QUANTITATIVE AND QUALITATIVE ANALYSIS OF PROTEINS IN RAT PERIPHERAL NERVES PREDEGENERATED FOR 7 DAYS

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Objectives: In contrast to peripheral nerves, central neurons do not regrow spontaneously after injury. Our previous studies showed that transplantation of degenerating peripheral nerves or their extracts can induce regeneration in the injured central nervous system. Non-predegenerated nerves show much weaker neurotrophic activity. The aim of the present work was to examine quantitatively and qualitatively the protein composition of rat sciatic nerve extracts.

Material and Methods: The experiments were carried out on male Wistar C rats. Distal fragments were collected immediately after transection or after 7 day-long predegeneration. The nerves were homogenized, centrifuged and ultracentrifuged. Extracts were analyzed by means of two-dimensional electrophoresis.

Results: The two-dimensional electrophoresis showed 69 protein subfractions with isoelectric points ranging from 4.2 to 7.0 pH and molecular weight ranging from 13.5 kDa to 335.4 kDa in extracts obtained from nonpredegenerated nerves. In predegenerated nerve extracts 114 subfractions with isoelectric points ranging from 4.2 to 7.4 pH and molecular weight from 21.1 kDa to 335.4 kDa were found. Fractions: 25.5 kDa, 31.6 kDa, 36 kDa, 38.4 kDa, 42.4 kDa, 46.6 kDa, and 50.5 kDa showed significant increase and two fractions: 68.5 kDa and 335.4 kDa demonstrated significant decrease in the number of subfractions in predegenerated nerves. Fractions 160.8 kDa, 236.1 kDa, and 5 fractions below 21.1 kDa were present only in extracts from non-predegenerated nerves.

Conclusions: In conclusion, the results of our study demonstrate that the most intense changes in protein composition in degenerating nerves take place in low molecular weight fractions.

INTRODUCTION

It is proven that the damaged neurons of the central nervous system (CNS) of adult mammals, in contrast to peripheral nervous system, do not have the capacity for spontaneous and successful regrowth. The inability of adult CNS neurons to regrow after injury do not stem from an intrinsic deficit of the neuron, but rather from a characteristic feature of the environment of damaged neurons that prevent successful regeneration. Recently it has been suggested that adult CNS neurons can regrow if the environment of central nervous system is modified suitably. Thus neurobiologists try to introduce into CNS regeneration, stimulators such as neurotrophic factors, substances blocking growth inhibitors, genetically modified cells, tissue extracts, fetal neurons, Schwann cells, fibroblasts, central glia, and olfactory epithelia or they manipulate intracellular signalling and modulate the immune response to obtain regrowth of damaged neurites^{10, 14, 35, 39}.

Implantation into injured parts of CNS fragments of peripheral nerves or extracts from such nerves is another strategy for inducing regeneration^{1, 9, 20, 24–26, 28, 29, 32}. It is known that distal part of injured peripheral nerve plays crucial role in nerve regeneration. Our previous studies revealed that purified extracts obtained from predegenerated nerves stimulate outgrowth of damaged neurites in

both PNS and CNS^{12, 24–26, 28, 29, 31}. Our group found that the neurotrophic activity of the above extracts depends on the predegeneration period and reaches three maxima at the 7th, 28th and 35th day after nerve transection. The greatest neurotrophic activity had fractions obtained from nerves predegenerated for 7 days^{24–26, 28}. Moreover it is possible to intensify their neurotrophic effect by extraction proteins of molecular weight from 10 to 100 kDa from the entire extract [in press – Neurotrophic activity of extracts from distal stumps of predegenerated peripheral rat nerves varies according to molecular mass spectrum⁵. This forms the rationale for selecting these extracts for further investigation.

The aim of the present work was to analyze quantitative and qualitative changes in protein composition of predegenerated peripheral nerves by means of two-dimensional gel electrophoresis.

MATERIALS AND METHODS

The experiment was carried out on male Wistar C rats, weighing 200–220 g according to the Polish Animal Protections Laws and the European Union Directives. All procedures were approved by the local Ethics Committee. The animals were housed in individual cages and received

a standard diet and water *ad libitum*. Forty animals were used to prepare postmicrosomal fractions from nonpredegenerated and predegenerated nerves (twenty animals for each kind). In our experiments we analysed the protein composition of extracts by means of two-dimensional gel electrophoresis.

Postmicrosomal Fraction Preparation

The animals were deeply anesthetized with a chloral hydrate intraperitoneal injection (420 mg/kg body wt). The right sciatic nerves were cut near the hip joint. Seven days later, the rats were sacrificed by decapitation and the distal stumps of cut sciatic nerves and whole left intact sciatic nerves were dissected and stored in cold Ringer solution for mammals. Nerves transected 7 days earlier served as a source of postmicrosomal fractions for the PD group, whereas fraction in the NPD group was obtained from intact nerves. Subsequently, nerves were homogenized (Ultrasonic Processor GE 50, Aldrich, USA) in a buffer (pH 6.4) consisting of 0.1 M morpholinoethane sulfonic acid (MES); 1mM ethyleneglycol bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA); 0.1 mM methylenediaminetetraacetic acid (EDTA) (all Serva, Heidelberg, Germany), using 2 ml/1g nerve tissue. Homogenates were filtered through cotton gauze and filtrates were sedimented by centrifugation at 700 x g for 10 min. Supernatants were centrifuged at 20 000 x g for 10 min and then ultracentrifuged at 105 000 x g for 120 min to obtain postmicrosomal fractions. All preparation procedures were carried out at 0 °C in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF) (Serva) as a protease inhibitor. Total protein concentration in the final extracts was determined by the method of Bradford³.

Two-dimensional electrophoresis

Postmicrosomal fractions were analysed by two-dimensional electrophoresis according to the method of O'Farrell³⁴. This method consists of two stages: isoelectric focusing and SDS-PAGE-electrophoresis.

Isoelectric focusing

Isoelectric focusing separated proteins according to their isoelectric points. Glass tubes were filled with urea gel [9 M urea (POCH), 3.77 % acrylics (Sigma), 0.22 % bis-acrylamid (Sigma), 0.5 % nonidet (Sigma), 25 M CHAPS (Sigma), 2 % Pharmalyt (Pharmacia)] with a pH gradient from 4.2 to 7.4 (achieved by means of 3–10 Pharmalyte). Gels were subjected to pre-focusing (preliminary focusing) lasting 45 min. Then 100 µl of protein fraction was placed at the top of the tubes. One of the tubes was left "empty" (without protein) and used for preparation of the standard curve for pH estimation. Proper focusing was performed for 18 hours at 300 V.

SDS-PAGE-electrophoresis

After focusing, the gel "noodles" were removed and stained in Coomassie Brilliant Blue R-250 [0.1% Coomassie Brilliant Blue R-250 (Serva), 10 % acetic acid (POCH), 40 % methanol (POCH)] for 15 min and were then joined

with SDS-polyacrylamide gels with help of 1 % agarose (Serva). We applied 8 % separating gel and 5 % concentrating gel. Electrophoresis was performed for 18 hours at 300 V followed with 400 V for 1 hour. After electrophoretic resolution gels were fixed and silvered according to the method of Wray⁴¹. Gels were photographed, molecular weights of proteins were estimated by the Southern method³⁸. Ten resolutions for each group were performed. For statistical analysis we used the Student t-test (we compared the number of the subfraction in protein fractions identified on electrophoregram of extracts from predegenerated nerves with nonpredegenerated). The level of significance was set at $p < 0.05$.

RESULTS

In extracts obtained from predegenerated nerves the total protein concentration was significantly higher ($p < 0.05$) than in the extracts from nonpredegenerated nerves. (Tab. 1)

The two-dimensional electrophoresis showed 28 protein fractions of different molecular weights (from 13.5 kDa to 335.4 kDa) containing 69 protein subfractions with pI ranging from 4.2 to 7.0 in extracts obtained from nonpredegenerated nerves. The widest range applies to the 68.5 kDa fraction, with pI 4.4–7.0. (Tab. 2)

In extracts obtained from predegenerated nerves 21 protein fractions and 114 subfractions were present, with pI ranging from 4.2 to 7.4 and molecular weight from 21.1 kDa to 335.4 kDa. Widest range showed fraction 42.4 kDa (4.4–7.4), whereas the narrowest – 335.4 kDa (4.6–4.9). (Tab. 3, Fig. 1) Seven fractions 25.5 kDa; 31.6 kDa; 36 kDa; 38.4 kDa; 42.4 kDa; 46.6 kDa; 50.5 kDa and showed significant increase in the number of subfractions as compared to extracts from nonpredegenerated nerves (Fig. 1 and 2). Only two fractions – 68.5 kDa and 335.4 kDa demonstrated significant decrease in the number of subfractions at the 7th day after transection. Fractions 160.8 kDa; 236.1 kDa and fractions below 21.1 kDa (13.5 kDa; 16.2 kDa; 17.2 kDa; 18.6 kDa; 20.0 kDa) were absent in extracts from predegenerated nerves.

On the basis of above observations it can be concluded that the most intensive changes in protein composition

Fig. 1. Histogram showing a number of subfractions with different isoelectric points in nonpredegenerated and predegenerated rat sciatic nerves.

Fig. 2. 2D-PAGE electrophoretogram of proteins from predegenerated distal stumps of rats' sciatic nerves.

of postmicrosomal fractions were observed in proteins between 25.5 kDa and 68 kDa in extracts from predegenerated nerves.

DISCUSSION

Our results demonstrate that a process of 7-days predegeneration of peripheral nerves leads to intensive changes in protein composition in the distal segment. We know that extracts from distal segments of peripheral nerves predegenerated for 7 days show the strongest neurotrophic activity on the damaged hippocampus in adult rats^{24, 25, 27, 28}. Moreover it is possible to intensify their neurotrophic effect by extraction from the entire extract proteins of molecular weight from 10 to 100 kDa (in press – Neurotrophic activity of extracts from distal stumps of predegenerated peripheral rat nerves varies according to molecular mass spectrum.).

After electrophoretic examination of extract from predegenerated nerves we observed a significant increase in the number of subfractions for fractions 25.5 kDa, 31.6 kDa, 36.0 kDa, 38.4 kDa, 42.4 kDa, 46.6 kDa and 50.4 kDa as compared to the extract from nonpredegenerated nerves. (Tab. 2 and 3; Fig. 1) Which of this fraction or combination of several fractions could participate in the neurotrophic activity?

The active subunit β of NGF has a molecular weight about 26 kDa²⁸. Perhaps in the mixture of subfractions from predegenerated extract it is NGF which plays an essential function in neurotrophic processes but probably it is not only factor. In an *in vivo* experiment we showed that introducing fractions below 30 kDa from predegener-

Table 1. Total protein concentration (mg/g tissue) in postmicrosomal fractions from the distal stumps of nonpredegenerated (NPD) and predegenerated (PD) rat sciatic nerves.

	NPD (n = 10)	PD (n = 10)
PROTEIN CONCENTRATION (mg/g tissue)	1.310 $\pm$ 0.810	3.210 $\pm$ 2.979

ated nerves into damaged hippocampus the outgrowth of neurites was not as intensive as in the case of complete extracts²².

Another interesting protein with a molecular weight of about 25 kDa is Bcl-2. Bcl-2 is the main controller of apoptosis in mammals, especially important in the nervous system⁸. Bcl-2 protein is known to prevent neurons from apoptosis induced by many different factors, including axotomy, but thus far, its role in axon regeneration has not been established. However our previous study suggested that Bcl-2 is important for the neurotrophic activity of peripheral nerve grafts during optic nerve regeneration²¹.

Comparing electrophoretograms from predegenerated nerves with nonpredegenerated ones we noticed lack of the protein fractions below 21.1 kDa (13.5 kDa, 16.2 kDa, 17.2 kDa, 18.6 kDa, 20.0 kDa). We assume that a reason for the absence of these proteins is degradation of the myelin during Wallerian degeneration induced by the nerve injury. Components of the myelin sheet have very similar molecular weights – protein A₁ – 18.4 kDa, MBP – 18 kDa and P₁ – 15 kDa. The proteins are degraded (resorpted) by macrophages and this might be the reason that they are absent in electrophoretograms. Perhaps among fractions with molecular weights from 25.5 kDa to 50.4 kDa which increases the number of subfractions, there are some proteins building the peripheral myelin sheet. The molecular weight of the main protein in peripheral myelin P₀ is 30 kDa and LG1 – 25 kDa, as well as Wolfgram proteins – 52 kDa and 55 kDa and two isoforms of CNP – 46 kDa and 48 kDa^{11, 16, 18, 21, 22, 39}. Myelin sheet proteins do not possess neurotrophic properties but as components of newly formed myelin, they might be able to influence the remyelination process of regenerating nerve fibres.

A protein which has aroused our interest is apolipoprotein E with a molecular weight of about 37 kDa and which participates in lipid and cholesterol metabolism and transport. Ignatius reports that synthesis and accumulation of apolipoprotein E increases in the distal segment of the damaged nerve. Probably this protein plays a role in the degradation of myelin sheet rich in lipids and is useful for incorporation of degradation products into membranes of newly formed axons^{4, 16, 33}. Since, we identified in electrophoretograms of extracts from predegenerated nerves two fractions with molecular weights similar to apolipoprotein E – 36.0 kDa and 38.4 kDa, we assume that the above hypothesis is likely to be true.

Table 2. Fractions with different molecular weight and pH range found in 2D-PAGE of postmicrosomal fractions from nonpredegenerated rat sciatic nerves.

Molecular weight (kDa)	pH Range																			
	4,2	4,4	4,6	4,9	5,1	5,4	5,5	5,8	5,9	6,3	6,4	6,5	6,7	6,9	7,0	7,3	7,4	7,6	7,8	
335,4 ± 1,70																				
236,1 ± 1,05																				
182,9 ± 5,98																				
160,8 ± 5,04																				
138,9 ± 5,56																				
119,5 ± 4,65																				
99,1 ± 4,21																				
80,3 ± 1,62																				
75,8 ± 2,50																				
68,5 ± 1,73																				
64,9 ± 0,96																				
60,0 ± 1,81																				
54,0 ± 1,17																				
50,4 ± 0,96																				
46,6 ± 0,88																				
42,4 ± 0,76																				
38,4 ± 0,76																				
36,0 ± 0,44																				
31,6 ± 0,66																				
25,5 ± 0,42																				
24,0 ± 0,37																				
22,5 ± 0,57																				
21,1 ± 0,13																				
20,0 ± 0,30																				
18,6 ± 0,37																				
17,2 ± 0,19																				
16,3 ± 0,29																				
13,5 ± 0,25																				

Table 3. Fractions with different molecular weight and pH range found in 2D-PAGE of postmicrosomal fractions from predegenerated rat sciatic nerves.

Molecular weight (kDa)	pH range																		
	4,2	4,4	4,6	4,9	5,1	5,4	5,5	5,8	5,9	6,3	6,4	6,5	6,7	6,9	7,0	7,3	7,4	7,6	7,8
335,4 ± 1,70*																			
236,1 ± 1,05*																			
182,9 ± 5,98																			
160,8 ± 5,04*																			
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25,5 ± 0,42*																			
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22,5 ± 0,57																			
21,1 ± 0,13																			
20,0 ± 0,30*																			
18,6 ± 0,37*																			
17,2 ± 0,19*																			
16,3 ± 0,29*																			
13,5 ± 0,25*																			

* fractions showed significant differences to compare with fractions from nonpredegenerated nerves
* absent fractions

After analysis of electrophoretograms, two further fractions – 42.4 kDa and 46.6 kDa were the focus of our attention. These fractions in extracts from predegenerated nerves contained a large number of subfractions with very wide pH range (42.4 kDa pH = 4.4 – 7.4; 46.6 kDa pH = 4.4–7.3). Presumably proteins known as GAPs (growth associated proteins) are located within the discussed range. GAPs are involved in the elongation of neuronal processes. After axotomy, synthesis of GAP-43 is especially increased. A molecular weight of GAP-43 is about 43 kDa. GAP-43 is considered as the marker of regeneration, because high expression of this protein takes place in growing axons of young animals and then is reduced in adults. The repeated increase of this protein expression occurs after breaking continuity of the nerve. GAP-43 is responsible for the polymerisation of actin¹⁸. Recently, GAP-43 has also been implicated in the regulation of synaptic transmission and plasticity in long-term potentiation¹⁵.

Structural elements of cytoskeletal microtubules – α and β tubulin (53 kDa, 55 kDa) and MAPs protein (30 kDa) are probably among fractions with a molecular weight from 25.5 kDa to 50.4 kDa. Phosphorylated forms of MAP1B participate in regeneration in the peripheral

nervous system, being responsible for dynamic the character of microtubules³⁶.

One of two fractions which have shown statistically significant decrease in the number of subfractions compared with extracts from nonpredegenerated nerves is fraction 68 kDa. (Tab. 2 and 3; Fig. 1) Molecular weight of L subunit of neurofilaments is about 57 – 70 kDa and it is possible that one of the reasons for the absence of 68 kDa proteins is degradation of this neurofilaments subunit³⁷. Bignami and co-workers, analyzing Wallerian degeneration in the optic and sciatic nerves observed that proteins with molecular weights of about 72 kDa and 150 kDa, and which build neurofilaments, were not detected two weeks after the beginning of the degeneration².

We observed lack of fractions 160.8 kDa and 236.1 kDa and statistically significant decrease in the number of the subfractions in 335.4 kDa fraction. (Tab. 2 and 3) Subunits of rats neurofilaments – M and H have molecular weights 110–160 kDa and 160–210 kDa and probably they break up into proteins with lower molecular weight seven days after transection^{13,30}. We assume that this is the reason that fractions 160.8 kDa and 236.1 kDa are not detected in electrophoretograms.

We should to remember, that in extracts from pre-

degenerated peripheral nerves, beside known proteins – the neuron degradation products or neurotrophic factors, there are probably also some completely new proteins. Chakraborty and co-workers found that in the distal segment of the damaged rat sciatic nerve proteins underwent posttranslational modifications. This process is characteristic for proteins with molecular weight from 80–90 kDa, 53–66 kDa, 22–46 kDa and 17 kDa, with isoelectric points from 5.0 to 7.9 (Ref.⁵⁻⁷). Because of this modification the properties of new proteins are changed and may become favourable for supporting outgrowth of the damaged nerve fibres.

In conclusion, the results of the present study demonstrate that the most intensive changes in protein composition of postmicrosomal fraction were observed in proteins between 25.5 kDa and 68 kDa. In extracts from predegenerated nerves fractions below 21.1 kDa (13.5 kDa, 16.2 kDa, 17.2 kDa, 18.6 kDa, 20.0 kDa) and fractions 160 kDa and 266.1 kDa these changes were absent. Among fractions described above, we should look for a factor or a combination of factors intensifying neurotrophic activity of extracts from predegenerated nerves. Identification of these molecules will help us to develop new strategies leading to the restoration of both structure and function of the damaged mature CNS.

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