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Myelination and demyelination processes in the rat cerebellum cell culture: an electron microscopic study

Вивчали особливості процесу мієлінізації у культурі клітин мозочка та зміни структури мієлінових оболонок під впливом демієлінізуючих факторів – сироваток крові хворих на розсіяний склероз. Згідно з отриманими результатами, на 26-у добу in vitro структура мієлінових оболонок була найбільш схожою до такої in vivo. Саме тому цей термін культивування було обрано для індукції демієлінізації. Додавання сироваток крові хворих на розсіяний склероз до поживного середовища досить швидко (протягом 3-6 год) викликало появу змін ультраструктури мієлінових оболонок, а саме: зменшення кількості ламел, їх розшарування та інвагінацію, формування везикул тощо. Сироватка крові хворих у стадії загострення викликала більш інтенсивну демієлінізацію, ніж сироватка крові хворих у ремісії.

INTRODUCTION

Myelin sheaths of the myelinated axons are specialized multilamellar structures formed by the membranes of myelin-producing glial cells, oligodendrocytes (OD) and Schwann cells in the central and peripheral nervous systems, respectively [10,13]. Damage to the myelin sheaths related to a number of pathological processes inevitably results in disorders of normal impulse propagation via myelinated fibers. One of the most widespread myelin-injuring diseases is multiple sclerosis (MS), which is characterized by the formation of numerous loci of inflammation and demyelination in the nerve tissue and development of sclerotic plaques in these sites. Multiple sclerosis is an autoimmune disease: autoantibodies against the protein components of myelin and proteins of OD are produced in the organism [4,2]. A number of aspects of etiology of this disease and its cellular mechanisms still remain unclear. Thus, the development of adequate experimental models allowing one to study in detail the processes of myelination/demyelination is obviously important [12,16]. Techniques of culturing of the nerve tissues possess certain crucial advantages with this respect: in this case there are (i) possibilities for direct application of various agents (including those produced in the organism under conditions of pathology) to the neurons and glial cells, (ii) favourable conditions for microscopic examination of these cells, and (iii) possibilities for the analysis of the dynamics of morphological and functional modifications in the course of culturing. The goal of our experiments was to study morphological manifestations of the myelination and demyelination processes in a dissociated culture of the cerebellum of newborn rats. Advantages and limitations of such a model will be discussed below.

MATERIALS AND METHODS

Cerebellar cells were cultured using an earlier described technique [5], but with some modifications of the medium composition, which allowed us to maintain an adequate state of the cells for a sufficiently long time. The cerebellum taken from newborn Wistar rats was mechanically dissociated, the cell suspension was plated on glasses

covered with poly-L-lysine ("Sigma", USA), and the cells were cultured in 5%CO_2-enriched air atmosphere at 37°C. The density of plating was $2x10^{-5}$ cells in 1 μl. The culturing medium initially contained 80% DMEM ("Gibco", USA), 10% bovine fetal serum ("Intergen", USA), and 10% horse serum ("Gibco", USA) with 6g/liter glucose added. From day *in vitro* (DIV) 5, the composition of the medium was changed to 80% DMEM, 20% horse serum, and 33,3 mM glucose. Electron microscopy examination of the state of cultured cells was performed on DIV 21, 26, and 31-35. Standard techniques for preparing ultrathin sections were used (5). Briefly, cultured cells were fixed with 1,5% glutaraldehyde and post-fixed with 1% osmium tetroxide, dehydrated in increasing concentrations of ethanol, and flat-embedded in epoxy resin (EPON-812, araldite M, DDSA, and DMP-30, "Fluka AG", Germany). Ultrathin sections were stained with uranyl acetate and lead citrate and examined using JEOL 100CX electron microscope at 60 kV.

As a demyelinating factor, we used blood serum obtained from patients suffering from MS in acute or remission stage, S_a and S_r respectively (courtesy of Dr. L.I. Sokolova, Bogomolets National Medical University, Kyiv, Ukraine). On DIV 26, horse serum (20%) in the medium for experimental samples was substituted by an equivalent amount of S_a or S_r. Cultures treated with the same amount of blood serum of healthy donors were used as the control. The complement of proteins obtained from healthy donors was added to the medium of all samples (to provide initiation of an immune response).

The nonparametric two-tailed Kolmogorov-Smirnov test was used to assess the differences between analysed samples. The level of statistical significance was set at $P<0,01$.

RESULTS

The myelination process in the culture became clearly observable on DIV 17-19, which in general agrees with earlier published data [11]. On DIV 21, myelin sheaths already covered most axons, and their pattern was rather close to that observed *in vivo*, in the cerebellum of rats of a comparable age. Dense (dark) and light lines within the sheaths were readily differentiable; they formed concentric (more correctly, spiral) structures. The axolemma was clearly outlined and contacted with an internal surface of the myelin sheath. Neurofilaments, microtubules, membrane structures, and dense bodies could be observed within the axoplasm. There was no OD cytoplasm on the internal side of the myelin sheath, but it could be noticed on the external surface. On DIV 21, most myelin sheaths consisted of 2 to 7 lamellae (some axons were covered even by 10 to 12 lamellae). Yet, within the above term most sheaths looked not absolutely compact; usually they included thin cytoplasm layers separating the lamellae. Therefore, on DIV 21 the pattern of the myelin sheaths should be considered to a certain extent immature.

On DIV 26, myelin sheaths contained 4 up to 17 (mean, $8,78 \pm 0,64$; $n=59$) compactly packed lamellae (Fig.1A.), whose pattern was most similar to that observed *in vivo*. On DIV 31-34, the number of myelin sheaths around individual axons of cultured neurons significantly decreased and in most cases did not exceed 9 (usually 6-7). However, within this term of culturing abnormal additional myelin structures could be observed: "secondary" and even "tertiary" myelin sheaths, surrounding one or several axons and in such a way forming axonal bundles were typical findings (Fig.1B.). Higher-order sheaths were separated from "normal" ones and from each other by some space; in many cases there was also spacing between individual lamellae. The latter frequently possessed a wavy shape. These phenomena should be obviously considered a clear deviation from the normal development of myelination observed under *in vivo* conditions. It is necessary to emphasize that the above late period of culturing was also characterized by clear manifestations of aging of the nerve and glial cells. Diameters of the axon cross-sections decreased, and the number of organelles within the axoplasm noticeably dropped. Vacuolisation

was also a typical finding both in the nerve cells and OD.

On DIV 26, neuronal cells were characterized by a typical ultrastructure of the cytoplasm and nucleus, and a standard set of the organelles was present in these units. Cultured neurons demonstrated certain specificity in their dimension and shape, dimension of the nucleus, and ultrastructure of the karyoplasm, which probably depended on a particular type of the cerebellar cell under study [9]. Comparison of the structural features of cultured cerebellar cells and their axons within various culturing periods allowed us to conclude that the maximum number of mature myelinated fibers was formed up to DIV 26. This is why we selected this time interval for examination of demyelination effects induced by sera obtained from the blood of MS patients. At the same time, within this period there were still no significant manifestations of pathological aging of the culture.

Substitution of horse serum in the culturing medium by S_a or S_r at DIV 26 resulted in rather rapid development (in 3 to 6 hours) of intensive structural modifications in the somata of cerebellar neurons, nerve fibers, and glial cells. Significant changes became also observable in a great majority of ultrathin sections of the myelinated axons (Fig.2). The compactness and integrity of the myelin sheaths were partially or completely disturbed. Contours of the lamellae in many cases looked "washed out". A number of the lamellae possessed a wavy pattern and looked separated from each other (Fig.2 A, B). A regular concentric pattern of the lamellae was also disturbed. Frequently, curved lamellae for-

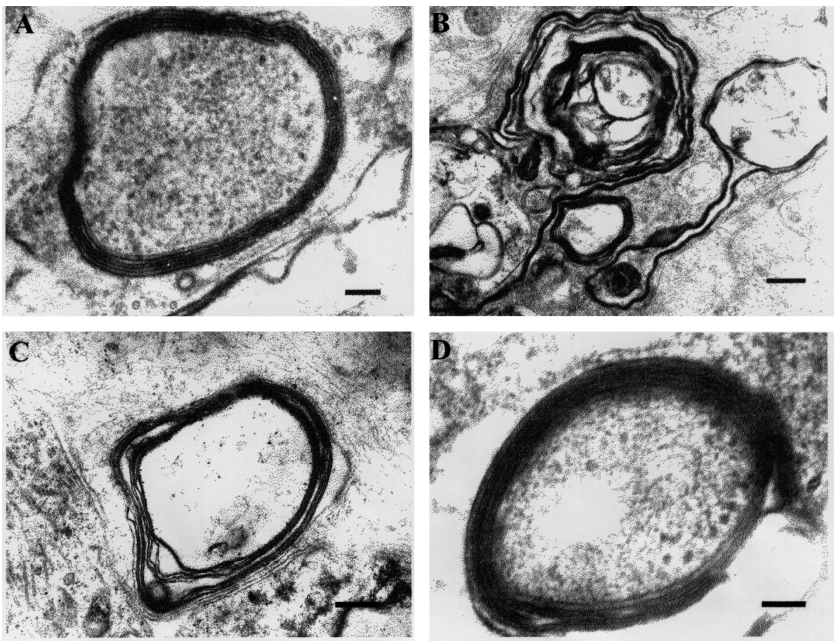

Fig. 1. Cross-sections of myelinated axons in dissociated cell cultures of the cerebellum of newborn rats.
A. Day *in vitro*, DIV, 26. The myelin sheath is formed by compactly packed lamellae, and its structure is rather similar to that observed *in vivo* within the comparable period of ontogenesis.
B. DIV 32. Manifestations of aging of the culture: the number of lamellae forming the sheaths decreases; the lamellae frequently possess a wavy shape; abnormal myelin configurations ("secondary" and even "tertiary" myelin sheaths covering several axons) are observed.
C and D. A control experiment. DIV 26, 6 hours after blood serum of a healthy donor was added to the culturing medium. Moderate disturbances in the structure of the myelin sheath of an axon (C), and no significant changes in the sheath of another axon (D).
Bar scale = 0.1μm (A, C, D) and 0.5μm (B)

med loops and cisterns within a sector of the fiber cross-section. In many cases, numerous vesicle-like structures could be observed within the above or adjacent regions of the sheath (Fig.2 C, D). Due to invaginations of the axolemma, cross-sections of many axons lost their regular oval or round shape. The axoplasm content looked more homogeneous than in the control, and differentiation of the organelles became more difficult. Six hours after addition of S_a or S_r, the mean number of the myelin lamellae covering axons in the S_a- and S_r- treated cultures significantly dropped (means, 5,07 ± 2,47, n=221 and 5,97 ± 1,24, n=210, respectively), as compared with that in the control samples. We should emphasize that 6 hours after the addition of S_a or S_r, when ultrastructural modifications of the axon cross-sections attained a dramatic level, round-up light microscopy of the culture samples failed to showed observable changes in the external view of the culture.

In general, the S_a-induced effects were somewhat more intensive than those related to the influence of S_r. In the former case, clear destructive changes were found in a greater proportion of the axons. In the S_a-treated cultures, isolated manifestations of "washing out" of the lamella boundaries and distortion of the shape of the lamellae were observed in 33% and 37% of the fiber sections, respectively. At the same time, in the S_r-treated cultures the respective proportions of fiber cross-sections with the above-mentioned "specific" impairments did not exceed 30%. Combination of the above pathological modifications in the same fiber was found in the S_a-treated cultures two times more frequently (18% vs. 9% in the S_r-treated samples). Similar intergroup differences were also characteristic of the

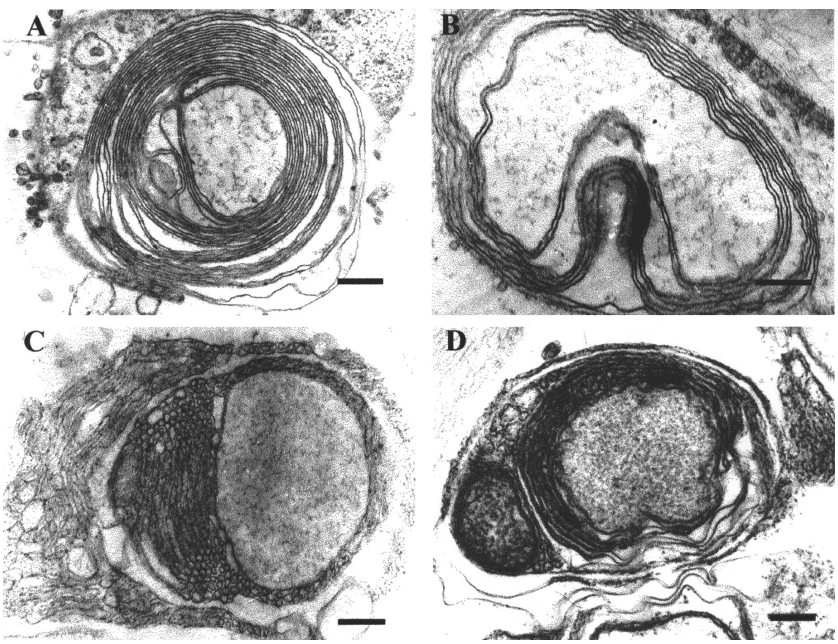

Fig. 2. Destructive changes in the myelin sheaths in dissociated cell cultures of the cerebellum treated with blood sera of patients with MS.
DIV 26, 6 hours after the addition of blood sera of the patients with MS in the remission stage (A and B) and in the acute stag e (C and D).
A and B. Loss of the compactness and integrity of the myelin sheaths of the axons; "washing-out" of the lamella contours within some sectors of the sheath; invagination of the lamellae (B).
C and D. Partial disintegration of the myelin sheaths; a concentric pattern of the lamellae is to great extent lost; curved lamellae form loops and cisterns on one side of the fiber cross-section; numerous vesicle-like structures are observed within the sections.
Scale bars = 0.2μm

presence of invaginations and "novel" structural elements (loops and/or vesicles) in the myelin sheaths. At least one type of the above-mentioned abnormal modifications was clearly manifested in 207 of 210 (98%) examined fibers in the S_a group, while the respective proportion in the S_r group was 181/221 (81%).

In the S_a- and S_r-treated cultures, OD were characterized by the presence of numerous lysosomes and vacuolisation. Sometimes, dark myelin-like formations and polysomes could be observed within these cells. Some manifestations could be interpreted as phagocytosis of the residues of disintegrated myelin (not illustrated).

In control experiments, when serum of neurologically healthy donors was added to the culturing medium, we also observed some disturbances in the myelin sheaths within the above-mentioned time intervals (Fig.1 C). Yet, clear changes could be found only in a rather limited proportion (not more than 4-5%) of the examined fiber sections, and they were, as a rule, much less intensive, as compared with those in the cultures treated with blood serum of MS patients.

DISCUSSION

We used a dissociated cell culture of the cerebellum taken from newborn rats to study the myelination and demyelination processes *in vitro.* The reasons for using this object were as follows. Intensive myelination in the rat cerebellum begins *in vivo* on postnatal days 5 to 7. An adequate modification of the culturing conditions allowed us to provide the development of cerebellar neurons, their axons, and glial cells for a sufficiently long period (about 35 days). Within early stages of the culturing process, our samples contained no myelinated axons; so, we can conclude that all crucial stages of formation of the myelin sheaths developed *in vitro*, and thus the entire dynamics of myelination was observable.

As was reported earlier [11], intensive myelination of the axons of cultured neurons taken from early postnatal rats begins approximately on DIV 19, and supposedly mature myelinated fibers could be observed on DIV 26–28. Although our results agree in general with these data, myelination developed in our experiments within somewhat earlier terms. We selected DIV 26 as the optimum term for observation of a greatest number of the myelinated axons, because longer culturing even with no additional influences resulted in clear degenerative changes of the cells and fibers (in particular, a considerable part of the myelin sheaths became thinner and possessed an abnormal pattern). Within late period (DIV 30-35), vacuoles and inclusions, which were absent *in vivo*, appeared in the cytoplasm of OD and some neurons, i.e., modifications similar to those observed *in vivo* in normal aging animals were identified [6].

Clinical observations show that the cerebellum is one of the CNS structures intensively damaged in MS [7,8,15]. It is known that manifestations of this disease include a typical syndrome of a serious cerebellar dysfunction: motor incoordination (ataxia), tremor, nistagm, and scanned speech. Destruction of the compact myelin and modification of metabolism of the myelin components are crucial phenomena related to MS (3,14), and such shifts could be readily detected in the cerebellum.

In our experiments, treatment of the cultures with blood sera of MS patients resulted in the rapid development of intensive changes clearly resembling manifestations of the demyelination process. Clear pathological ultrastructural modifications of the myelin sheaths became readily visible even in a few hours after the addition of the sera (S_a or S_r) to the culturing medium. These were signs of degradation of the myelinated axons: complete or partial destruction of myelin, spacing of the myelin lamellae from each other, and appearance of abnormal "novel" structures (clearly outlined vesicle-like compartments, either scattered between separated lamellae or concentrated on one pole of the fiber cross-section). In addition, we could observe cistern-like myelin structures probably formed from curved lamellae. As formation of the vesicular compartments is considered to be one of the crucial phenomena

related to damage to any membrane structure [1], we can suppose that the above-described "novel" microstructures within the periaxonal space should be considered derivatives of the myelin lamellae affected by the demyelinating factor. Control experiments demonstrated that the observed changes are undoubtedly specific. Blood serum obtained from healthy persons exerted no dramatic effect on the ultrastructure of the myelin sheaths: the observed modifications were mild or negligible. Thus, we can conclude that antibodies to protein components of the myelin sheaths and to OD proteins, which are present in the blood sera of MS patients and absent in the sera of healthy individuals, are responsible for the induction of destructive changes in the myelin sheaths. Quantitative estimates showed that S_a evokes somewhat more intensive damage to the myelin sheaths as compared to S_r; this difference is probably related to a higher amount of anti-myelin antibodies in S_a.

In general, protein-lipid complexes forming the myelin sheaths are supposed rather conservative structures. This is why an extremely rapid development of the demyelination process in the S_a- and S_r - treated cerebellar cultures looks somewhat surprising. We can speculate that such a dynamics is to a great extent related to the period of culturing we have selected for our tests. This was practically a climax stage of the culture development, when even in the absence of any negative factor the culture was close to an unstable state. Within this time interval, the presence of a specific demyelinating agent (MS-related antibodies) in the culturing medium could play the role of an extremely potent destructive factor and provide a high rate of the respective ultrastructural modifications.

Our experiments demonstrated that cultured nerve and glial cells of the cerebellum of newborn rats could be used as a sufficiently adequate model for studying the process of myelination. Moreover, this cell system allowed us to successfully model the process of demyelination using blood serum of MS patients as a specific demyelinating agent. We believe that this model can be in future used for testing the effects of pharmacological factors, which are capable of preventing demyelination, promoting remyelination, and stabilizing the myelin structures.

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MYELINATION AND DEMYELINATION PROCESSES IN THE RAT CEREBELLUM CELL CULTURE: AN ELECTRON MICROSCOPIC STUDY.

We observed manifestations of the myelination process in dissociated culture of the cerebellar tissue of newborn rats and modifications of the structure of myelin sheaths after treating the culture with a demyelinating factor, blood serum of patients suffering from multiple sclerosis (MS). On day *in vitro* (DIV) 26, in the control myelin sheaths of the axons demonstrated the closest resemblance to those observed *in vivo*, and we selected this term for inducing demyelination. Addition of the serum of MS patients to the culturing medium evoked rapid (in 3-6 hours) dramatic changes in the ultrastructure of myelin sheaths; these were a decrease in the number of the lamellae, their splitting and invagination, formation of vesicles, etc. The serum of MS patients in an acute stage of the disease exerted more intensive demyelination effects than that of patients in a remission stage.

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