学 位 論 文 の 要 旨

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学　位　論　文　名	Blocking Insulin-Like Growth Factor 1 Receptor Signaling Pathway Inhibits Neuromuscular Junction Regeneration After Botulinum Toxin-A Treatment
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論 文 内 容 の 要 旨

INTRODUCTION

Botulinum toxin-A (BTX) administration into muscle is an established treatment for conditions with excessive muscle contraction. BTX has effects at the neuromuscular junction (NMJ) which is a specialized synapse between motor nerve endings and their muscle fibers. However, botulinum therapy has short-term effectiveness, and high-dose injection of BTX could induce neutralizing antibodies against BTX. Therefore, prolonging its effects could be beneficial in a clinical situation. Insulin-like growth factor-1 receptor (IGF1R) and its ligands, insulin-like growth factor (IGF) -I and II, regulate the physiological and pathological processes of the nervous system. It has been suggested that IGF1R is involved in the process after BTX administration, but the specific regeneration mechanism remains unclear. Therefore, this study aimed to determine how inhibition of the IGF1R signaling pathway affects BTX-induced muscle paralysis.

MATERIALS AND METHODS

Male B6.Cg-Tg(Thy1-YFP)16jrs/j mice (Thy1-YFP mice) (The Jackson Laboratory, Bar Harbor, ME, USA) and C57BL/6J mice (SLC Co., Shizuoka, Japan) aged 6–8 weeks were used in this study. Thy1-YFP mice were used for analysis of the NMJ and C57BL/6J mice were used for muscular and satellite cells (SCs) analysis. All experiments with animals in this study were approved by the Animal Care and Use Committee of Shimane University and Shimane University Safety Committee for Recombinant DNA Experiments (IZ31-57, 58, IZ2-124, IZ4-11and protocol No.595).

BTX and anti-IGF1R antibodies were administered to the gastrocnemius (GC) muscle of mice. The control-IgG (cont IgG) antibody combination group was used for comparison. Muscle

paralysis was evaluated using a footprint test (FPT) and the tibial functional index (TFI). After the FPT procedure, the GC muscle was collected for immunochemical staining to evaluate the NMJ, muscle fiber area, and SCs. We quantified the protein and gene expression levels involved in the process of NMJ regeneration using immunoblotting and quantitative reverse transcription-polymerase chain reaction (qPCR). Statistical analyses were performed with unpaired t-tests, one-way or two-way analysis of variance (ANOVA) method followed by post-hoc Tukey's multiple tests using PRISM 9 software (GraphPad Software, La Jolla, CA, USA).

RESULTS AND DISCUSSION

Western blot analyses were performed to examine the protein levels of IGF-I, IGF-II, IGF1R, and phosphorylated-IGF1R in the GC muscle 2 weeks following BTX treatment. All the protein levels compared to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) increased after BTX administration.

BTX treatment in the GC muscle induced nearly complete paralysis at 2 or 4 weeks, followed by a spontaneous recovery over 12 weeks. Paralysis lasted significantly longer with the addition of anti-IGF1R antibody, compared to BTX alone or BTX with cont IgG. With respect to the effects of sequential injections of anti-IGF1R or cont IgG, with or without a half dose of BTX administration, the TFI of the anti-IGF1R antibody alone group was not significantly different from that of the cont IgG alone group. In contrast, at 6 weeks after BTX injection, the TFI of the BTX+anti-IGF1R antibody group remained low, whereas that of the BTX cont IgG-treated group spontaneously recovered.

Immunohistochemical analysis of the GC muscle and NMJ revealed that IGF1R was highly expressed in the membrane of muscle fibers, $Pax7^{+}$-SCs, and NMJ. However, it was weakly expressed in the growing tip of the sprouting fiber.

The treatment of BTX caused morphological changes in the NMJ, leading to an increase in axonal sprouting fibers. Both presynaptic and postsynaptic components were significantly lower in the BTX-treated samples compared to naïve controls. The group treated with BTX+ anti-IGF1R antibody showed a lack of regeneration of both presynaptic and postsynaptic components compared with BTX+cont IgG group. However, the number of axonal inputs to the NMJ did not show significant differences between BTX+cont IgG and BTX+anti-IGF1R antibody groups. These results suggested that the NMJ, but not sprouting fibers, was regulated by the IGF1R signaling pathway.

The weight of the denervated GC muscle was significantly lower by approximately 40% than that of the contralateral control GC muscle at 2 weeks following BTX administration. At 6 weeks after BTX treatment, muscle weight in the cont IgG group was spontaneously recovered

to 64.2 ± 2.3% of the contralateral control muscle weight. In contrast, the anti-IGF1R antibody group did not show any recovery in muscle weight and maintained 44.7±1.5% of the contralateral muscle weight.

We subsequently performed a histological analysis of the cross-sectional area of the muscle fibers. The BTX-treated group had fewer muscle fibers with a larger cross-sectional area at 2 weeks. Interestingly, the distribution was spontaneously recovered after 6 weeks in the BTX+cont IgG group, while the BTX+anti-IGF1R antibody group showed a higher frequency of small muscle fibers with cross-sectional areas of 51–150 and 151–300 μm^2 and a lower frequency of muscle fibers with cross-sectional areas of >451 μm^2. These results suggested that BTX treatment induced neurogenic atrophy and that sequential treatment with anti-IGF1R antibody maintained the neurogenic atrophy induced by BTX.

The ratio of SCs in the GC muscle was not significantly different between the two groups at 2 weeks after treatment. However, the ratio of SCs was significantly higher in the BTX+anti-IGF1R antibody group than in the BTX+cont IgG group 6 weeks after treatment.

Denervation by BTX strongly activated the expression of total mTOR and Akt. In addition, phosphorylated mTOR (Ser2448) and S6 kinase were more significantly activated than total mTOR and S6 kinase. However, the light chain 3BII/light chain 3BI (LC3BII/ LC3BI) ratio was not changed. The relative expression of phosphorylated mTOR and S6 kinase was significantly affected by anti-IGF1R antibody administration. Also, the genes related to neural recovery, except Trim63/ MuRF1 and Chrne, were upregulated by BTX treatment, although none of them were affected by anti-IGF1R antibody treatment.

These results showed that anti-IGF1R antibody administration inhibited the recovery from BTX-induced neurogenic paralysis, and the synaptic components at the NMJ, mainly post-synaptic components, were significantly affected by the antibody. In addition, the wet weight or frequency distribution of the cross-sectional area of the muscle fibers was regulated by IGF1R, and sequential antibody administration following BTX treatment increased the number of $Pax7^+$-SCs in the GC muscle, independent of NMJ recovery. Moreover, BTX treatment upregulated the mTOR/S6 kinase signaling pathway, HDAC4, Myog, Fbxo32/MAFbx/Atrogin-1 pathway, and transcription of synaptic components, but not autophagy. Finally, IGF1R inhibition affected only mTOR/S6 kinase translational signaling in the GC muscle.

CONCLUSION

Anti-IGF1R antibody administration prolongs the effects of BTX by regulating the mTOR/S6 kinase signaling pathway and NMJ regeneration. The IGF1R signaling inhibition could be highly beneficial in clinical practice.

論文審査及び最終試験又は学力の確認の結果の要旨

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論文審査の結果の要旨

ボツリヌス療法はボツリヌス毒素(以下BTX)を用いた治療方法で、脳卒中後に生じる痙縮をはじめ、過度に緊張した筋肉を弛緩させて症状を改善させる。BTXは可逆的な作用をもち、3～4ヶ月で効果が減弱する。効果を保つためには治療の反復が必要であり、侵襲・費用・通院などの問題から十分な治療を受けられない場合がある。今回、ボツリヌス療法の効果延長を目指し、神経・筋再生に関与する成長因子Insulin-like growth factorの受容体IGF1Rに対する機能阻害抗体の併用投与を行った。正常マウスを用いた行動学的実験、組織学的解析、タンパク質・遺伝子の発現変化を解析した。結果、抗IGF1R抗体併用群ではBTX単独投与群と比較して筋弛緩作用の効果が有意に延長した。免疫組織学的解析から、BTX投与による神経筋接合部のシナプス異常が、抗IGF1R抗体の併用により持続した。神経原性筋萎縮の機序に関わるタンパク質分解・合成経路、シナプス関連遺伝子の発現は、BTX投与後2週で活性化が確認され、抗IGF1R抗体の併用により、mTOR/S6キナーゼ経路の活性化のみが有意に抑制された。従って、ボツリヌス療法に抗IGF1R抗体を併用投与することで、タンパク質の合成経路の阻害により神経筋接合部再生が抑制され、筋弛緩効果が延長することが示唆された。本研究は臨床問題の解決を目指した重要な基礎研究であり、博士（医学）の学位授与に値すると判断した。

最終試験又は学力の確認の結果の要旨

ボツリヌス毒素（BTX)は痙性麻痺に対する治療法としてもちいられるが、痛みや薬価から、頻回投与が難しい場合がある。候補者は、低容量のBTXと抗IGF1R抗体の併用効果について明らかにした。行動試験、組織学的解析、分子メカニズム解析等、多様な解析手法を用いたデータから結論を導いており、博士の学位授与に値すると判断した。(主査：藤田　幸)

申請者は、神経筋接合部におけるBTX効果延長をもたらす機序についてタンパクおよび遺伝子発現レベルでの解析を行い、抗IGF1R抗体の有効性を確認した。本研究結果は臨床的意義を含む貴重な研究であり、申請者の豊富な関連知識も確認できたことより学位に値すると判断した。(副査：長井　篤)

申請者は、ボツリヌス毒素療法の効果を延長するための治療戦略として抗IGF1R抗体の投与が有効であることを示し、さらにその作用機序や分子基盤も明らかにした。本研究成果は、臨床応用につながる重要な知見であり、また、申請者は関連知識も豊富であることから十分に学位授与に値すると判断した。（副査：桑子　賢一郎）

（備考）要旨は、それぞれ４００字程度とする。