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Rapamycin activates autophagy in Hutchinson-Gilford progeria syndrome

Implications for normal aging and age-dependent neurodegenerative disorders

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While rapamycin has been in use for years in transplant patients as an antirejection drug, more recently it has shown promise in treating diseases of aging, such as neurodegenerative disorders and atherosclerosis. We recently reported that rapamycin reverses the cellular phenotype of fibroblasts from children with the premature aging disease Hutchinson-Gilford progeria syndrome (HGPS). We found that the causative aberrant protein, progerin, was cleared through autophagic mechanisms when the cells were treated with rapamycin, suggesting a new potential treatment for HGPS. Recent evidence shows that progerin is also present in aged tissues of healthy individuals, suggesting that progerin may contribute to physiological aging. While it is intriguing to speculate that rapamycin may affect normal aging in humans, as it does in lower organisms, it will be important to identify safer analogs of rapamycin for chronic treatments in humans in order to minimize toxicity. In addition to its role in HGPS and normal aging, we discuss the potential of rapamycin for the treatment of age-dependent neurodegenerative diseases.

Keywords: progerin, rapamycin, autophagy, aging, neurodegeneration, progeria

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HGPS

Hutchinson-Gilford progeria syndrome (HGPS) results from a de novo mutation in the gene for the nuclear lamina protein, lamin A, and is observed in about 1 in 4 million live births.[1,2] Patients with HGPS appear normal at birth, but begin to display alopecia, growth retardation, bone abnormalities, osteoporosis and scleroderma tous skin by one year of age.[1,3] The cardiovascular system is severely affected and, sadly, HGPS patients most often succumb to myocardial infarction or stroke in their early teens.[1] The most common form of HGPS is caused by a de novo mutation in exon 11 of the lamin A gene. This mutation activates a splice donor, leading to production of a lamin A variant with an internal deletion of 50 amino acids. This protein is termed progerin[4] and is defective in a critical post-translational processing step. The normal processing of the lamin A protein involves farnesylation of the C terminus by farnesyltransferase, followed by cleavage by the metalloproteinase Zmpste24, which removes 18 amino acids at the C terminus and yields mature lamin A.[5,6] The farnesylation of lamin A is an essential step in this process, as it targets the protein to the inner nuclear membrane surface where it is finally cleaved by Zmpste24 to yield mature lamin A.[7] In HGPS, the mutant progerin protein has lost this cleavage site, resulting in permanent farnesylation. The retention of the farnesylated C terminus is thought to cause the progerin protein to remain anchored in the nuclear membrane, with other proteins of the normally fluid nuclear scaffold attached to it. During mitosis, when the scaffold has to disassemble and reassemble, the mutant protein is highly disruptive. With increasing numbers of cell divisions, several phenotypes can be observed including nuclear blebbing, which is seen in primary

fibroblasts from HGPS patients, along with a reduced growth rate and increased senescence in culture.[8,9] Of note, mutations in humans and mice that disrupt Zmpste24, resulting in permanent farnesylation of an otherwise normal lamin A, also give rise to phenotypes similar to HGPS, supporting the hypothesis that retention of the farnesylated C terminus leads to the cellular phenotype.[6,10,11]

Therapeutic Strategies for HGPS

Without treatment, HGPS is a uniformly fatal disorder. A promising new therapy, now being subjected to a clinical trial, is the use of farnesyltransferase inhibitors (FTIs). FTIs are experimental drugs originally developed for cancer therapy, because of their predicted effect on the oncoprotein Ras, which also requires farnesylation for its function.[12] While FTIs have been somewhat disappointing in their effectiveness against cancer, FTI treatment reduces nuclear blebbing in HGPS fibroblasts and cell lines transfected with progerin.[13,14] Furthermore FTIs improve phenotype and life span in mouse models of progeria.[9,15] It is thought that FTIs, by preventing farnesylation of progerin, reduce the accumulation of progerin at the nuclear rim, reducing the damaging effects of the mutant protein on the nucleus.[13] The outcome of an open-label HGPS clinical trial of the FTI lonafarnib is awaited with great interest.

Rapamycin Promotes Clearance of Progerin

Rapamycin is an FDA-approved drug that has been used historically to suppress the rejection of transplanted organs, usually in combination with other immunosuppressants. In addition to its historical use as an immunosuppressant, there is mounting pre-clinical evidence that rapamycin or analogs of rapamycin extend life span in a number of species including flies and mice.[16-18] The life-span extending effect in mice is observed even if treatment is not started until the mice are already aged.[19] Furthermore, there is evidence that rapamycin and rapamycin derivatives are useful in delaying or treating age-related conditions, including cancers and neurodegenerative diseases. These effects are presumed to be due to the inhibition of mTOR by rapamycin, and are at least in part dependent on autophagy.[17,20,21]

We recently found that the mTOR inhibitor rapamycin rescues the cellular phenotype of HGPS fibroblasts and decreases the amount of progerin protein through autophagic clearance.[22] Co-treatment with rapamycin and the autophagy inhibitors bafilomycin A1 or 3-methyladenine slow the enhanced clearance, suggesting that progerin is being cleared through autophagy. Further experiments using genetic inhibition of autophagy by *ATG7* knockdown confirmed this result.

To examine the mechanism of progerin clearance in further detail, we next examined the ubiquitination status of progerin. Protein ubiquitination has recently emerged as an important signal for selective autophagic clearance that involves autophagy adaptor proteins that bind both ubiquitinated cargo and autophagosomes (e.g., p62). Specifically, different effects have been described for different types of polyubiquitin chains; for example, the K48 polyubiquitin (polyUb) chain is recognized by, and promotes degradation of proteins through, the proteasome, whereas the K63 polyUb chain promotes autophagic clearance of oligomeric and aggregated proteins.[23-25] We found that progerin is preferentially ubiquitinated by K63-linked polyUb chains, suggesting that it is a substrate for autophagy. Additionally, progerin co-immunoprecipitates with the autophagic adaptor protein p62, further demonstrating that the clearance of progerin is mediated by autophagy. Finally, via immunofluorescence microscopy, we found that progerin colocalizes with both p62 and the autophagy linked FYVE protein, ALFY, in the presence of rapamycin. Interestingly, upon treatment with rapamycin, less progerin is seen in the nuclei, and it instead colocalizes with p62 and ALFY in the juxtanuclear cytoplasm, suggesting that ALFY and p62 are instrumental in the clearance of progerin. Importantly, because p62 and ALFY can also interact with the mammalian homolog of Atg8 (LC3), they therefore represent a functional link between ubiquitinated cargo and autophagosomes.[26-28] Since ALFY normally resides in the cell nucleus, it will be of particular interest to further examine its role in clearance of nuclear progerin.

Interestingly, in the context of HGPS, rapamycin also has been reported to have a beneficial effect on mouse models prone to cardiovascular disease phenotypes. In three separate studies involving apoE knockout mice or LDLR knockout mice, rapamycin reduces arteriosclerotic lesions despite the severe hypercholesterolemia in these mice, even when fed a high-fat diet.[29-31] Because the cardiovascular disease component of HGPS leads to devastating heart attacks or strokes, it will be of interest to examine if rapamycin confers any benefit on the cardiovascular aspects of HGPS by promoting clearance of progerin in cardiovascular tissues. The G608G mouse model of HGPS has a cardiovascular phenotype that shows improvement with FTI treatment.[9] Experiments are now underway with this mouse model of HGPS to see whether everolimus (an analog of rapamycin) is capable of preventing the cardiovascular disease phenotype.

Rapamycin and Autophagy in Neurodegenerative Diseases

It is thought that the accumulation of the progerin protein in HGPS underlies the progression of the disease phenotype. This is true for other proteinopathies that are caused by accumulation of mutated or improperly processed protein. Among them are several neurodegenerative diseases, including Huntington, Parkinson and Alzheimer diseases. Recent evidence suggests that boosting autophagy in models of these disorders can reduce the accumulation of the disease protein and protect against toxicity associated with protein aggregation.

For example, Huntington disease is caused by an abnormal expansion of the polyglutamine tract in the huntingtin protein. This mutant form of huntingtin accumulates inside neurons, forms aggregates and eventually causes cell death. Cell culture models overexpressing mutant huntingtin are protected from toxicity and clear huntingtin faster when treated with the autophagy-enhancing drug rapamycin,

leading to less aggregation.[32] This finding has been extended in vivo, in both flies and mice. Fruit flies expressing mutant huntingtin in photoreceptor neurons are protected from rhabdomere degeneration when fed rapamycin.[33,34] Mouse models expressing mutant huntingtin in the brain also demonstrate less aggregation in the striatum when treated with the rapamycin analog temsirolimus.[33] Huntingtin is a substrate for autophagic clearance,[27] and the protective effects of rapamycin in these models occur at least in part through the activation of autophagy,[35-37] and the subsequent clearance through degradation of unnecessary or toxic components inside the cell.[38,39]

A characteristic feature of Parkinson disease (PD) is the presence of intracellular inclusions known as Lewy bodies. These inclusions contain α-synuclein, which accumulates in PD. Accumulation of toxic α-synuclein species further affects the lysosomes' ability to clear α-synuclein.[40] Increasing autophagy increases clearance of α-synuclein in cell models that overexpress the mutant proteins,[41] and mouse models which overexpress and accumulate α-synuclein also show less accumulation when treated with rapamycin because of increased autophagy and lysosomal activation.[42] Furthermore, in mouse models of PD treated with the toxin MPTP, autophagosomes accumulate due to a decrease in lysosomal function. Accumulation of autophagosomes and loss of lysosomes is also found in postmortem PD brain, and Lewy bodies are positive for autophagosomal markers. In the MPTP mouse model, rapamycin treatment is able to restore lysosomal levels, decrease autophagosome accumulation, and protect against dopaminergic cell death.[43]

Alzheimer disease is characterized pathologically by the presence of amyloid plaques, consisting of amyloid-β and neurofibrillary tangles, which are intracellular inclusions composed of hyperphosphorylated tau. In several mouse and fly models of Alzheimer disease, upregulation of autophagy improves some of the behavioral and pathological phenotypes, and reduces amyloid β and tau related toxicities.[38,44,45] Interestingly in Alzheimer models, mTOR is activated, potentially by the presence of amyloid-β, which therefore inhibits autophagy. Rapamycin restores autophagy and reduces amyloid-β despite this baseline inhibition of autophagy.

Together, these studies demonstrate that in neurodegenerative diseases where misfolded proteins accumulate, boosting autophagy with pharmacological agents can prove beneficial. As in HGPS, rapamycin shows promise in various models of neurodegenerative disorders; however, it will be important to identify safer analogs of rapamycin for chronic treatments in patients.

Progerin and Normal Aging

In HGPS, progerin is produced as a result of a mutation that activates a cryptic splice site in the lamin A gene. However, in normal cells, this unmutated cryptic splice site is also used sporadically, leading to production of progerin protein in non-HGPS individuals.[46,47] Increased activation of this cryptic splice site appears to correlate with incipient cell senescence, and the progerin protein appears to accumulate with increasing age in skin biopsies,[47] as well as in culture. Interestingly, an early report showed that oligonucleotide based inhibition of the cryptic splice site in *LMNA* reduces the changes in nuclear architecture found in cells from aged individuals, and reduces markers of senescence as well,[46] raising the hypothesis that these low levels of sporadic progerin production could contribute to the aging process.

Another recent study by Olive et al.[48] looked for the presence of progerin in non-HGPS arteries in 29 individuals ranging in age from 1 mo to 97 y. They found a statistically significant increase in the amount of progerin-positive cells with age, ranging from an average of 1 in 1,000 cells at 1 mo, to ~20 in 1,000 cells at 97 y in the adventitia. This represents an increase of 3.34% per year.[48] In cells where progerin was present, it was found as progerin-positive cytoplasmic puncta, suggesting that it accumulates in certain cells over time after repeated mitoses.

Progerin accumulation in normal cells would presumably have negative consequences, particularly in cell populations that divide rapidly, including stem cell populations responsible for tissue homeostasis. Indeed, Scaffidi et al.[49] showed that progerin expression interferes with the differentiation potential of human mesenchymal stem cells (hMSC), which in turn gives rise to many of the affected tissues in HGPS. This raises the possibility that sporadic expression of aberrant progerin in physiological aging could affect tissue homeostasis by inhibiting the ability of stem cells to regenerate damaged cells.

Most recently, activation of progerin expression in normal cells has been linked to telomere dysfunction. Non-HGPS cells expressing progerin have shorter telomeres than non-progerin-expressing cells, and inducing telomere damage experimentally also upregulates progerin expression through increased usage of the cryptic splice site in *LMNA* (and many other changes in alternative splicing).[50] Interestingly, there is also evidence that progerin expression actually induces telomere damage,[51] raising the possibility of the existence of a positive feedback loop between telomere damage and progerin expression that is capable of driving programmed senescence.

As a whole, these findings raise the likelihood that splicing of *LMNA*, leading to increasing progerin production after multiple cell divisions, affects physiological aging in normal individuals and contributes functionally to programmed cell senescence. Although progerin accumulation is just one part of the many physiological changes that occur in the aging process, treatments that enhance clearance of progerin or prevent its production could have aging-related health benefits over the long-term.

It will therefore be of interest to examine whether upregulation of autophagy by rapamycin could enhance clearance of progerin, as well as other proteins that accumulate during normal aging.

Therapeutic Limitations of Rapamycin

Rapamycin treatment is associated with side effects, some of which require discontinuation of treatment (in up to 39% of patients), at least at the doses used in

transplant patients. Side effects include gastrointestinal symptoms, edema, infection, delayed wound healing, high cholesterol and triglyceride levels, anemia, and interstitial pneumonitis.[52,53] The interstitial pneumonitis is a particularly severe side effect and can be life threatening. Some of these effects may be due to off-target effects of rapamycin. The use of alternative, more specific inhibitors of the mTORC1 pathway, such as everolimus (RAD001) may be safer. Everolimus also has better solubility in aqueous solution and is more stable compared with rapamycin. Several case reports indicate resolution of pneumonitis upon switching from sirolimus to everolimus.[54]

In cases where the potential benefits outweigh the potential for side effects, such as in devastating diseases like HGPS, neurodegenerative disease and cancer, rapamycin and rapamycin derivatives may offer a new treatment strategy. However, the use of rapamycin in otherwise healthy people to slow the aging process would require safer alternatives and/or lower doses than those typically given to transplant patients to mitigate the chance of side effects.

Another potential drawback for the use of rapamycin in children with HGPS is the potential effect on growth and development. This could pose a problem for drugs that inhibit mTOR, which is a master regulator of cellular growth and proliferation. A recent study involving pediatric kidney transplant recipients undergoing treatment with rapamycin suggests that growth rate is reduced in the treated group, at least for the 2 y of data available.[55] Nevertheless, considering the severity of the disease, the benefits may outweigh the potential side effects, and perhaps they can be controlled with careful dosing schedules, for instance, intermittent periods of treatment, followed by recovery periods, rather than chronic treatment.

Alternatively, mTOR-independent stimulators of autophagy such as trehalose or lithium could be explored to sidestep the potential developmental issues. It is not known, however, if these approaches will be effective in clearing the progerin protein. In neurodegenerative disease models, these mTOR-independent compounds have an additive effect on autophagy induction when co-administered with rapamycin, so it is possible that they might help increase progerin clearance even more than rapamycin alone.[41,56]

Finally, co-administration of rapamycin with the FTIs currently in clinical trials for HGPS could also have an additive effect on progerin clearance. While rapamycin clears farnesylated progerin through autophagy, FTIs presumably help by limiting the amount of farnesylated progerin produced in the first place. Perhaps a combination treatment involving both FTIs and rapamycin would be even more efficacious in clearing progerin and eventually treating HGPS.

Conclusion

The last several years of studies on HGPS have yielded important insights about the role of progerin in this disease and in the normal aging process. Our recent finding that rapamycin clears the progerin protein through autophagy and reverses the cellular phenotypes of HGPS fibroblasts provides a new target for treatment of HGPS: the clearance of progerin. Given the recent findings that progerin is also expressed during physiological aging, this finding possibly has implications for the normal aging process as well.

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