THE TRANSCRIPTOME OF CELL CULTURES DERIVED FROM MUSCLE BIOPSIES OF CONTROLS AND SPASTIC PATIENTS IS AFFECTED BY BOTULINUM TOXIN TYPE A
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ABSTRACT
Botulinum toxin (BTK) is widely used for treating skeletal muscle spasticity. Experimental reports on BTK treatment have been mainly focused on the neuromuscular junction, while relatively little is known about toxin effects on the muscle cell itself. We investigated possible impact of BTK type A on skeletal muscle cell transcriptome by microarray analysis in muscle-derived cell cultures (fibroblasts, myoblasts and myotubes) from controls and spastic patients, and validated results at transcript and protein level by Real-Time PCR and by immunocytochemistry and western blot, respectively.

BTK-A treatment of control cells induced major changes in the myogenic component of the transcriptome, whereas the same treatment had a negligible effect in the fibrogenic component. BTK-A treatment of cells from spastic patients induced an increased number of genes differentially expressed both in the fibrogenic and myogenic components. Specifically, BTK-A had a major effect on cell cycle-related genes in myoblasts, on muscle contraction-related genes in myotubes, and on extracellular-matrix-related genes in fibroblasts from spastic patients.

Our findings showing that in vitro BTK-A treatment differentially affects transcript expression in muscle cells from spastic patients compared to those from controls suggest a direct effect of BTK-A on muscle-specific functional pathways.

RESULTS
BTK-A treatment induced no significant variations in cell cycle distribution in spastic fibroblasts (Fig. 1D), while it induced significant changes, in all three phases of the cell cycle, in spastic myoblasts. MTT assay (A,B). Dose-response in control fibroblasts (A) and myoblasts (B) treated by different concentrations and at an exposure time of 48 h by the MTT assay.

FACS analysis (C-H). Cell cycle distribution in control fibroblasts (C), spas2c fibroblasts (D), control myoblasts (E), and spas2c myoblasts (F) before and after 48 h of treatment with BTK-A. Representative images from FACS of a control myoblast cell line (G) and a spas2c myoblast cell line (H) after BTK-A treatment are shown.

In total, 1362 unique probe sets were identified in control cells, reduced to 376 DEGs after amore stringent selection by using a cut-off of two-fold change. In spastic cells 44,533 coding gene transcripts were identified after a first filtering, and to 1246 DEGs, after using a cut-off of FC ≥ 2.

METHODS
Primary cell cultures were obtained from muscle biopsies of 5 controls (aged 14-35 years) and 5 patients (38-56 years) with past stroke severity of variable duration (6 months - 3 years).

To separate myoblast and fibroblast-enriched populations, primary mixed cell cultures were sorted by immunomagnetic selection using the CDS5 surface marker.

To obtain myotubes, myoblasts at 70% confluence were allowed to differentiate to myotubes over 10 days in differentiating medium.

The cyotonic action of BTK-A on fibroblast and myoblast cell populations was assessed by the MTT assay. BTK-A was tested at different concentrations as dose-response curve.

Distribution of fibroblasts and myoblasts in the cell cycle phases was analysed by flow cytometry before and after BTK-A treatment (15 SU for 48 h).

Total RNA was extracted from fibroblasts, myoblasts and differentiated myotubes using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. RNA was quantified, quality-checked and reverse-transcribed with a cDNA synthesis kit in the presence of SuperScript II RT (Invitrogen Life Technologies, Inc.) and an oligo (dT) primer. Ten μg of purified cDNA were used for the in vitro transcription amplification reaction, in the presence of biotinylated dUTP (Enzymatic Rhodamine Labelling Kit, US Biochemicals) and 1 μg of T7 oligo(dT)16 primer. The cRNA product was fragmented by incubation at 94°C for 35 min in fragmentation buffer and hybridized competitively against the Affymetrix Human Transcriptome Array 2.0 microarray set. Arrays were scanned using a GeneArray 2500 scanner (Affymetrix) and analyzed using MicroArray Suite 5.0 (Affymetrix).

Validation was performed by quantitative RT-PCR of selected genes. These were chosen based on their p-value and fold change.

Alquots of protein extract from myoblast, myotubes or fibroblasts cultures from control and spas2c patients, before and after BTK-A treatment, were analysed by western blot or immunocytochemistry of selected proteins.

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In cells from control muscles, KEGG and GO analysis of genes altered after BTK-A treatment showed no changes in fibroblasts; down-regulation of genes mainly involved in the cell cycle, in myoblasts; up-regulation of genes involved in inflammation and down-regulation of genes involved in muscle contraction, in myotubes.

In cells from spas2c muscles, KEGG and GO analysis of genes altered after BTK-A treatment showed variation of genes related to complement activation, cell cycle and collagen metabolism, in fibroblasts; down-regulation of genes involved in the cell cycle and up-regulation of genes involved in sarcoem organisation, in myoblasts; and up-regulation of genes involved in inflammation and down-regulation of genes involved in muscle contraction, in myotubes.

CONCLUSIONS
BTK-A induced modulation of the cell cycle causes muscle satellite cells to enter a differentiation phase, thus providing a regenerative pool upon toxin treatment; modulation of inflammation-related genes would translate into increased stress response.

The down-regulation of collagen metabolism-related genes, in the fibrogenic component, is likely to mean a major contribution to reduction of the passive component of muscle stiffness, thus potentiating the toxin effect on stiffness mediated by the neuromuscular transmission.

The down-regulation of contraction and sarcomere formation-related genes in the myotubes, may suggest a direct effect of the toxin on muscle fibre remodelling.

All such different effects in the fibrogenic and myogenic components are probably related to different interaction of the toxin with cellular surface receptors and to different intracellular targets and signalling pathways.

The main clue of our results is that botulinum toxin has a direct action on skeletal muscle, independent from neural transmission, and this could have implications for the rehabilitative procedures aimed at enhancing BTK-A effect, such as stretching or use of casting or guardians that induce mechanic stress by elongating muscle fibres immediately after inoculation of the toxin.