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Research article

Changes in the connective tissue sheath of Wistar rat nerve with aging

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ABSTRACT

The alterations due to aging in the peripheral nerves can affect the physiology of these structures. Thus, the purpose of the present study was to describe the activity of the MMP-2 and MMP-9, as well as the structure and composition of the extracellular matrix of the rat sciatic nerve during maturation and aging. Our results have shown that the extracellular matrix of the sciatic nerve of 30-, 180- and 730-day-old Wistar rats present ultrastructural, morphometrical and biochemical changes during aging. The perineurium was the structure most affected by age, as evidenced by a decrease in thickness and in collagen fibril content. Cytochemical analysis detected proteoglycans in the basal membrane of Schwann cells and around perineural cells, as well as on the collagen fibrils of the perineurium and endoneurium at all ages. Biochemical analyses showed that the quantity of non-collagenous proteins was higher in 730-day-old animals compared to other ages, while the uronic acid content was higher in 30-day-old animals. Morphometrical analysis detected greater numbers of myelinated fibers and increased myelin thickness in 180-day-old animals. Zymography analysis detected greater amounts and activity of MMP-2 and MMP-9 in 180- and 730-day-old animals compared to younger rats. In conclusion, our results showed changes in the structural organization and composition of extracellular matrix of the sciatic nerve during aging, such as increase in the non-collagenous protein content and higher MMP-2 and MMP-9 activity, decrease in uronic acid concentration and in collagen fibril content in the perineurium, as well as degeneration of nerve fibers.

1. Introduction

The peripheral nerve has three connective tissue sheaths called the epineurium, perineurium and endoneurium, which create a microenvironment providing support to the nerve fibers and maintaining their physiology (Cavallotti et al., 2003). The endoneurial connective tissue and the Schwann cell-axon units of peripheral nerves are surrounded by the perineurium (Sladjana et al., 2008). The endoneurium contains capillaries, mast cells and collagen fibrils produced by fibroblasts and Schwann cells, which release neurotrophic factors during the development and regeneration of the nerve (Micheal et al., 2003). The perineurium isolates the individual nerve fascicles from the epineurial connective tissue, and it is composed of basement membranes that surround the perineurial cell layers on both sides (Cavallotti et al., 2003; Micheal et al., 2003). Basement membranes are specialized extracellular matrix (ECM) structures composed of type IV collagen associated with proteoglycans (PGs), which are constituted of long-chain glycosaminoglycans (heteropolymers of uronic acid and n-acetyl-glucosamines) (Barros et al., 2011); and non-collagenous proteins (NCPs), such as laminin and fibronectin which provide tissue architectonics and influence cell behavior (Podratz et al., 2001; El-Sayyad et al., 2014). The concentric layers of the perineurium are divided by collagen fibril bundles that maintain intrafascicular pressure, preserving endoneurial osmotic homeostasis. The perineurium also contains blood vessels, numerous pinocytotic vesicles and phosphorylated enzymes, indicating their role as an active metabolic diffusion barrier. The outer connective tissue sheath is the epineurium, which is composed of dense irregular connective tissue with abundant collagen fibrils and elastic fibrils, fibroblasts, mast cells and adipose cells (Barros et al., 2011).

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The ECM in the peripheral nerves plays an important role in the migration and proliferation of cells, as well as the preservation of nerve structure (Luo et al., 2002; Barros et al., 2011). Changes in peripheral nerves have been reported both in relation to pathology and in non-pathological aging (King, 2001; Platt et al., 2003; Yasuda et al., 2003). The literature describes some morphological and functional age-dependent changes in peripheral nerves, such as abnormalities in myelination (Verdú et al., 1996), axonal loss (Sharma and Thomas, 1975) and alterations in connective tissue and vascularization (Ceballos et al., 1999), as well as changes in target organs, such as reduction of muscle mass (Aniansson et al., 1981) and loss of sensory receptors (Schimirgk and Ruttinger, 1980). The regenerative ability of peripheral nerves can also be affected by aging (Ceballos et al., 1999). According to Shen et al. (2011), the degenerative changes caused by aging may lead to a progressive decline in conductive functioning, resulting in a disruption of the neuronal circuits.

The matrix metalloproteinases (MMPs) are a large family of zinc-dependent enzymes which are able to degrade the protein components of the ECM. Considering that these enzymes can be placed into subgroups based on structural similarities and substrate specificity, MMPs traditionally are subdivided into collagenases, gelatinases, stromelysins, and membrane-type MMPs. Although MMPs are increasingly being implicated in several pathologies of the nervous system, it is not yet clear what role they play in some normal neurobiological processes, mainly during aging (Yong et al., 2001; Lemaître and D'Armiento, 2006).

Nowadays, there is a lack of data regarding age-related changes focusing on the ECM of the peripheral nerves and the presence of MMPs. Thus, considering the diversity of the alterations caused by aging in the peripheral nerves and its implication in physiology of these structures, the purpose of the present study was to describe the activity of the MMP-2 and -9, composition and ultrastructure of the ECM in the rat sciatic nerve during aging.

2. Materials and methods

2.1. Biological material

Fifteen animals divided into three groups according to age ($n = 5$ by age); i.e. 30-day-old (sexually immature, young, 110–130 g), 180-day-old (sexually mature, adult, 430–480 g) and 730-day-old (adult senile, 685–725 g) male Wistar rats were obtained from CEMIB – Multidisciplinary Center for Biological Research (State University of Campinas, Sao Paulo, Brazil). All procedures were conducted in accordance with the guidelines of the institutional animal ethics committee. All specimens were sacrificed using pentobarbital overdose (Pfizer, New York, NY, USA) to removal the sciatic nerve. Samples from different groups were removed and immediately fixed for ultrastructural and morphometrical analysis or frozen for biochemical analysis.

2.2. Extracellular matrix extraction

Samples ($n = 5$) of fresh tissue of the same quantity (250 mg) from each group were cut into pieces and immersed in 25 volumes of 4 M guanidine hydrochloride (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 0.05 M EDTA and 1 mM PMSF in 0.05 M acetate buffer, pH 5.8 (Sigma–Aldrich, St. Louis, MO, USA) (Heinegård and Sommarin, 1987). The extraction mixture was gently stirred for 24 h at 4 °C, followed by centrifugation at 27,000 × g for 50 min with a Beckman (Fullerton, CA, USA). The supernatant (total extract) was used for biochemical analyses.

2.3. Analytical methods

The Bradford method (Bradford, 1976) and the Dische method (Dische, 1947) were used to quantify NCPs and uronic acid, respectively, in the samples of the total nerve extract. The quantification of NCPs from guanidine extract allows us to infer the available content of these molecules for metabolic or structural functions in the tissue. The uronic acid content in the extract is related to glycosaminoglycan (GAGs) quantification. This component amount is associated to proteoglycan amount in nerve tissue (Morita and Kamada, 1993). The results, obtained from extracts processed of the same tissue amount, were compared using ANOVA and the Tukey post-test ($p < 0.05$) and are reported as the mean and respective standard deviation.

2.4. Chromatography and SDS-PAGE analysis

The total extracts from the sciatic nerve were fractionated using DEAE-Sephacel (Sigma–Aldrich, St. Louis, MO, USA) chromatography and the NaCl-eluted aliquots (anionic molecules) were analyzed by SDS-PAGE using gradient gels (4–16%). The relative molecular masses were estimated by comparison with protein standard molecular mass markers. Some gels (10%) were fixed in solution (50% (v/v) methanol, 12% (v/v) acetic acid, 0.05% (v/v) formaldehyde) (Sigma–Aldrich, St. Louis, MO, USA), washed in solution I (50% (v/v) ethanol) and in pretreated by solution II (0.02% (w/v) sodium thiosulfate). Then, the gels were impregnated by silver solution (0.2% (w/v) silver nitrate, 0.075% (v/v) formaldehyde) for 20 min, washed and exposed to solution II (revelation treatment) (6% (w/v) sodium carbonate, 0.0004% (w/v) sodium thiosulfate, 0.05% (v/v) formaldehyde). To stop the process, solution II was decanted and solution III (50% (v/v) methanol, 12% (v/v) acetic acid) was immediately added (Blum et al., 1987). Others gels were submitted to Alcian blue method for GAGs detection, according Wall and Gyi (1988).

2.5. Zymography for gelatinases

The total extract (50 μg of proteins) of the nerves was used for metalloproteinase types 2 (MMP-2) and 9 (MMP-9) detection and evaluation of activity. The samples were analyzed by gel electrophoresis performed at 4 °C using 10% polyacrylamide (Sigma–Aldrich, St. Louis, MO, USA) containing 0.1% gelatin. After electrophoresis, the gels were washed with 2.5% Triton X-100 (Sigma–Aldrich, St. Louis, MO, USA) and incubated for 21 h in a solution of 50 mM Tris–HCl (pH 7.4), 0.1 M NaCl and 0.03% sodium azide (Sigma–Aldrich, St. Louis, MO, USA) at 37 °C. The gels were stained with Coomassie brilliant blue R-250 (Sigma–Aldrich, St. Louis, MO, USA) for 1 h for visualization of the negative bands of protein corresponding to gelatinolytic activity (Kleiner and Stetlerstevenson, 1994). Additionally, as a positive control, 20 mM EDTA was used in the incubation buffer to inhibit gelatinase activity (Aro et al., 2008).

2.6. Ultrastructure

Fragments ($n = 5$) of the sciatic nerves from different ages were fixed in 2.5% glutaraldehyde and 1% tannic acid dissolved in 0.1 M cacodylate buffer (Sigma–Aldrich, St. Louis, MO, USA), pH 7.3, for 2 h at room temperature. After fixation, the fragments were rinsed in cacodylate buffer and post-fixed in 1% osmium tetroxide (Sigma–Aldrich, St. Louis, MO, USA) in the same buffer for 1 h at 4 °C. The fragments were then washed in glucose saline, and treated with 1% uranyl acetate (Sigma–Aldrich, St. Louis, MO, USA) in 1.2% NaCl and 7.3% sucrose in water overnight at 4 °C (Esquisatto et al., 2007). Other fragments were treated with cuprolinic basic blue (Chemos GmbH, Regenstauf, Germany) for the detection of