Sparing of Extraocular Muscles in Muscular Dystrophies: A Role for Retinoic Acid Signaling

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Introduction
Duchenne muscular dystrophy (DMD) is a muscular degenerative disease that is characterized by an age-related loss of muscle mass and function (Blake et al. 2012). It is caused by a mutation in the dystrophin gene. This mutation results in the absence of the dystrophin protein - a cytoskeletal protein that plays an important role in muscle function (Blake et al. 2012). DMD patients experience decreased lower limb muscle strength and joint contractures and most often die at an early age of respiratory complications due to intercostal muscle weakness (Blake et al. 2012).

Interestingly, the extraocular muscles (EOM) that function in eye movement, primary gaze position and motor fusion are functionally and morphologically spared in DMD patients (McLoon EOM chapter, Kaminski et al. 1992, Karpati et al. 1988 and Kallstad et al. 2011). No studies have been able to find why EOM are spared while limb skeletal muscles degenerate in DMD patients. We hypothesize that two differences between EOM and limb muscle contribute to the sparing of EOM in DMD. First, unlike limb muscles, EOM undergo continuous myofiber remodelling (McLoon and Wirschafter 2002). Second, EOM and limb muscles have different requirements for their development. The transcription factor Pitx2 is required for the development of EOM but not for the development of limb muscle (Diehl et al. 2006 and Zhou et al. 2011). Retinoic acid, a derivative of vitamin A, controls the expression of Pitx2 during development. A loss of retinoic acid signaling results in absence of EOM as well (Matt et al. 2008 and Duester 2012).

One way to study the role of retinoic acid signaling in the sparing of EOM could be to investigate the number of the retinoic acid receptor alpha (RARα) - positive nuclei per myofiber in EOM and tibialis anterior (TA) limb muscle. We predict that EOM would possess more RARα than limb muscle, thereby indicating that retinoic acid signaling is involved in the sparing of EOM in DMD.

Objective
The aim of this project was to find the best optimized condition for staining for RARα and dystrophin.

Method
1. All tissue samples were prepared by following the same protocol. The mouse globes with the EOM attached or tibialis anterior from the limb were removed, embedded in tragacan gum, and frozen in methylbutane chilled to slurry on liquid nitrogen.
2. The tissue samples were then subjected to different immunostaining conditions to find the best one.

Immunostaining for RARα in EOM
1. The EOM sections were incubated in primary antibody against RARα at a concentration of 1:100.
2. The sections were reacted with secondary antibody, Elite ABC reagents.
3. The stained sections were developed using DAB (3,3’ diaminobenzidine tetrahydrochloride). The staining for the RARα was black.

Conclusion: DAB staining was able to locate RARα in EOM. Next, we stained for RARα in EOM and TA.

Immunostaining for RARα in EOM and TA
1. Similar procedure were followed for immunostaining as before, using primary antibody concentration of 1:250.

Conclusion: Immunostaining for RARα in EOM and TA was successful. However, we can not make a count of RARα per myofiber in these tissue samples as the outline of each myofiber is not specified.

Next, we doubled stained for RARα and dystrophin (myofiber membrane cytoskeleton) using fluorescent immunohistochemistry.

Fluorescent IHC for RARα and dystrophin
1. The EOM sections were incubated in primary antibodies against dystrophin and RARα at a concentration of 1:500 and 1:100-1:1000, respectively.
2. The sections were reacted with secondary antibodies, anti-rabbit AlexaFluor 488 and Elite ABC reagents.
3. The peroxidase was developed using substrate Amplex UltraRed. The staining for dystrophin was red, and the RARα should be green.

Conclusion: Fluorescent IHC gave no staining for RARα in the subsequent staining procedures too. We tried to double stain for Aldehyde dehydrogenase (ALDH, an enzyme involved in converting retinol to retinoic acid), however, it wasn’t successful either.

Summary
Immunostaining for RARα using DAB gave no information about the number of RARα-positive nuclei per myofiber, which is critical for understanding the role of RA signaling in the sparing of EOM in DMD. Unfortunately, double staining for RARα (or ALDH) and dystrophin using IHC was not successful either.

In the future, try different double staining conditions, such as varied antibody concentrations or varied incubation times to find out the best condition.

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References