THE EFFECTS OF ANTI-AGING PROTEIN, KLOTHO, ON SKELETAL MUSCLE

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INTRODUCTION

The physiological and societal impacts of aging represent a significant source of concern. Aging is characterized by several distinct physiological phenotypes, such as sarcopenia, osteopenia, growth retardation, impaired cognitive abilities, and pulmonary emphysema. Aging also greatly impacts the healthcare system, and is expected to cause an 18% increase in healthcare costs in the United States by 2050. Since aging-related consequences have such a great impact on society, one can see why understanding the mechanisms underlying the aging process is important.

Several mechanisms underlying the aging process can be attributed to the actions of a single gene, klotho. Klotho was originally discovered by an accidental gene deletion in mice, which resulted in the early onset of all aging-related phenotypes. Further studies revealed that the klotho gene acts as a powerful aging suppressor gene in vivo. The klotho gene is expressed predominantly as a transmembrane protein in the renal tubules of the kidney and in the choroid plexus. Klotho can also be cleaved from these sites to form a soluble form of klotho that can act in other tissues. Although some of the mechanisms underlying klotho’s potent aging suppression abilities have been elucidated, there remain many tissues where klotho’s mechanisms of action remain unknown.

Muscular regeneration is a well-studied and flourishing field, with much of current research easily translating into a clinical setting. However, no research group has looked at how klotho expresses itself within the muscular tissue. Additionally, it is known that exercise can promote the longevity of skeletal muscle, while injury can induce sarcopenia and loss of functionality in the damaged muscle. While many of the mechanisms mediating the homeostasis of muscle following exercise or injury are well understood, klotho’s role has not been established in either exercise or injury models.

OBJECTIVE

The objective of this research was to investigate the differences in klotho expression patterns between control, exercise, and injury groups using immunohistochemical analysis of total klotho expression area in vivo.

HYPOTHESIS/SUCCESS CRITERIA

Exercise and injury both have profound effects on muscle physiology and functionality. Thus, one would expect to see changes in klotho expression across a control group and an exercise or injury experimental group. Therefore, the success criteria for this experiment would be met if one could show statistically significant differences (p < 0.05) in total area of klotho expression across experimental groups.

METHOD

A murine model was chosen to study the klotho expression patterns across the control and experimental groupings in vivo. The mice were divided up and placed into one of the experimental groupings, with each experimental group containing four mice (n=4). The control mice received no treatment, the exercise group underwent a bout of treadmill running at 40% VO2 MAX, and the injury group received a cardiotoxin injection to simulate muscular injury. Each of the mice was sacrificed at the end of their experimental treatments, and the tibialis anterior was collected from each of the mice and prepped for histological analysis.

The next step was to optimize an immunofluorescence protocol to facilitate collecting and quantifying klotho data for the muscle sections. The immunohistochemical stain was performed using several different antibody concentrations of 1:20, 1:40, and 1:100 of primary antibody to bovine serum albumin (BSA). The same procedure was done for the secondary antibody concentrations, except that concentrations of 1:500 and 1:1000 were tested. It was also necessary to determine the appropriate amount of time to leave the primary antibody on the muscle section. The time points tested for the primary antibody were 1 hour at room temperature, 4 hours at room temperature, and overnight at 4°C Celsius.

Once the immunohistochemical protocol was optimized, the muscle samples were then sectioned and prepped for immunohistochemical staining. A primary antibody conjugated against murine klotho (Sigma-Aldrich, SAB3500604) was used to tag any klotho present within the muscle sample. A secondary antibody - which was conjugated with fluorescent protein, Cy3 – was applied to the muscle section to tag any klotho present within the muscle sample. A macro was then written in Nikon AR to analyze each image, mask any background noise, and determine the total area of klotho expression within a given image. After all the images had been quantified, the data was analyzed for statistical significance using Student’s t-test. A p value of less than 0.05 was defined as the criteria for statistical significance.

RESULTS

For the stain optimization, the primary time point that yielded the best signal was four hours at room temperature. The one hour and overnight time points yielded no immunofluorescent signal. The optimal primary antibody was determined to be 1:20 (1°C:BSA), with the concentrations of 1:40 and 1:100 yielding a weaker signal when imaged. For the
secondary antibody, a concentration of 1:1000 (2°: BSA) was determined to give the strongest fluorescent signal, however the differences in signal strength between the 1:500 and 1:1000 secondary concentrations were minimal.

Qualitative analysis of the images collected showed slight increases in klotho expression from the control to the exercise group, and a large increase in klotho expression from the control to injury groups. Quantification of this data yielded similar results (Figure 1). Klotho expression increased, on average, by 179 μm² between the control and exercise group. For the injury group, klotho expression increased, on average, by 1020 μm². Although there existed differences amongst the average area of klotho expression between the three experimental groups, statistical analysis of the data did not yield any significant results based on a p value of p<0.05.

DISCUSSION

The results of the experiment can yield insight into several phenomena surrounding klotho’s actions in vivo. First, the relatively low levels of klotho observed in the muscle are consistent with what one would expect, since the klotho gene is expressed primarily in the kidneys and in the choroid plexus. The relatively small increase in klotho expression area from the control to the exercise group suggests a lack of klotho dependence in exercise mediated muscular longevity. However, the large increase in klotho area expression from control to injury suggests klotho’s role in the muscular regenerative cascade following significant muscular trauma.

While one can hypothesize about the implications of the klotho expression trends observed in the experiment, the lack of statistical significance for both control vs. exercise and control vs. injury is a severe limiting factor. In order to further any hypotheses, one would need to perform a power analysis, and determine the appropriate number of animals with which to repeat the study, so that one can achieve statistical significance within the data.

In addition to the lack of statistical significance, the experiment had several other limitations. First, a murine model was used to establish klotho expression patterns. While many of the murine in vivo observations are still valid, one cannot assume that all of the observations will carry over to higher animals. Also, the experimental injury model utilized was only one form of muscular injury model, and does not characterize all possible muscular injuries.

CONCLUSION

Aging represents both a significant physiological risk to all persons as well as a significant burden on the health care system. Therefore, one can see why fully understanding aging and working to optimize treatment methods is essential. The effects of anti-aging gene klotho offer great promise to accomplish that goal. In this experiment, the klotho expression patterns in control, exercise, and injury groups were investigated. It was determined that klotho is slightly upregulated from control to exercise groups, and greatly upregulated from control to injury groups. However, due to a lack of statistical significance, further experiments need to be performed to confirm the observations and fully elucidate the klotho expression patterns in vivo.

ACKNOWLEDGMENTS

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