Quantifying dystrophin in cell culture: a method to accelerate preclinical assessment of DMD treatments.

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INTRODUCTION

Restoring dystrophin expression in Duchenne muscular Dystrophy (DMD) is the goal of many potential therapies in development. Due to the mutation-specific nature of many of these therapies, it is necessary to test them in patients’ cell cultures. However, preclinical development of these therapies does not rely on accurate dystrophin quantification: western blotting, the current gold standard for protein quantification in cell culture models, requires a large amount of cultured cells and patients’ cultures are scarce. This means that less compounds are routinely screened as thoroughly as would be advisable.

We are developing a method to quantify dystrophin in cell culture to tackle this problem.

METHODS

Samples
To develop this method, we used immortalised myoblast cultures from one control and 3 DMD patients, with 2 different DMD deletions. These cultures were requested from the MRC Centre for Neuromuscular Diseases (MRC CNMD) Biobank, London.

Cytoblots or in-cell westerns (ICW)

A cytoblot, also known as “in-cell western (ICW)” is a quantitative immunofluorescence assay performed in microplates that permits the quantification of proteins directly in cell culture combining the specificity of western blotting with the reproducibility and throughput of ELISA.

METHOD OPTIMISATION

Cell density
Prior to the cytoblot experiments, the cultures used in this project were carefully characterised. This allowed us to modify the number of cells that were seeded per well, according to their particular doubling times.

Antibodies
A panel of dystrophin and other dystrophin associated complex (DAC) protein antibodies were tested by standard fluorescent microscopy methods, before being used as part of our cytoblot protocol.

RESULTS

Preliminary experiments in control cultures

Myosin Heavy Chain (MF20 antibody), a muscle specific protein and marker of differentiation, was used in preliminary experiments in control myoblast cultures to normalise the results. A good correlation was found between differentiation and number of cells, as well as dystrophin expression (normalised with MF20) versus the number of cells.

Variations in differentiation amongst cultures.

When the differentiation of several cultures from DMD patients was studied with MF20, it was decided that further normalisation should be performed with a cell number marker, using the manufacturer’s suggested marker, CellTag 700 stain (LI-COR, US).

Dystrophin expression in controls/DMD patients.

Several dystrophin and dystrophin associated proteins have been studied in control and patient’s samples with this method. In all cases, control and patients were clearly identified according to their protein expression.

Detection of dystrophin expression after AON treatment.

Our preliminary experiments, using our ICW method to study patient cultures after treatment with exon skipping antisense oligonucleotides, only showed a restoration of dystrophin expression at the highest concentration tested. We are currently developing this further.

CONCLUSIONS

• In-cell western blots are a useful tool that should be explored to further analyse culture samples.
• ICW shows good discrimination of control and DMD samples.
• Differentiation of cultures correlates with dystrophin expression, and could be a limiting factor to study dystrophin restoration in some patients’ cultures.
• We are developing this method further and working on its validation against stabilised methods.