RNA/MBNL1-containing foci in myoblast nuclei from patients affected by DM2: immunocytochemical characterization

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INTRODUCTION

Myotonic dystrophy type 2 (DM2) is an autosomal dominantly inherited disease due to the CCTG repeat expansion in intron 1 of the zinc finger protein 9 (ZNF9) gene, and exhibiting multi-systemic clinical features. The expanded-CCTGCCUG-containing transcripts are retained in the cell nucleus and accumulate as discrete ribonucleoprotein (RNP)-containing foci, creating a toxic RNA effect that acts as dominant gain of novel function mechanism. RNA binding proteins such as Muscleblind-like (MBNL) proteins which are sequestered into the foci are splicing regulators that cannot accomplish their physiological function: this results in the alteration of additional downstream gene transcripts that are normally processed by these RNA binding proteins. The splicing factor MBNL1 proved to be a good marker for nuclear RNP foci typical of DM2. In this investigation, we aimed at testing whether other pre-mRNA processing factors might be sequestered in the RNP foci: to do this, we first attempted to identify these foci at electron microscopy (EM) in the muscle of DM2 patients, then we analysed in situ their molecular composition in cultured myoblasts using immunocytochemistry at both confocal and EM.

MATERIALS AND METHODS

Muscle fragments from DM2 biopsies and from normal controls, as well as cultured myoblasts from the same individuals were used for the morphological and immuno-cytochemical analyses at fluorescence and EM. For fluorescence microscopy, myoblasts were planted on glass coverslips, fixed with 2% formaldehyde in PBS, and post fixed with cold 70% ethanol. For EM morphology, the tissues and cultured cells were fixed with 2% glutaraldehyde in PBS, post-fixed in 1% OsO_4 in PBS, then dehydrated and embedded in Epon; the thin sections were stained with uranyl acetate and lead citrate. For EM immunocytochemistry, fixation was performed with a mixture of 4% paraformaldehyde and 0.2% glutaraldehyde, and the specimens were dehydrated and embedded in LR White resin. In situ hybridization and immunolabeling for MBNL1 were performed at fluorescence microscopy. In addition, to get information on the intranuclear location of the RNA-containing foci and their composition, a panel of antibodies directed against transcription, splicing and cleavage factors were used; secondary antibodies labeled with different fluorochromes were used to reveal the primary antibodies. At EM, anti- MBNL1 antibodies reveled by gold-particle-conjugated probes and the EDTA regressive staining for RNPs were used to recognize specifically the nuclear foci.

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RESULTS & CONCLUSIONS

Dual-labeling experiments of in situ hybridization and immunolabeling on muscle sections or myoblasts in culture showed that nuclear foci of mutant mRNA containing CCUG repeats sequester the splicing factor MBNL1, thus demonstrating that the molecular features of the DM2 dystrophy are reproduced in the in vitro system. Consistent with previously reported findings [1], no alterations in the differentiation pattern were observed in these cultures: in fact, DM myoblasts differentiate into myotubes expressing markers of muscle differentiation, as the controls.

The RNA-containing nuclear bodies observed at fluorescence microscopy, can be also recognized at EM as roundish electron-dense domains which were stained for RNPs and proved to be immunopositive for MBNL1. These structures morphologically resemble the so-called *amorphous bodies*, i.e. the RNP-containing nuclear aggregates which accumulate in several Vertebrate tissues during hibernation and were found to be storage sites for many protein factors involved in pre-mRNA processing [2-4]. The diffuse immunolabeling for MBNL1 observed in myocyte and myoblast nuclei at fluorescence microscopy was found to correspond to the weak EM labeling of perichromatin fibrils, where transcription and early splicing of pre-mRNA takes place [5].

Fluorescence dual-immunolabeling experiments demonstrated that RNA polymerase II, the non-snRNP splicing factor SC35, the RNP-associated protein PANA, the cleavage factor CStF and the PML protein do not co-locate with MBNL1 in the foci, whereas a partial co-location was observed for the hnRNP core protein and the snRNPs' Sm antigen, which are both involved in the early events of pre-mRNA splicing.

These preliminary observation indicate that the DM2-specific nuclear foci differ, in their molecular composition, from both the *amorphous bodies* and the heterogeneous ectopic RNP-derived structures (HERDS, [6]) where a large amounts of many different nuclear RNA-associated protein factors accumulate. However, the partial co-location with MBNL1 of the snRNA-associated Sm antigen suggests that specific pre-mRNA processing factors might be also sequestered in the RNP foci, possibly inducing a general alteration in the expression of mRNAs, which could lead to the multiple pathological dysfunctions observed in dystrophic patients.

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