proximity with blood vessels. Cavanagh[19] reports that mast cells proliferate in any damage to peripheral nerves, although he can not explain their actual function within the tissue. In the present investigation they might be reactive to the non-specific toxic effects of EB or to the injury induced by the needle trauma. Their participation in EB induced lesions deserves further investigation since mast cells have been described in multiple sclerosis lesions[23] and it was suggested that their presence was related to persistently marked blood-brain-barrier, degenerative and immune-inflammatory changes[24].

Repaired myelin sheaths had numerous redundant loops (tomacula). Tomacula are consistently reported in lower species[25] and they were described after EB injection in the sciatic nerve of rats[17]. Tomacula are prominent areas of peripheral myelin sheaths, considered as hypermyelination in many diseases and experimental models, including those where mutation of genes that codify myelin proteins are the main change[26].

The regenerative capacity of peripheral nerve fibers and peripheral myelin is notorius in the PNS[22] and characterizes the tight relationship among the fibers, Schwann cells and the extracellular matrix. After regeneration both fibers and myelin sheaths undergo extensive remodelling that ends with few or no myelin excess as observed in tomacula. This remodeling is described as incomplete in those animals with genetic defects of myelin, which show difficulty even in prior adequate myelination of their fibers[26]. An alternative method to study the morphology of repaired PNS myelin and fibers is that of teased fibers[27,28]; the study of individual fibers facilitates the observation of morphological changes and improves morphometric evaluation.

Onion bulb formation was not observed in the EB-induced lesions in either normal or KO for Cx 32 mice. Onion bulbs are characteristic changes in CMT1X and in 4-6-month-old knockout mice and in a few experimental models of demyelination[18]. This absence suggests that the irregular communication between the axon and its Schwann cell in KO mice is a late event when those formations are reported[18]. Thus we suspect that the molecules that activate these Cx 32 genes occur in restricted numbers and that some putative alternative mechanisms for myelin maintenance are no longer available in 4-6-month-old mice when tissue decay starts in short-lived animals. For instance, the increased presence of TGFβ2 detected in Cx 32 KO mice[29] appears to be enough for myelination and myelin maintenance in young mice; changes in the biological clockwork of the involved cells however would determine the loss of function during repair[6].

Additionally, normal morphology of the myelin sheaths of EB non-injected KO mice and the repaired sheaths of the EB injected KO mice differs from the described in other myelin proteins deficient mice such as P0, PNP22 and MAG[18]. In those models, myelination itself is severely impaired (dysmyelination) and there is progressive demyelination of the ill-formed sheaths, aspects not observed in KO mice during this investigation. It would be expected that KO mice would show myelination-remyelination changes with aging.

Our findings indicate that Cx 32 absence does not change the pattern of remyelination of PNS fibers demyelinated by EB. We may speculate that either Cx 32 is not that critical for those processes, or other proteins are expressed instead; the more serious candidates would be Cx 46 and Cx 43. Cx 46 has been so far detected in denervated Schwann cells, whereas Cx 43 has not yet been found in paranodal regions and Schmidt-Lantermann of PNS fibers[20].

Alternatively, in very young individuals during peripheral myelination, mRNA for Cx 32 is very scantly detected, whereas mRNA for Cx 29 is conspicuous. This relationship between the two connexins is inverted during degeneration/regeneration[30]. Thus, Cx 29 could be expressed in the sciatic nerve of KO mice after EB-induced demyelination leading to a classic pattern of remyelination. It would be interesting to label injected fascicles in search of Cx 29 to test this hypothesis.

From the data above it may be concluded that Schwann cells from Cx 32 KO mice are able to remyelinate EB-demyelinated peripheral fibers, at least when the animals are young. The pattern and the timing of the regeneration of lost myelin sheaths in this investigation is the same as previously described for normal mice and Wistar rats. It is suggested that the process is mediated by connexons composed by connexins other than Cx 32 or alternatively by some so far unknown mechanisms that may lead to myelin repair.

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