Contrasting Effects of Rapamycin and 3-Methyladenine on Cellular Autophagy, Apoptosis, Senescence, and Extracellular Matrix Metabolism Are Linked to Akt Phosphorylation in Intervertebral Disc Cells

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Disclosures:

Introduction: Intervertebral disc degeneration is associated with back pain, which is characterized by matrix degradation and decreased cellularity. Disc cells are subject to diverse stresses during their lifetime, suggesting a possible role of autophagy, an important cell survival mechanism by which cells break down and recycle damaged components. However, the impact of autophagy regulation on disc cells are poorly unstudied. Therefore, an in vitro study was designed to elucidate the effect of an autophagy inducer, rapamycin, and an inhibitor, 3-methyladenine (3-MA), on disc cell fate and matrix homeostasis.

Methods: All experiments were performed under the approval and guidance of the University of Pittsburgh Institutional Animal Care and Use Committee. The first passage, 80% confluent, monolayer fibrochondrocytes from the annulus fibrosus of 6-month-old female New Zealand White rabbits (~2.5 kg) cultured in a 37 °C, 5% CO₂, and 5% O₂ incubator were used.

Dehydrogenase activity, DNA amount, and cell metabolic activity assays: Cells were cultured for 48 h in 1% fetal bovine serum (FBS)-supplemented Dulbecco’s modified Eagle’s medium (DMEM) with 0-50 μM rapamycin or 0-50 mM 3-MA. Dehydrogenase activity and DNA amount were measured by CCK-8 and PicoGreen assays, respectively. Cell metabolic activity was calculated as dehydrogenase activity normalized to DNA amount.

Proliferation assay: Cells were cultured up to 14 days in 10% FBS DMEM with rapamycin (100 nM or 1 μM) or 3-MA (2.5 or 5 mM) and trypsinized for counting every other day.

Imaging cytometry: Cells were cultured in 1% FBS DMEM with 100 nM rapamycin or 2.5 mM 3-MA. Markers of autophagy, LC3 spot number and cytoplasmic HMGB1 intensity, were measured longitudinally at 0-48 h.

Western blotting: Total protein extracts were collected at 0, 12, 24, and 48 h. Expression of LC3-II (autophagosome-bound form), HMGB1, and a negatively correlated autophagy marker, p62/SQSTM1, was assessed. Total induction of autophagic flux was examined using chloroquine (autophagosome degradation inhibitor). Expression and phosphorylation of a negative autophagy regulator, mammalian target of rapamycin (mTOR), a mTOR effector (regulating protein synthesis), p70/S6K, and a mTOR regulator mediated by class I PI3K, Akt, were similarly evaluated.

Immunostaining: Cells were cultured for 48 h in 1% FBS DMEM with 100 nM rapamycin or 2.5 mM 3-MA. Apoptotic and senescent cells were determined by TUNEL and SA-β-gal staining, respectively. Immunofluorescence for apoptotic cleaved caspase-3 and senescent p16/INK4A was performed.

Real-time RT-PCR and matrix synthesis assay: Cells were cultured for 48 h in 1% FBS DMEM with 100 nM rapamycin or 2.5 mM 3-MA. The mRNA levels of MMP-3, -13, TIMP-1, aggrecan-1, and collagen types 1-α1 and 2-α1 normalized to 18S rRNA were measured. Newly synthesized proteoglycans, collagens, and total proteins were detected using radioisotopic 35S-sulfate and 3H-proline.

Statistical analysis: Multi-way ANOVA with the Tukey-Kramer post-hoc test was used. P-values were set at 0.05.

Results: Rapamycin and 3-MA both reduce disc cell metabolic activity and proliferation but with different dose-response relationships.

Rapamycin reduced total dehydrogenase and cell metabolic activity from 100 nM but DNA amount from 1 μM. 3-MA decreased total dehydrogenase and cell metabolic activity from 5 mM but DNA amount from 2.5 mM. Cell proliferation decreased in response to the concentration of rapamycin but not of 3-MA. Based on these results, we selected 100 nM rapamycin and 2.5 mM 3-MA for the following assessments.

Rapamycin and 3-MA both activate disc cellular autophagy with different kinetics.

Imaging cytometry demonstrated that rapamycin increased the number of LC3 puncta with a peak at 6-12 h more remarkably than the control but 3-MA did not show it; however, 3-MA subsequently showed increased LC3 puncta (Fig. 1AB). Cytoplasmic intensity of HMGB1 increased with time by both agents (Fig. 1AB). Longitudinal Western blotting showed that LC3-II expression increased with a peak at 12-24 h by rapamycin but with time by 3-MA (Fig. 2A). HMGB1 showed increased expression at 12-24 h by rapamycin but not by 3-MA (Fig. 2A). p62/SQSTM1 expression decreased time-dependently by both agents (Fig. 2A). Furthermore, in LC3 turnover assay using chloroquine, rapamycin and 3-MA both elevated LC3-II accumulation (Fig. 2B). Rapamycin decelerates but 3-MA accelerates disc cellular apoptosis and senescence.
TUNEL- and cleaved caspase-3-positive cells decreased by rapamycin but increased by 3-MA (Fig. 1C). SA-β-gal- and p16/INK4A-positive cells also decreased by rapamycin but increased by 3-MA (Fig. 1C).

**Rapamycin and 3-MA play contrasting anti-catabolic and catabolic roles, respectively, in matrix metabolism**

In quantitative PCR, rapamycin down-regulated MMPs and up-regulated TIMP-1 while 3-MA up-regulated MMPs and down-regulated TIMP-1. However, aggrecan-1 and collagens were down-regulated by both agents. Radioisotopic assay also exhibited decreased new synthesis of proteoglycans, collagens, and total proteins by both agents.

**Rapamycin suppresses mTOR but activates Akt while 3-MA suppresses both mTOR and Akt**

Rapamycin induces autophagy by inhibiting mTOR. 3-MA inhibits autophagy by inhibiting class III PI3K. Western blotting displayed rapamycin decreased mTOR and S6K but increased Akt phosphorylation whereas 3-MA decreased mTOR, p70/S6K, and Akt phosphorylation (Fig. 2C). Autophagy activation by both agents is consistent with mTOR suppression. Reduced matrix protein synthesis and mRNA transcription by both agents is consistent with p70/S6K suppression. Contrasting effects of rapamycin and 3-MA may be explained by up-regulation versus down-regulation of Akt (Fig. 2D).

**Discussion:** Rapamycin demonstrated protective effects while 3-MA showed harmful effects on disc cell fate and matrix homeostasis; however, both agents induced autophagy activation. Recent evidence has shown 3-MA inhibits class III PI3K transiently but class I PI3K persistently, resulting in autophagy induction by class I PI3K inhibition-mediated mTOR suppression. Suppressed mTOR and p70/S6K by rapamycin loses the S6K-mediated negative feedback loop for class I PI3K, resulting in induction of class I PI3K and Akt and providing Akt-mediated pro-survival and proliferative signals. This study suggests rapamycin is a promising drug to slow degenerative disc disease; however, its beneficial effect may result from activated Akt signals as well as from activated autophagy.

**Significance:** This study demonstrates the effects of rapamycin and 3-MA on disc cells, suggesting a potential application of rapamycin to slow disc degeneration.

**Acknowledgments:** This work was supported by The Albert B. Ferguson, Jr., M.D. Orthopaedic Fund of The Pittsburgh Foundation and The Uehara Memorial Foundation.
