Vol. 90, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 12, 1979 Pages 130-134

LEVEL OF NERVE GROWTH FACTOR-LIKE IMMUNOREACTIVITY IN THE LOWER LIMB MUSCLES OF MUSCULAR DYSTROPHIC MICE

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Received July 20,1979

SUMMARY The level of nerve growth factor-like immunoreactivity in the lower limb muscles, the site where the dystrophic mice are effected, of both normal and dystrophic mice was studied by solid-phase radioimmunoassay. Nerve growth factor-like immunoreactivity levels of the homozygous dystrophic mice were about two times lower than those of the heterozygous dystrophic mice at 10-11 weeks and 7-8 weeks of age. The levels in 4-5 week old homozygous mice were too low for detection and remarkable differences between the homozygous and the heterozygous mice were observed at this age. These differences in the level of nerve growth factor-like immunoreactivity suggest that the factor may have some relation to the disease.

INTRODUCTION

Muscular dystrophy is a collective term applied to a large number of inherited diseases of the skeletal muscle. This disease occurs not only in man, but also in many other animals. The disease of mice is characterized by progressive muscular weakness and gross atrophy of muscles (1). The symptoms are recognized when the mice are 2-3 weeks old. Inheritance has been shown to follow the pattern of an autosomal recessive gene.

Until recently, muscular dystrophy of mice was considered to result from a genetic defect within the muscle fibers themselves. Accumulated evidence (2-4) suggests that dystrophic individuals have an abnormality within the nervous system which may itself be largely responsible for the muscular disorder probably due to an abnormal trophic influence on the affected muscle.

Nerve growth factor has been detected in many vertebrates and plays an important role in the development and maintenance of the sympathetic neurons and in the development of sensory ganglionic neurons as well (5). In the course of our study on the relationship between nerve growth factor and some neurological disorders, we found that the level of nerve growth factor

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0006-291X/79/170130-05$01.00/0
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activity in submaxillary glands of homozygous dystrophic mice was less than that of heterozygous mice by more than two orders (6).

This paper deals with the level of nerve growth factor-like immunoreactivity in the lower limb muscles, the site where the dystrophic mice are effected, by the use of the solid-phase radioimmunoassay.

MATERIALS AND METHODS

Dystrophic mice: The mice used were genetically dystrophic mice designated C57BL/6J strain of 4-5 weeks, 7-8 weeks and 10-11 weeks old obtained from Central Institute for Experimental Animals, Kawasaki, Japan. The lower limb muscles of the dystrophic mice were homogenized in cold phosphate buffered saline (20% w/v). Composition of the phosphate buffered saline is as follows: NaCl 8.0 g, KCl 0.2 g, Na_2HPO_4 1.15 g, KH_2PO_4 0.2 g, $CaCl_2$ 0.1 g and $MgCl_2 \cdot 6H_2O$ 0.1 g in 1 liter of water. The homogenate was centrifuged at 3,500 rpm for 20 min and the supernatant was measured for nerve growth factor-like immunoreactivity by radioimmunoassay system.

Nerve growth factor: Mouse β nerve growth factor was purified by CM-Sephadex C-50 chromatography from mouse 7S nerve growth factor obtained according to the method by Varon et al. (7). The purified β nerve growth factor was labeled with [^{125}I] by the method of Fabricant et al. (8). The specific radioactivity of the preparation was 50-100 μCi/μg.

β Nerve growth factor specific antibodies: Rabbit anti β nerve growth factor serum was prepared by the method of Vaitukaitis et al. (9). Pure β nerve growth factor antibodies were isolated from antiserum by affinity chromatography according to the method of Stoeckel et al. (10).

Radioimmunoassay: Procedures were based on the technique introduced by Catt and Tregear (11). Polystyrene tubes were coated with mouse β nerve growth factor antibodies as follows; 200 μl of mouse β nerve growth factor specific antibodies (5 μg/ml) in 0.05 M Tris-Cl buffer, pH 8.5, was poured into the tubes to coat the surface with the specific antibodies and allowed to stand overnight. The solution containing excess antibody was removed by aspiration. The tubes coated with mouse β nerve growth factor specific antibodies were washed twice with 0.05 M Tris-Cl buffer, pH 8.0, containing 0.075 M NaCl, 0.1% bovine serum albumin and 0.02% NaN_3. The sample (200 μl) was poured into the tubes to be assayed and allowed to stand for 24 h at 4°C. After washing twice with the same buffer as described above, 200 μl of [^{125}I]-labeled β nerve growth factor (20,000-30,000 cpm) was added to the tubes. After incubation overnight at 4°C, the tubes were washed twice with the same buffer and counted by a Dainabot Auto-Logic Gamma Counter. For each assay, a standard curve was prepared using known amounts of mouse β nerve growth factor. From the displacement of the radioactivity, it was possible to make accurate measurements of the amount of β nerve growth factor antigen present in the sample.

RESULTS

The body weights of homozygous dystrophic mice (dy/dy) were about half of those of heterozygous dystrophic mice (dy/+) or normal mice (+/+). The gross atrophy was observed in the lower limb muscles of homozygous dystrophic mice.

The radioimmunoassay was sensitive to 2-10 ng β nerve growth factor/ml, and we found a good correlation in the results obtained with the different concentrations at which supernatants of muscles homogenates were tested,

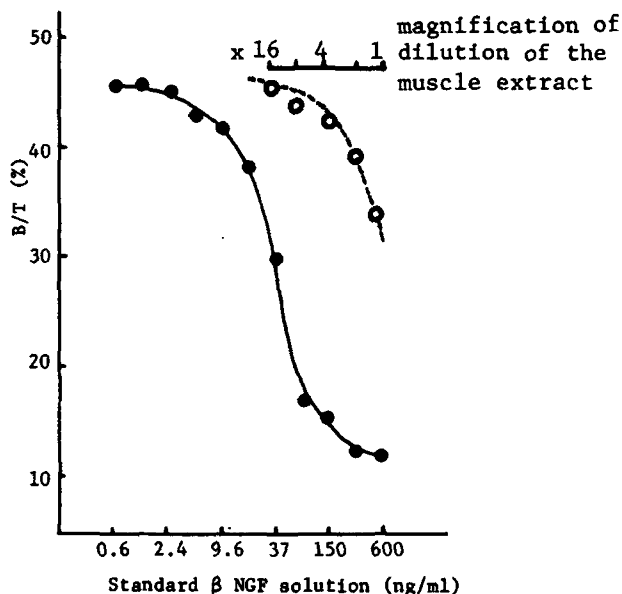

Fig. 1. Calibration Curve Using a Varying Concentration of Unlabeled Mouse β Nerve Growth Factor (•) and of the Muscle Extract of Heterozygous Dystrophic Mouse (o). Concentrations of mouse β nerve growth factor and dilution of lower limb muscle extract are plotted against bound [^{125}I]-β nerve growth factor/total [^{125}I]-β nerve growth factor (%). Values are means of duplicate determinations.

proving the immunological cross reactivity between β nerve growth factor and nerve growth factor-like materials in the muscles (Fig. 1). Estimations were carried out in three groups of mice with different ages; 4-5 weeks, 7-8 weeks and 10-11 weeks old.

Fig. 2 shows the nerve growth factor-like immunoreactivity levels in the lower limb muscles of homozygous and heterozygous dystrophic and normal mice of C57BL/6J strain. A difference in nerve growth factor-like immunoreactivity levels between male and female mice was not observed. The nerve growth factor-like immunoreactivity levels of the muscle supernatant of heterozygous dystrophic mice was not significantly different at three different ages. The nerve growth factor-like immunoreactivity levels of the homozygous dystrophic mice were about two times lower than those of heterozygous dystrophic mice at 10-11 weeks and 7-8 weeks of age. Nerve growth factor-like immunoreactivity levels of 4-5 weeks homozygous mice was too low for detection.

DISCUSSION

The results demonstrate that the levels of nerve growth factor-like immunoreactivity in the homozygous mice lower limb muscles are significantly

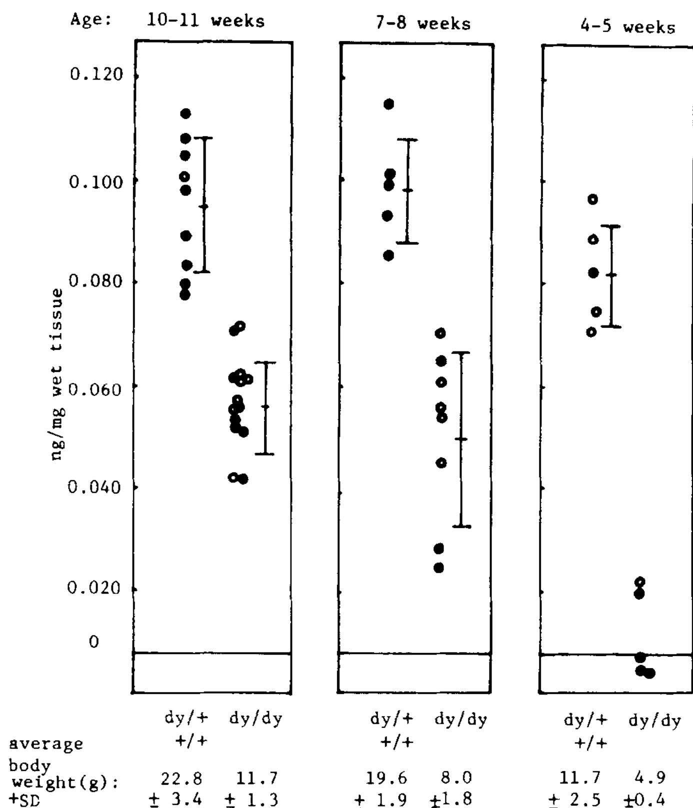

Fig. 2. Level of the Immunoreactivity with β Nerve Growth Factor in the Lower Limb Muscles of Dystrophic Mice (C57BL/6J). Values represent means ± S.E.M. (vertical bars) for indicated groups. The lower limb muscles from each mouse were homogenized in a cold phosphate buffered saline (20% w/v). The homogenate was centrifuged at 3,500 rpm for 20 min, and the supernatant fluid was measured in duplicate for nerve growth factor-like immunoreactivity by solid-phase radioimmunoassay using mouse β nerve growth factor as standard. •, male mouse; o, female mouse; dy/dy, homozygous dystrophic mouse; dy/+, heterozygous dystrophic mouse; +/+, normal mouse.

lower than those of heterozygous mice. The greatest concentration differences between homozygous and heterozygous mice were observed in the young mice (4-5 weeks). As the symptoms are recognized when the mice were 2-3 weeks old, the fact that the nerve growth factor-like immunoreactivity was almost completely lacking in the lower limbs muscles of the homozygous dystrophic mice in 4-5 weeks old appears to relate to the symptoms.

Many investigators have reported the existence of nerve growth factor activity in the skeletal muscles (12, 13). In 1977, Murphy et al. (14) reported that the rat skeletal muscle cell and a cloned myogenic cell line synthesize and secrete nerve growth factor-like material in culture. This

factor is biologically and immunologically indistinguishable from the active form of mouse submaxillary gland nerve growth factor. It has been suggested that neurotrophic factors are produced by peripheral cells, including muscle, to enhance the growth of developing neurons (15). Thus, it could be supposed that nerve growth factor is an informational factor that muscle cells produced to communicate with the nervous system, and that the disorder or abnormality of this system is related to dysfunction of muscle. However, because the role of nerve growth factor in the skeletal muscle is not clear, it is difficult at this time to conclude that the absence of nerve growth factor-like immunoreactivity found in the muscles of the very young homozygous dystrophic mice is the cause of this disease.

ACKNOWLEDGEMENTS

This work was aided in part by research grants from the Ministry of Education and from the National Center for Nervous, Mental and Muscular Disorders (NCNMMD) of the Ministry of Health and Welfare, Japan.

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