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NERVE GROWTH FACTOR HOMOLOGY IN THE NERVE REGENERATION-CONDITIONED FLUID OF RABBITS AND RATS

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We analyzed the homology of nerve growth factor (NGF) in the nerve regeneration-conditioned fluid (NRCF) obtained from New Zealand white rabbits and SD rats. A nerve regeneration chamber bridged distal and proximal ends of the severed rabbit sciatic nerve, and the NRCF was extracted from such chamber. The supernatants and precipitates of nerve tissue of rabbits and rats were extracted from the sciatic nerves by grinding and centrifugation. An antigen-antibody reaction was detected by the ELISA technique using rabbit anti-mouse NGF as primary antibody and goat anti-rabbit IgG as secondary antibody. Antigen-antibody reactions were detected in all the above-mentioned samples obtained from rabbits and rats. The intensity rank of an antigen-antibody response for different samples was the following: rabbit NRCF > rabbit nerve tissue precipitation fluid > rabbit nerve tissue fluid supernatant > > rat nerve tissue fluid supernatant. Thus, there is high homology of the molecular structure of NGF in the NRCFs obtained from rabbits and rats (i.e., animals belonging to different taxonomic groups of mammals).

Keywords: nerve growth factor, nerve regeneration-conditioned fluid (NRCF), peripheral nerve, homology of proteins.

INTRODUCTION

A nerve regeneration chamber, NRC, consisting of a silicone tube bridging distal and proximal ends of the transected sciatic nerve, has been proposed as a device that helps to study the sequence of events accompanying axonal regeneration *in vivo* following nerve injury [1, 2]. The chamber provides an appropriate microenvironment for survival of all types of fibers found in the sciatic nerve, including axons of sympathetic neurons, sensory fibers, and motor fibers (axons of spinal motoneurons) [3, 4]. The microenvironment within the nerve regeneration chamber contains regeneration-conditioned fluid, a fibrous matrix, macrophages, fibroblasts, Schwann cells, and endothelial cells. Nerve regeneration-conditioned fluid (NRCF) contains many proteins related to neural regeneration, and research of these active proteins in NRCF is important to understand the mechanism of neural regeneration [5-7]. Thus, selection and functional studies of these proteins are promising research directions [6, 8]. In a number of previous studies of the NRCF, rats were extensively used as donors in research with protein purification and structural analysis; rats, however, cannot provide sufficiently large NRCF sample sizes. It seems that using bigger experimental animals (rabbits) as donors in the respective studies is more practical, but there is a problem needing to be considered: What is the level of homology of proteins in the NRCF obtained from rats and rabbits?

Nerve growth factor (NGF) is one of the main active proteins in the NRCF. It is a potent growth and survival factor for sympathetic neurons [9, 10]. Previous studies showed that NGF is essential for enhanced post-infarct sympathetic sprouting and other events related to nerve regeneration [5, 11-13]. So, it is reasonable and important to detect the homology of NGF in the NRCF obtained from rabbits and rats.

In our study, we carried out a comparative analysis of the homology of NGF in the NRCF between New Zealand white rabbits and SD rats through detecting an antigen-antibody reaction using the ELISA technique. The results showed that homology of this factor in the NRCF of these two species is rather high.

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METHODS

Experimental Animals and Groups. Five New Zealand white rabbits (body mass 1.8 to 2.5 kg) were chosen for NRCF extraction. The animals were obtained from the Department of Laboratory Animal Science (Medical College of Shanghai Jiao Tong University, Shanghai, Chaina). Albino SD rats were also used.

Extraction of the Sciatic Nerve NRCF from Rabbits. NRCF samples of New Zealand white rabbits were extracted using a nerve regeneration chamber model. Rabbits were anesthetized by i.m. injection of xylazine-sailaqin (500 mg/kg) and ketamine (0.2-0.3 ml per rabbit). Skin preparation was performed at the outsides of two hindlimbs, and the skin outside of the right hindlimb was cut about 5 cm after disinfection three times by benzalkonium. We separated muscle fibers of the *m. gluteus* and exposed the sciatic nerve. Then, the nerve was transected at its middle level, and a 20-mm-long fragment of the nerve was removed. Finally, two ends of the sciatic nerve were sutured to a silicone tube (length 4 cm, diameter 3 cm) by an atraumatic needle with 8/0 nylon to form the nerve regeneration chamber model, and then muscles and skin were sutured gradually. After one week, the rabbits were dissected in the same way, and the liquid (NRCF) was collected into a silicone tube (using a micro-injector) and stored at –20°C.

Extraction of the Sciatic Nerve Tissue Fluid from Rabbits and Rats. After anesthesia, fragments of the sciatic nerves of rabbits and rats (length 2.5 cm and 2.0 cm, respectively) were cut off from the hindlimbs and stored in normal saline. The tissue fluid of the sciatic nerves was obtained (by triturating and homogenizing using a homogenizer after washing off with PBS, 0.05 M, pH 7.2) and stored at 4°C.

Comparison of NGF Homology between Rabbits and Rats. We studied the homology of NGF under the following three conditions by the method of ELISA according to the respective manual:

Supernatant samples of the sciatic nerve tissue fluid of rabbits and rats were obtained by centrifugation and diluted in carbonate buffer (pH 9.5) to 1:5, 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, and 1:640. The primary antibody of rabbit anti-mouse NGF (M-20, Santa Cruz Biotech, USA; 100 µl in each well) was diluted to 1:20 and 1:50 in 0.3% BSA-PBS buffer, and the secondary antibody of goat anti-rabbit IgG was diluted to 1:1000.

The ammonium sulfate technique was applied to separate the sciatic nerve tissue fluid of rabbits and rats using a 50% saturated ammonium sulfate overnight at 4°C, and then the supernatant and precipitate were extracted by centrifugation. These samples were diluted to 1:2, 1:4, 1:8, and 1:16 in carbonate buffer (pH 9.5) for ELISA detection, and primary antibody (rabbit anti-mouse NGF, E-Ab) was diluted to 1:1000.

The ELISA detection was performed again for samples of the rabbit NRCF and for supernatants and precipitates of the rabbit and rat sciatic nerve tissue fluids. The coating buffer was diluted separately to 1:10, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$, $1:10^6$, $1:10^7$, and control. Primary antibody (rabbit to mouse NGF) was diluted to 1:500, and secondary antibody (HRP-labeled goat to rabbit IgG) was diluted to 1:1000.

RESULTS

A total of 500 µl NRCF from five rabbits (50-150 µl per rabbit) was extracted, and ELISA experiments were performed with different samples from New Zealand white rabbits and SD rats to detect their antigen-antibody reactions to NGF antibody under three different conditions. Results can be summarized in the following two paragraphs:

(i) Antigen-antibody reactions could be observed in all samples (nerve tissue fluid, supernatants, and precipitates treated by saturated ammonium sulfate) of rabbits and rats, which indicated that there is rather high homology between nerve tissue fluids of these animals (Tables 1 and 2).

(ii) In accordance with the optic density, the rank of the immune response intensity of antigen-antibody reactions was the following: rabbit NRCF > rabbit nerve tissue precipitation fluid > rabbit nerve tissue fluid supernatant > rat nerve tissue fluid supernatant. This indicated that proteins in the NRCF of New Zealand white rabbits demonstrate the highest activity in the reaction to NGF antibody (Table 3).

DISCUSSION

We detected the antigen-antibody reaction of the NRCF and nerve tissue fluids from rabbits and rats with rabbit anti-mouse NGF antibody by ELISA under different conditions in order to obtain more reliable results. These results showed that all samples of the supernatants and precipitates that were extracted by centrifugation or 50% saturated ammonium sulfate precipitation technique from the rabbit and rat nerve

TABLE 1. ELISA indices for the sciatic nerve tissue fluid of New Zealand white rabbits and SD rats

Т а б л и ц я 1. Показники, отримані з використанням методики ELISA, для рідини з тканини сідничного нерва новозеландських білих кролів та щурів лінії SD

Coating buffer dilution	1	2	3	4
1:5	1.952	1.501	1.515	0.866
1:10	1.859	1.050	0.852	0.882
1:20	1.890	1.416	1.465	0.895
1:40	1.938	2.047	1.724	1.072
1:80	1.851	1.718	1.958	1.490
1:160	1.569	2.123	1.966	2.417
1:320	1.386	2.024	1.577	1.974
1:640	1.351	1.691	1.205	1.459

Footnotes: Columns 1 and 2 are samples of the nerve tissue fluid of rabbits, while columns 3 and 4 are those of the fluid of rats. Primary antibodies for samples 1 and 3 were diluted to 1:20, while those for samples 2 and 4 were dilute to 1:50; secondary antibody was diluted to 1:1000.

TABLE 2. ELISA indices for the sciatic nerve tissue fluid of New Zealand white rabbits and SD rats treated with saturated ammonium sulfate

Т а б л и ц я 2. Показники, отримані з використанням методики ELISA, для рідини з тканини сідничного нерва кролів та щурів, обробленої насиченим розчином амонію сульфату

Coating buffer dilution	1	2	3	4
1:2	2.830	0.350	3.304	0.727
1:4	2.918	0.497	3.394	0.712
1:8	2.932	0.530	3.808	0.746
1:16	2.703	0.325	3.900	0.838

Footnotes: Columns 1 and 3 are for samples of rabbits; column 1 is for the supernatant, and column 3 is for the precipitate; columns 2 and 4 are for samples of rats; column 2 is for the supernatant, and column 4 is for the precipitate.

TABLE 3. ELISA indices for the NRCF and sciatic nerve tissue fluid of New Zealand white rabbits and SD rats

Т а б л и ц я 3. Показники, отримані з використанням методики ELISA, для рідини, кондиційованої процесом регенерації нерва, та рідини з тканини сідничного нерва кролів та щурів

Coating buffer dilution	1	2	3	4
1:10	2.318	2.165	3.330	0.363
$1:10^2$	2.272	2.685	0.735	0.261
$1:10^3$	3.328	2.115	0.068	0.044
$1:10^4$	3.202	0.208	0.178	0.039
$1:10^5$	2.023	0.067	0.062	0.151
$1:10^6$	0.323	0.107	0.041	0.145
$1:10^7$	0.073	0.060	0.045	0.087
Control	0.018	0.126	0.009	0.134

Footnotes: Column 1 is for the NRCF of rabbits, column 2 is for the supernatant of the nerve tissue fluid of rabbits, column 3 is for the precipitate of the rabbit nerve tissue fluid, and column 4 is for the precipitate of the rat nerve tissue fluid. Primary antibody was diluted to 1:500, and secondary antibody was dilutев ещ 1:1000.

tissue stock solution, the sciatic nerve tissue fluid of rabbits and rats, and the NRCF of rabbits can react with rabbit anti-mouse NGF, indicating that the NRCF of rabbits demonstrates high homology with the rat nerve fluid. The rank of the immune response intensity indicated that the rabbit NRCF showed the strongest protein activity in reacting with NGF antibody, which might be related to the higher concentration of the protein in rabbit samples.

The nerve regeneration chamber is implanted between the transected ends of the nerve, and this is the main convenient model to study the nerve regeneration microenvironment [5, 8, 14]. Researchers found that the chemotaxis and specificity of nerve regeneration could be fully tapped when the renewable regeneration chamber helped to repair the peripheral nerve, and there was no (or the smallest) scar after the process of nerve regeneration. This indicates that the regenerative microenvironment (and NRCF as its main ingredient) formed in the regeneration chamber played an important role in self-regulation and self-improvement of the process of nerve regeneration [15-17]. Thus, research on the NRCF provides us with an important way to study the nerve regeneration mechanisms [18, 19].

Our research has focused mainly on the extraction, purification, and testing of active proteins in the NRCF in order to understand the structures, sources, and biological activity of these active ingredients. These researches are mostly based on chromatography and immunohistochemistry, thus requiring a sufficiently large NRCF sample size [20, 21]. Naturally, the size of NRCF sample extracted from the traditional rat model is too small to be suitable for the requirements of experimental research. Recently, the rabbit model has been used to acquire larger NRCF sample sizes [22, 23]. So, our data on the immune homology of the NRCF between rabbits and rats are, probably, rather significant for comparing and studying the results obtained using different models. Our results showed that NGF in the NRCF obtained from rabbits demonstrated high homology with that in the rat samples. Probably, it can be stated that the molecular protein structures of NGFs of mammals belonging to different taxonomic groups (rabbits as *Lagomorpha* and rats as rodents) are rather similar. These data suggest that the rabbit model can be successfully used for further experimental studies of nerve regeneration mechanisms.

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The authors of this study, Y. Ye, Q. F. Li, and L. Li, confirm that the research and publication of the results were not associated with any conflicts regarding commercial or financial relations, relations with organizations and/or individuals who may have been related to the study, and interrelations between co-authors of the article.

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ГОМОЛОГІЯ ФАКТОРА РОСТУ НЕРВІВ (NGF) У РІДИНІ, КОНДИЦІЙОВАНІЙ ПРОЦЕСОМ РЕГЕНЕРАЦІЇ НЕРВА, У КРОЛІВ ТА ЩУРІВ

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Р е з ю м е

Ми аналізували молекулярну гомологію росту нервів (NGF) у кондиційованій процесом нервової регенерації рідині (NRCF), котру отримували від новозеландських білих кролів та щурів лінії SD. Нейрорегенеративна камера з'єднувала дистальний та проксимальний кінці перерізаного сідничного нерва у кролів, і NRCF відбирали з такої камери. За допомогою гомогенізації та центрифугування з тканини сідничних нервів кролів та щурів готували супернатанти та преципітати. Реакцію антиген–антитіло виявляли за допомогою методики ELISA, використовуючи первинні антитіла щодо мишачого NGF та вторинні козячі антитіла щодо кролячого імуноглобуліну. Реакції антиген–антитіло виявлялися в усіх вищезгаданих зразках, отриманих від кролів та щурів. Послідовність інтенсивностей відповідей антиген-антитіло у різних зразках була такою: кроляча NRCF > преципітат із нервової тканини кроля > супернатант із нервової тканини кроля > супернатант із нервової тканини щура. Отже, молекулярна структура зразків NGF, що знаходяться в NRCF, отриманій від кролів та щурів (тобто тварин, котрі належать до різних таксономічних груп ссавців), демонструє високий рівень гомології.

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