Evaluating the Relationship Between Keratin Biomaterials and the Autophagy Pathway in Human Embryonic Kidney 293 Cells

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ABSTRACT

Biomaterials are biologically-based substances that are used to support or replace tissue function in living organisms. Keratin is a commonly used structural biomaterial in the field of cell culture research due to its abundance and cost efficiency, but studies investigating keratin's ability to influence cell behavior are few. In this study, we investigated the relationship between keratin biomaterials and the autophagy pathway in Human Embryonic Kidney 293 (HEK 293) cells. We treated HEK 293 cells with crude keratin extract derived from human hair and autophagy controls such as rapamycin, chloroquine, and 3-MA to test keratin's relationship to the autophagy pathway in HEK 293 cells.

INTRODUCTION

Keratin is a protein found in skin and hair and is a common biomaterial used in tissue research (Hill et al., 2010). Several studies suggest that keratin extracted from human hair possesses the ability to modulate cell viability and cell proliferation (Sierpinski et al., 2007; Poranki & Van Dyke, 2014). In addition, a study by Poranki & Van Dyke (2014) found that keratin treatment was associated with an upregulation of autophagy genes in heat shocked fibroblasts. This finding suggests a relationship between keratin and the autophagy pathway that may underlie keratin's cytoprotective effects.

Autophagy is a catabolic process that is essential for the maintenance of cellular homeostasis. During autophagy, cells create membrane-bound compartments called autophagosomes from phagophores. The autophagosomes engulf structures such as protein complexes, aggregates, and organelles. Autophagosomes then fuse with lysosomes, which break down the contents of the autophagolysosomes in order to provide energy, recycle metabolic building blocks, and ameliorate damage due to reactive oxidant species or toxic protein aggregates (Pohl & Dikic, 2019).

One important protein implicated in the autophagy pathway is the microtubule associated protein light chain 3 (MAPLC3, or LC3). A common approach to track autophagic activity in cells involves monitoring the expression and modification of the LC3 protein as it goes back and forth between two forms: LC3-I to LC3-II. When autophagy levels in a cell are low, LC3 is distributed throughout the cytosol as LC3-I. When autophagy begins, LC3-I is converted to LC3-II and joins the membrane of autophagosomes (Velazquez et al., 2018). During this time, LC3-II on the interior of the autophagolysosome is degraded and LC3-II on the exterior of the autolysosome is recycled. Figure 1 on the next column demonstrates a brief overview of the autophagy process in cells.

Figure 1: The Autophagy Process in Mammalian Cells

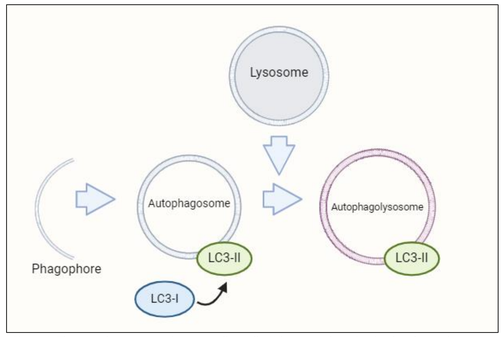

Figure 1: The autophagy process in mammalian cells. When autophagy is stimulated, membrane sites at the endoplasmic reticulum bud to form phagophores. These phagophores mature into autophagosomes. Autophagosomes recruit the LC3 protein to their membranes, which allows organelles to be brought to the autophagosome and engulfed. Once the autophagosome matures, it fuses with a lysosome to form an autolysosome. The low pH of the autolysosome then degraded the inner contents.

The ratio between LC3-II to LC3-I serves as an indicator of the level of autophagy occurring in a cell population. An increase in LC3-II relative to LC3-I can be interpreted as an increase in autophagic activity. However, an increase in LC3-II relative to LC3-I can also indicate a blockage in autophagosome-lysosome fusion. Therefore, researchers must use compounds that control for such a blockage in order to make judgements about the level of autophagy occurring in cells. One example of such as compound is chloroquine.

Our lab has performed fluorescent imaging experiments to explore the link between keratin biomaterials and autophagy activity, but more research was needed. Here, we use western blotting to evaluate keratin's relationship to autophagy. We incorporate autophagy controls such as the autophagosome-lysosome fusion inhibitor chloroquine alongside crude keratin extract to further probe the relationship between keratin and autophagy.

METHODS

Human Embryonic Kidney 293 (HEK 293) cells were grown according to standard cell culture protocols and seeded to a density of $1.5x10^5$ cells/mL. After 24 hours of incubation at 37 °C, cells were given the following treatments at the indicated concentrations:

- 0.2 µM Rapamycin (an autophagy stimulator)
- 50% starvation (1 mL Phosphate buffered saline and 1 mL Dulbecco's Modified Eagle Medium (an autophagy stimulator)
- 50 µM Chloroquine (an autophagosome-lysosome fusion)
- 10 mM 3-methyladenine (an early autophagy inhibitor)
- 0.1 mg/mL crude keratin extract

Cells were incubated for 24 hours after treatment, then lysed and stored until further analysis. The total protein concentration of each lysate was quantified using a Coomassie blue G-250 dye assay. SDS-PAGE and western blotting were then used to resolve proteins and quantify LC3 levels in cells. A densitometry analysis of each western blot was performed using ImageJ, and results were analyzed with a T-test.

RESULTS

Figure 2 below shows a western blot of the LC3 protein in its two forms: LC3-I and LC3-II. These proteins are present in both untreated cells and cells treated with 0.2 µM rapamycin. Rapamycin is an autophagy stimulator. An increase in LC3-II relative to LC3-I is apparent in each group treated with rapamycin below (Figure 1.A). A densitometry analysis was used to calculate the ratio between LC3-II and LC3-I (Figure 1.B). Increased ratios in the rapamycin treated group indicate an increased autophagic state in the cells. These results are statistically significant and serve as a positive control for the induction of autophagy in our experiments.

Figure 2: Rapamycin Induces Autophagy in HEK 293 Cells

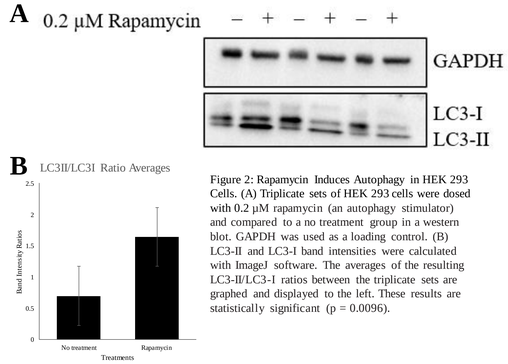

Figure 2: Rapamycin Induces Autophagy in HEK 293 Cells. (A) Triplicate sets of HEK 293 cells were dosed with 0.2 µM rapamycin (an autophagy stimulator) and compared to a no treatment group in a western blot. GAPDH was used as a loading control. (B) LC3-II and LC3-I band intensities were calculated with ImageJ software. The averages of the resulting LC3-II/LC3-I ratios between the triplicate sets are graphed and displayed to the left. These results are statistically significant ($p = 0.0096$).

Figure 3 is a western blot of cells treated with various compounds used to stimulate or block autophagy (see methods) in combination with crude keratin extract (Figure 3.A). We hypothesized that cells treated with 50% starvation and keratin would show an increased level of autophagy compared to cells treated with 50% starvation alone or keratin alone. Densitometry analysis and further statistical testing reveal that there is no significant difference between the average LC3-II/LC3-I ratios (Figure 3.B).

Figure 3: Impact of Keratin on Autophagy in HEK 293 Cells

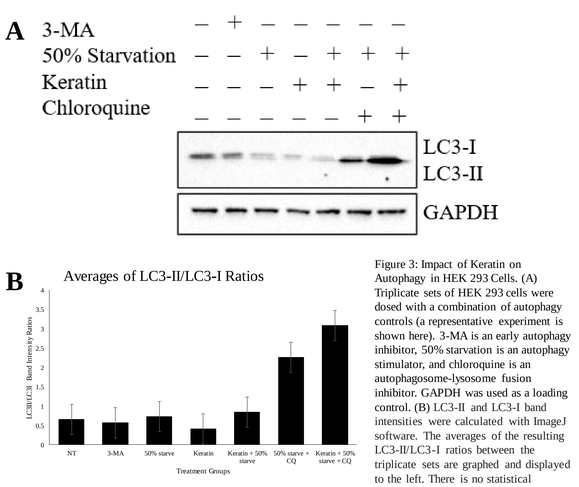

Figure 3: Impact of Keratin on Autophagy in HEK 293 Cells. (A) Triplicate sets of HEK 293 cells were dosed with a combination of autophagy controls (a representative experiment is shown here). 3-MA is an early autophagy inhibitor, 50% starvation is an autophagy stimulator, and chloroquine is an autophagosome-lysosome fusion inhibitor. GAPDH was used as a loading control. (B) LC3-II and LC3-I band intensities were calculated with ImageJ software. The averages of the resulting LC3-II/LC3-I ratios between the triplicate sets are graphed and displayed to the left. There is no statistical significance between the no treatment, 3-MA, 50% starvation, keratin, and keratin + 50% starvation groups.

DISCUSSION AND CONCLUSION

In the past, our lab has performed heat shock experiments and starvation experiments to evaluate keratin's relationship to autophagy. In these experiments, HEK 293 cells were stressed, dosed with keratin, and monitored for autophagy activity. We observed a potential increase in autophagy activity at certain early time points in cells that were stressed and grown with keratin extract, as opposed to cells that were stressed without keratin. We hypothesized that keratin may increase autophagy activity in stressed cells and thus serve as a cytoprotective mechanism. However, more experimentation was necessary to confirm this link. In this current study, we follow up on this previous work by using western blotting to evaluate autophagy levels in cells stressed with starvation and treated with crude keratin extract. We also expand upon our previous work by using several autophagy control drugs (rapamycin, 3-MA, and chloroquine) to provide a framework for evaluating autophagy activity in our samples. Cells treated with 50% starvation for 24 hours in addition to crude keratin extract did not show an increase in autophagy activity as compared to our rapamycin-treated positive control, 3-MA negative control group, no treatment group, and 50% starvation positive control group (Figure 3.B). We speculate that 24 hours post treatment may not be an optimal time point for assessing autophagy in these cells. 24 hours post treatment was chosen for this study because it allowed for comparison to rapamycin treated cells (rapamycin has the greatest effect at 24 hours post treatment). Although we are unable to conclude based on the conditions used in this study whether keratin increases autophagy activity, we speculate that earlier timepoints may be a more appropriate measure for evaluating autophagy with starvation. Future studies would revisit these earlier time points with the current western blot methods to further explore this relationship.

References

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