Pathology – Research and Practice 211 (2015) 493–504

Contents lists available at ScienceDirect

Pathology – Research and Practice

journal homepage: www.elsevier.com/locate/prp

Original Article

Skeletal muscle findings in experimental autoimmune encephalomyelitis

Evelio Luque[a,e,1], Ignacio Ruz-Caracuel[a,1], Francisco J. Medina[b], Fernando Leiva-Cepas[a], Eduardo Agüera[c,e], Fernando Sánchez-López[c,e], Rafael Lillo[d,e], Macarena Aguilar-Luque[b], Ignacio Jimena[a,e], Isaac Túnez[b,e], José Peña[a,e,*]

[a] Departamento de Ciencias Morfológicas (Sección de Histología), Facultad de Medicina y Enfermería, Universidad de Córdoba, Spain
[b] Departamento de Bioquímica y Biología Molecular, Facultad de Medicina y Enfermería, Universidad de Córdoba, Spain
[c] Unidad de Gestión Clínica de Neurología, Hospital Universitario Reina Sofía, Córdoba, Spain
[d] Departamento de Ciencias Sociosanitarias, Radiología y Medicina Física (Sección de Psiquiatría), Facultad de Medicina y Enfermería, Universidad de Córdoba, Spain
[e] Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), Spain

ARTICLE INFO

Article history:
Received 5 September 2014
Received in revised form 23 January 2015
Accepted 6 February 2015

Keywords:
Experimental autoimmune encephalomyelitis
Multiple sclerosis
Skeletal muscle
Ragged-red fibers
Mitochondria

ABSTRACT

Introduction: Skeletal muscle is a target organ in multiple sclerosis, a chronic debilitating disease of the central nervous system caused by demyelination and axonal deterioration. Since the experimental autoimmune encephalomyelitis model reproduces the relapsing-remitting course found in most multiple sclerosis patients, this model was used to compare the histological features of skeletal muscle at onset with those observed at the start of the second relapse.
Material and methods: Histological, histochemical and ultrastructural changes, as well as biochemical oxidative damage and antioxidant-system markers, were examined in the soleus and extensor digitorum longus muscles of Dark Agouti rats in which experimental autoimmune encephalomyelitis had been induced by active immunization using myelin oligodendrocyte glycoprotein.
Results: Histological examination at disease onset revealed *ragged-red* fibers and ultrastructural evidence of mitochondrial degeneration. At the second relapse, neurogenic changes included a wide range of cytoarchitectural lesions, skeletal muscle atrophy and the appearance of intermediate fibers; however, differences were observed between soleus and extensor digitorum longus lesions. Biochemical tests disclosed an increase in oxidative stress markers at onset, which was more pronounced at the second relapse.
Conclusions: Microscopic findings suggest that two patterns can be distinguished at disease onset: an initial phase characterized by muscle mitochondrial alterations, and a second phase dominated by a histological muscle pattern of clearly neurogenic origin.

Introduction

Skeletal muscle is a target organ in multiple sclerosis (MS), a chronic neurodegenerative disease in which 65–97% of patients display neuromuscular symptoms including weakness, fatigue and spasticity [1,2]. However, published muscle biopsy findings in MS patients are both limited and contradictory. They include: muscle fiber atrophy [3,4]; change in fiber type [3,4]; reduced activity of succinate dehydrogenase (E.C. 1.3.5.1), an enzyme linking the Krebs cycle and the electron transport chain (ETC) of which it forms Complex II [3]; a greater tendency to express the fast myosin heavy chain (MHC) isoform [5]; and a reduction in ETC complex I (NADH: *ubiquinone oxidoreductase*, EC 1.6.5.3) activity [6]. By contrast, other authors report no changes [7].

Abbreviations: MS, multiple sclerosis; ETC, electron transport chain; MHC, myosin heavy chain; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; EDL, extensor digitorum longus; PBS, phosphate buffered saline; OG, onset group; RG, relapsing group; ATPase, adenosine triphosphatase; NADH-tr, nicotinamide adenine dinucleotide-tetrazolium reductase; SDH, succinate dehydrogenase; GSH, reduced glutathione; GSSG, oxidized glutathione; H-E, hematoxylin-eosin.

* Corresponding author at: Departamento de Ciencias Morfológicas (Sección de Histología), Facultad de Medicina y Enfermería, Universidad de Córdoba, Spain. Tel.: +34 957218264; fax: +34 957218246.
E-mail address: cm1peamj@uco.es (J. Peña).
[1] Both authors contributed equally to this work.

http://dx.doi.org/10.1016/j.prp.2015.02.004
0344-0338/

Although experimental autoimmune encephalomyelitis (EAE) is among the most widely used animal disease models for the study of MS, very little published research has focussed on the impact of this disease on skeletal muscle. To our knowledge, only two studies have addressed this issue, reporting – in the acute phase – evidence of muscle fiber atrophy 2–3 days after the first symptoms appear [8], and small irregular muscle fibers together with fibrosis and apoptosis 2–5 days after the onset of EAE [9]. Since MS is a progressive disease, it would be interesting to establish, using the EAE model, whether skeletal muscle might be affected at a more advanced stage. Vogt et al. [10] observed a remarkable reduction in the number of lower motor neurons (by between 47 and 74%) in some EAE models; this finding, coupled with the demyelination and axonal degeneration known to occur in EAE [11], suggests that histological neurogenic changes in the skeletal muscle may take place at any stage in the course of the disease. The present paper sought to test that hypothesis.

Histological, histochemical and electron-microscopy techniques were used to examine potential muscle pathological reactions in EAE induced by active immunization with myelin oligodendrocyte glycoprotein (MOG), this being among the most widely used approaches [12]. Regardless of the complex variety of clinical forms found in MS, and although a number of experimental models have been tested [13,14] this EAE model was used because it reproduces the relapsing-remitting course observed in most MS patients [15]. The aim was to compare skeletal muscle histology at disease onset with that observed at the start of the second relapse after a remission phase. Given that denervation can affect differently the muscle fibers types in both human and rat muscle [16,17], this study compared two muscles with radically different fiber-type composition: the soleus muscle contains 85–100% slow fibers, while the *extensor digitorum longus* (EDL) muscle comprises 95% fast fibers [18].

Materials and methods

Experimental design

A total of 28 male Dark Agouti rats (2 months old, weight 190–220 g) were used, since EAE in this strain bears the closest clinical and pathological resemblance to MS [19]. Experiments were approved by the University of Cordoba Bioethics Committee and were carried out in accordance with European Directive 86/609/ECC as approved by the Council of the European Communities, and with Spanish Law RD 53/2013, approved by the Minister of the Presidency (BOE 08, February 2013).

EAE induction was performed by injecting subcutaneously, at the dorsal base of the tail, 100 μl of a solution containing 150 μg MOG (fragment 35–55; Sigma–Aldrich, Madrid, Spain) in phosphate buffered saline (PBS) emulsified 1:1 in complete Freund's adjuvant (Sigma–Aldrich, St. Louis, USA). To complete the adjuvant, 400 μg of heat-inactivated *Mycobacterium tuberculosis* (H37Ra, DIFCO, Detroit, MI, USA) were added. Sham induction was performed in control-group (Sham) animals by subcutaneous injection of 100 μl of complete Freund's adjuvant.

Animals were monitored and clinical signs were scored as follows; 0: no sign, 1: limp tail, 2: hind limb paresis, 3: hind limb paralysis, 4: front limb paresis, 5: quadriplegic [9]. Based on this clinical monitoring, a first group of animals, *onset group* (OG) ($n = 4$), was sacrificed just after the appearance of the first clinical signs, at 14 days post-induction. In order to study skeletal muscle at a more advanced and presumably more severe stage, the other rats were allowed to continue until a second relapse was recorded on the basis of clinical scoring. At this point (day 35 post-induction), animals in this *relapsing group* (RG) ($n = 8$) were sacrificed. This study design enabled comparison of skeletal muscle features in the acute phase (OG) with those occuring at mid- to long-term (RG).

Two control groups were used: a first *control group* (Control) undergoing no intervention ($n = 8$) and a second *sham group* (Sham) inoculated with complete Freund's adjuvant alone and sacrificed at day 35 post-sham induction ($n = 8$). Animals were weighed at the start of the experiment (induction day), and at days 14 and 35 post-induction, depending on the group. Animals were kept in standard conditions with controlled temperature at 25 °C in a 12 h light/12 h dark cycle with water and food *ad libitum*.

Specimen collection

Animals were anesthetized with an intraperitoneal injection of 75 mg/kg ketamine (Imalgene® 100 mg/ml, Merial Laboratorios, Lyon, France) and killed by decapitation. EDL and soleus muscles were excised and weighed. Four random animals from Control, Sham and RG groups were used for histological examination and 4 for biochemical analysis. Muscles from OG rats were distributed as follows: the right hind limb was used for histology and the left hind limb for biochemistry. The belly of the muscles used for histological examination was sectioned for light and electron microscopy. Specimens for biochemical analysis were immediately collected and frozen at −80 °C until measurements were performed.

Histological and histochemical examination

Specimens for histological and histochemical examination were embedded in OCT (Tissue-Tek®, Miles INC), flash-frozen in isopentane cooled with liquid nitrogen, and transversely sectioned at 7 μm using a cryostat (Leica CM1850 UV) at −20 °C. Sections were stained with hematoxylin–eosin (H–E) and modified Gomori trichrome for morphological analysis. Histochemical specimens were stained for adenosine triphosphatase pH 9.6 (ATPase), nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-tr), succinate dehydrogenase (SDH) and acid phosphatase. Standard staining procedures were used [20].

Electron microscopy

Small fragments of soleus and EDL muscles were fixed by immersion in 2.5% glutaraldehyde in 0.1 M PBS buffer (pH 7.4) for 48 h at 4 °C. Tissues were postfixed in 1% osmium tetroxide for 1 h at 4 °C, dehydrated through an acetone series and embedded in epoxy resin (Durcupan ACM, Fluka, Switzerland). Ultrathin sections were obtained with an LKB 8800 Ultratome III microtome (Sweden), placed on copper grids and stained with uranyl acetate and lead citrate. Observation was performed with a JEM 1400 high-resolution transmission electron microscope (JEOL, USA) installed at the Central Research Support Service (SCAI, University of Córdoba, Spain).

Histomorphometry

Histological sections were photographed with a Sony Exwaved HAD digital camera mounted on a Nikon Eclipse E1000 microscope (Nikon, Tokyo, Japan) and a Leica MC170 HD digital camera mounted on a Leica DM2000 microscope (Leica Microsystems, Germany). Morphometric analysis was performed using the Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MA, USA) image analysis program.

Five random fields at 40× were selected in each cross-section, covering a total area of 263.200 μm^2/muscle. *Ragged-blue* and *ragged-red* fibers were quantified on sections stained with SDH or modified Gomori trichrome, respectively. ATPase pH 9.6 was used to distinguish light-staining type 1 fibers from dark-staining type 2