4-P26

SOME CHARACTERISTICS OF S-MYOTROPHIN, THE MOTOR-NERVE DEPENDENT MUSCLE CELL GROWTH FACTOR IN PORCINE SKELETAL MUSCLE

Toshiya Hayashi[1], Takehiro Ogawa[1], Masamichi Sato[1], Nobuyuki Tsuchida[1], Abbas Fotovati[1], Kazumasa Nodake[1], Shun-Ichiro Kawabata[2] and Tatsumi Ito[1]

1) Laboratory of Biological and Functional Chemistry, Division of Bioresource and Bioenvironmental Sciences, Graduate School of Kyushu University, Fukuoka 812-8581, Japan

2) Department of Biology, Faculty of Science, Kyushu University, 812-8581, Japan

Keywords: muscle cell growth factor, s-myotrophin, characterization, motor nerve.

Backgrounds and Objectives

The growth of skeletal muscle cells is promoted by several growth factors, such as growth hormone, insulin-like growth factor-1 (IGF-1) and steroids hormones, and so on. During the course of our preliminary study with regard to the effect of denervation of motor nerves on muscle protein behavior, we found that the distribution of a water-soluble factor in skeletal muscle having almost 12kDa molecular mass was decreased, and the specific activity in promoting muscle cell growth of the crude extraction from denerved the muscles was also reduced compared to that of control muscles. This evidence suggests that the decrease of the water-soluble substance is related to the atrophy of skeletal muscle by denervation. Therefore, we tentatively hypothesized that a water-soluble growth factor which mediates a signal from motor nerves to muscles exists in the sarcoplasmic fraction, and we could successfully purify a water soluble growth factor, named s-myotrophin, from porcine skeletal muscle (Hayashi et al., 1998). The novel factor has 12 kDa molecular mass almost the same molecular weight of myotrophin previously discovered in hypertrophied cardiac muscle. However, myotrophin mRNA hasn't been detected in rat skeletal muscle (Mukherjee et al., 1992). S-myotrophin has a strong growth promoting activity of cultured skeletal muscle cells. Unfortunately, the N-terminal end of s-myotrophin was blocked, and no fragmentations of it were observed after the digestion of s-myotrophin by lysyl-endopeptidase, trypsin, chymotrypsin and thermolysin. Crude preparation of this factor could be stained by periodic acid / Schiff (PAS) detection on SDS-polyacrylamide gel electrophoresis. Moreover, chemically deglycosylated s-myotrophin appeared as a single band having molecular weight of 6.8 kDa.

The objective of the present study was to characterize a partial sequence of the deglycosylated peptide and its growth promoting effect *in vivo*.

Materials and Methods

Modification of the purification method of s-myotrophin : Purification of s-myotrophin of skeletal muscle was made fundamentally according to the original method described in Hayashi et al. (1998) with several modifications. i. e., CM-cellulofine chromatography (batch adsorption method) which was effective to eliminate coexisting myoglobin in the crude extraction of this factor was made at the first step after preparation of crude water-soluble extract from porcine skeletal muscle. As a result of this modification, one of two gel filtration steps by Sephadex G-75 could be omitted from purification steps reported previously (Hayashi et al., 1998). Residual purification steps were the same as the original method.

Bioassay : The effect of this factor on protein synthesis of muscle cell culture was examined as follows. Dispersed muscle cells free from fibroblasts was prepared from thigh muscle by collagenase and dispase treatment and by the following filtration and differential cell adhesion. Growth promoting activity of crude and purified s-myotrophin on the primary muscle culture or rat skeletal muscle L6 cells purchased from ATCC was examined in DME medium containing 2% fetal bovine serum. Confluent monolayers of muscle cells in the medium in gelatin-coated plastic dishes were incubated at 37°C for 2-4 hr after the addition of ^{3}H-leucine (5 μCi /ml) and crude/purified s-myotrophin to the medium. The incorporation of ^{3}H-leucine into the muscle cells was counted with a scintillation counter. Protein composition of cultured skeletal muscle cells after the exposure to the factor was made as followed. After the number of cells reached to confluent in the 60 mm dishes in diameter, the cultures were incubated for two days in the presence or absence of the factor in the DME medium including 2% FBS. Then, the monolayers were washed with PBS (-) three times, and solubilized for electrophoresis in sample buffer (1% SDS, 10 mM Tris-HCl, pH 6.8, 1mM 2-mercaptoethanol, 1 mg/ml bromphenol blue, 20% glycerol). The resulting cell lysates were subjected to SDS-polyacrylamide gel electrophoresis as described below.

SDS-polyacrylamide gel electrophoresis : SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) or Tricine-SDS-PAGE (Schägger et al., 1987). Gels were stained with CBB or a silver-staining kit (Silver stain Kanto II).

Deglycosylation of s-myotrophin by chemical treatment and enzymatic treatment : The freeze-dried purified s-myotrophin (or incomplete deglycosylated factor) was treated with a glyco-free deglycosylation kit (K-500, Oxford GlycoSysem Inc.). After the treatment, the reaction mixture was subjected to gel filtration on a Sephadex G-75 column with 0.1 % TFA. The resulting void volume fraction was used as deglycosylated s-myotrophin. To examine the effect of enzymatic digestion on the deglycosylation of s-myotrophin, the factor was treated by N-glycanase (Oxford GlycoSysem Inc.) according to the method of Hirani et al. (1987).

Digestion of the substance with proteases and peptide sequencing : Deglycosylated *s*-myotrophin (2 μg) was digested with the mixture of trypsin and chymotrypsin (1 + 1μg) for 12 hr. Then, the reaction mixture were directly applied onto a reverse phase HPLC (μ Bondasphere) to separate the fragmented peptides, and resulting peptide fractions were subjected to peptide sequencer (ABI, 473).

Results and Discussion

In the present study, we prepared s-myotrophin by the original and the modified methods, and the yield of s-myotrophin was increased by the modification of purification procedure ; i. e., the yield of s-myotrophin was approximately 3μg from 500g porcine muscle in the the original method, whereas it was approximately 5μg from 500g porcine muscle in the modified method. Again, the purified s-myotrophin was highly resistant against the proteolysis, i.e., lysyl-end peptidase, trypsin, chymotrypsin and thermolysin couldn't hydrolyze s-myotrophin, although it has proteinaceous nature. Such characteristic of s-myotrophin molecule might be dependent on its oligosaccharide moiety in the molecule (Hayashi et al., 1998). To elucidate the carbohydrate content of s-myotrophin, s-myotrophin was treated by N-glycanase which can specifically cleave N-linked oligosaccharide in the presence of detergent, and then subjected to tricine-SDS-polyacrylamide gel electrophoresis. As a result, the bands having approximately 10kDa and 7kDa molecular mass were detected respectively. This result indicated that s-myotrophin contains the N-linked oligosaccharide moiety in the molecule. Furthermore, chemical deglycosylation which can release both N- and O-linked oligosaccharides moiety was conducted to remove the saccharide from the glycopeptide. Tricine-SDS-polyacrylamide gel electrophoresis clearly showed that the band having molecular weight of 12kDa completely disappeared, and new band having 6.8kDa appeared after the cleavage with the chemical deglycosylation treatment. This result suggests that this 6.8kDa molecule is core peptide of s-myotrophin. In order to determine the partial sequence of the peptide moiety of s-myotrophin, deglycosylated s-myotrophin was digested with the mixture of trypsin and chymotrypsin as described above. The resulting peptide fragments separated by a reverse phase HPLC were subjected to peptide sequencer. As a result, partial sequence composed of 9 amino acids was obtained from one fraction of enzymatic fragments. This sequence was not, at least, consistent with that of trypsin and chymotrypsin. This result suggests that the sequence could be the peptide moiety of s-myotrophin. Further analysis of the detailed structure of s-myotrophin is now under investigation.

In our preliminary study, on the other hand, regarding the physiological function of s-myotrophin *in vivo*, the morphological observations of hind leg muscles of male mice (C57BL / 6J) showed that the areas of cross section of myofiber in the muscle of mice subjected to subcutaneous s-myotrophin injection were appreciably increased compared to that of control mice (PBS injection), although the s-myotrophin used for this experiment was crude obtained immediately after ion-exchange HPLC on mono-Q column. Therefore, the present result suggests that more clear difference in weight gain and muscle growth will be able to be observed in such animal experiments by using the purified factor having higher specific activity.

References

1) Hayashi, T., Takeshita, K., Tsuchida, N., Kitano, K., Kawabata, Kawabata, S., Iwanaga, S. and Ito, T., Purification of a novel muscle cell growth factor s-myotrophin from porcine skeletal muscle., *Int. J. Biochem. Cell Biol.*, **30**, 897-908 (1998).

2) Hirani, S., Bernasconi, R. J. and Rasmussen, J. R. Use of N-glycanase to release asparagine-linked oligosaccharides for structural analysis., *Anal. Biochem.*, **162**, 485-492 (1987).

3) Laemmli, U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4., *Nature*, **227**, 680-685 (1970)

4) Mukherjee, D., Thekkumakara, T., Sen, G. C. and Sen, S., Ontogeny of myotrophin mRNA in spontaneously hypertensive rat (SHR) heart., Circulation 86 (Suppl. 1) (4) I-96 (1992).

5) Schägger, H. and Jagow, G. V., Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa., *Anal. Biochem.*, **166**, 368-379 (1987).

6) Sen, S., Kundu, G., Mekhail, N., Castel, J., Misono, K. and Healy, B. Myotrophin : purification of a novel peptide from spontaneously hypertensive rat heart that influences myocardial growth., *J. Biol. Chem.*, **265**, 16635-16643 (1990).