Aging and Oxidative Stress

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Life Span and Oxidative Stress in Nematode

Naoaki Ishii*

Department of Molecular Life Science, Tokai University School of Medicine, Isehara, Kanagawa 259–1193, Japan

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Summary The nematode *Caenorhabditis elegans* has proven a robust genetic model for studies of aging and the roles of oxidative stress. In this review we focus on the genetics of select long-lived and short-lived mutants of *C. elegans* that have proven useful in revealing the relationships that exist between oxidative stress and life span. The former are known to be controlled by an insulin/insulin-like growth factor (IGF -1)-like pathway, while the latter are affected by mitochondrial functions.

Key Words: nematodes, oxidative stress, life span, insulin/IGF-1-like signal pathway, mitochondria

Introduction

Aging is controlled by a complex interplay of genetic and environmental factors. In common aging, one of the most interesting relationships to emerge from aging research is that the product of the standard metabolic rate and the maximum life span is roughly constant for various animals. Thus, animals with lower standard metabolic rates generally live longer than animals with higher rates [1]. This concept, called the Lifespan Energy Potential (LEP), suggests that metabolic rate is closely related to aging. Metabolic rates can be experimentally manipulated. For example caloric restriction extends life span in rodents, nematodes, yeast and probably primates [2–7]. The mechanism is still unclear, but one hypothesis is that caloric restriction slows energy metabolism. Related to these studies is the positive

correlation between antioxidant concentrations in the tissues of mammalian species and their maximum lifespan potential (MLSP) [1]. Reactive oxygen species (ROS) such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($\cdot OH$) are produced as byproducts of energy metabolism. Such endogenously generated molecules can readily attack a wide variety of cellular entities, resulting in damage that compromises cell integrity and function [8, 9]. This can cause or at least contribute to a variety of pathologies, including some in humans [10]. To lessen the consequences of this damage, cells have evolved complex defense mechanisms, including enzymes, *e.g.*, superoxide dismutase (SOD) and catalase as well as various non-enzymatic antioxidants, *e.g.*, vitamins C and E and glutathione that act to detoxify the offending molecules [9, 11, 12]. Tolmasoff *et al.* found that the ratio of SOD specific activity to specific metabolic rate increased with increasing MLSP for all species examined [13]. These data show an important correlation between life span and energy metabolism as well as between oxidative stress and antioxidant defense.

* To whom correspondence should be addressed.

Tel: +81-463-93-1121 ext. 2650 Fax: +81-463-94-8884
E-mail: nishii@is.icc.u-tokai.ac.jp

To learn about the genes that affect aging, the use of animals with established genetics is essential. This is a limited list (e.g. human, mice, fruit flies and so on). One of these animals, the free-living nematode *Caenorhabditis elegans* (*C. elegans*), has been employed widely to explore the genetics of aging. One extremely successful approach has been to isolate mutants that extend life span. Conversely, genetic analyses of mutants that shorten life span have also provided insights into aging as it occurs in wild type. In this review, we discuss insights provided by the genetic analysis of long- and short-lived mutants that are affected by insulin-like signaling and mitochondrial functions in energy metabolism, respectively.

***Caenorhabditis elegans* as a Model System for Aging Research**

C. elegans can be readily cultured in petri plates on a simple diet of *Escherichia coli* and reproduces with a rapid life cycle of approximately 3.5 days at 20°C. Embryonic development is rapid, taking only 13 h at 20°C [14, 15]. The cell lineage has been traced from single-celled zygote to adult and the entire cell lineage has been determined [14–17]. The first half of embryogenesis is characterized by extensive and rapid cell divisions, with embryos reaching 558 cells after 5 h. During the second half, cell divisions cease and the body elongates. After hatching, larval development proceeds through four molts, which punctuate larval stages L1 through L4. About 10% of the cells in the first larval stage undergo further cell divisions contributing to the hypodermis, neurons, musculature, and somatic gonadal structures. This culminates in adults that contain fewer than 1,000 somatic cells and have been generated from a largely invariant set of cell divisions. Under adverse environmental conditions such as a high population density or starvation, larvae develop into an arrested and quiescent stage called dauer larvae [18]. Dauer larvae consume energy but do not feed, and their metabolism differs markedly from all other stages [19].

C. elegans offers several distinct advantages for aging research, one of which is a short maximum life span of approximately 30 days. In addition, dauer larvae can easily survive for over three months with no effect on post-dauer life span, and, as such, have been described as non-aging [20]. As described below, the genetics of dauer formation overlaps with that of adult life span, mitochondrial functions, and

oxidative stress responses. Genetic approaches have proven useful for identifying the genes and pathways that regulate aging [21]. In part this derives from its self-fertilizing, hermaphroditic mode of reproduction, which facilitates mutant isolation (<http://www.wormbase.org/>). Literally thousands of mutants have been isolated, each affecting virtually any all biological processes including aging [22]. This classical approach is complemented by some powerful molecular genetics. For example, *C. elegans* holds the distinction of being the first metazoan to have its genome completely sequenced. In addition, germ-line transformation is readily accomplished through microinjection, enabling the creation of transgenics, including those with reporter genes. *C. elegans* has also proven susceptible to the phenomena of RNAi. By exposing animals to double-stranded RNA, an organismal response is triggered that mimics the null phenotype of the gene corresponding to that particular RNA [23]. Because all putative genes have been identified *in silico*, RNAi provides the opportunity to at least crudely determine the phenotypes resulting from inactivation of literally every *C. elegans* gene. In addition to these advantages, a soma consisting of fewer than 1,000 cells, all of which are postmitotic in adults, offers the opportunity to detect cumulative age-related cellular alterations [24, 25]. In addition to these biological advantages, there exists a general spirit of cooperativity and free exchange of information within the “worm community.” For example the *Caenorhabditis* Genetics Center at the University of Minnesota distributes a large number of strains free of charge. Finally, there are a number of Internet sites (most notably <http://elegans.swmed.edu>) that serve as excellent resources.

Regulation of Aging and Oxidative Stress by the Insulin/IGF-1-like Signaling Pathway

Studies in *C. elegans* have led to the identification of genes that have major effects on life span. These genes associate with the insulin/IGF-1-like signaling pathway. For example, *age-1* and *daf-2* extend life span by up to 100% [26, 27]. This increased life span is known as the Age phenotype. The *daf-2* gene encodes a protein of the mammalian insulin/IGF-1 like receptor and growth factor receptor [28]. DAF-2 activates a conserved phosphatidylinositol-3-OH kinase (PI3K)/3-phosphoinositide-dependent kinase-1 (PDK1)/Akt signaling pathway [29–36]. The *age-1* encodes a catalytic subunit of PI3K [29] and the

pdk-1 gene encodes a PDK1 homologue [30]. Mutations of *daf-16* and *daf-18* suppress the Age phenotype of *age-1* and *daf-2* mutations [37–41]. The *daf-16* gene encodes a forkhead transcription factor [31, 36]. The *daf-18* gene encodes a protein similar to the human PTEN protein that has phosphatidylinositol 3,4,5-triphosphate (PIP₃) 3-phosphatase activity [33, 34], thereby possessing antagonist actions to that to the *age-1* gene. *daf-2* acts cell non-autonomously and cell lineages included neurons are involved in life span regulation [42]. Wolkow *et al.* showed that the limiting expression of *daf-2* and *age-1* to neurons is sufficient to cause major effects on longevity [43]. DAF-16 might regulate the genes that are involved in the biosynthesis of a secondary signal that emanates from *daf-2*-expressing cells. On the other hand, the mutation of *daf-12* that encodes an orphan nuclear hormone receptor enhances the longevity of some *daf-2* alleles [37, 44]. The longevity induced by germ line ablation requires functional *daf-16* and *daf-12*, suggesting that the gonad also might affect aging [45].

Mutations of components in the insulin/IGF-1-like signaling pathway also confer resistance to heavy metals [46], heat stress [47], ultraviolet light (UV) [48], and ROS [49–52]. This suggests that the life span extension results from the coordinate over expression of stress-response genes [49]. *daf-16* mutation also suppresses the phenotype. Insulin/IGF-1-like signaling is thought to lead to phosphorylation of DAF-16 by AKT activity. DAF-2 pathway prevents DAF-16 accumulation in nuclei [53]. It is known that the DAF-16 protein is located in cytosol under an atmospheric condition, while it moves to nuclei under hyperoxia [54]. Recently, we also found that, even under an atmospheric condition, the DAF-16 protein remains in nuclei of *mev-1* mutant as shown below, which has abnormal energy metabolism and then overproduces ROS from mitochondria (unpublished data). These results suggest that energy metabolism is intimately related with oxidative stress and the *daf-16* gene is thought to regulate not only energy metabolism but also resistance to oxidative stress. Lin *et al.* have shown that both sensory neurons and germline activity regulate DAF-16 accumulation in nuclei, but the nuclear localization patterns are different [53]. This reveals the unexpected complexity of the DAF-16-dependent pathway that regulates aging and oxidative stress.

The current state of our understanding is summarized by Fig. 1.

Fig. 1. Insulin/IGF-1-like signaling pathway in nematode. P, phosphate; PIP₂, phosphatidylinositol 3,4-di-phosphate; PIP₃, phosphatidylinositol 3,4,5-triphosphate.

rized by Fig. 1.

Mitochondrial Contribution to Aging and Oxidative Stress

Many short-lived mutants from *C. elegans* have been isolated. It has been claimed upon occasion that short-lived mutants may not provide significant insights into the normal aging process. This concern is founded on the premise that, at least theoretically, mutations in a large number of genes can negatively impact life span in ways that do not contribute to normal aging. However, molecular characterization of short-lived *C. elegans* mutants (*e.g.*, *mev-1* and *gas-1*) indicates that this reservation has not been realized. Because these mutants exhibit normal patterns of aging, except at accelerated rates, and because they are affected by a known determinant of aging in wild-type organisms, namely oxidative stress, it seems reasonable to assume that their study will illuminate mechanisms important in normal aging as opposed to aberrant processes irrelevant to wild-type organisms.

The *mev-1* and *gas-1* genes encode different subunits in electron transport complexes in mitochondria. The energy metabolism in aerobic organisms is almost exclusively the result of glycolysis, the Krebs cycle and electron transport. With respect to electron transport, five membrane-bound complexes within mitochondria form the respiratory chain that sequentially transfers electrons through a series of

donor/acceptors, with O_2 as the final acceptor. Electrons enter the electron transport system through either complex I (NADH-CoQ oxidoreductase) or complex II (succinate-CoQ oxidoreductase), which are capable of using metabolic products of the Krebs cycle to start the flow of electrons in the respiratory chain. The electrons are transferred *via* two, single-electron reductions to CoQ (Coenzyme Q or ubiquinone), thereby reducing CoQ first to ubisemiquinone and then to ubiquinol (dihydrubiquinone). At complex III (CoQH₂-cytochrome *c* oxidoreductase), ubiquinol undergoes two sequential and spatially separate one-electron oxidations, a process called the Q cycle. These reducing equivalents are then transferred to cytochrome *c* and complex IV (cytochrome *c* oxidase). In the final step in the process, complex V (ATP synthase) synthesizes ATP.

Mutations in *gas-1* were isolated based upon their ability to confer hypersensitivity to volatile anesthetics such as halothane or diethyl ether [55]. We have recently determined that *gas-1* mutants are hypersensitive to hyperoxia in a temperature-dependent fashion and have a shorter life span. The *gas-1* encodes a subunit (Ip49 kDa) of complex I of the electron transport system [56]. The *mev-1* mutant was isolated based upon its hypersensitivity to the superoxide-generating chemical methyl viologen [57]. In addition to its methyl viologen hypersensitivity, *mev-1* mutants are oxygen-hypersensitive with respect to its effects on both development and aging. The *mev-1* mutation causes a decrease in life span depending on oxygen concentration [58]. In addition, *mev-1* mutants accumulate fluorescent materials and protein-carbonyl derivatives at significantly higher rates than do wild-type cohorts [24, 25]. Thus, the aging process in *mev-1* animals approximates that of wild type except for its precocious nature. The *mev-1* mutation has been identified as residing in the putative gene *cyt-1*, which is homologous to succinate dehydrogenase (SDH) cytochrome *b*₅₆₀ in complex II [59]. This results in a greater than 80% reduction in complex II activity in the mitochondrial membrane fraction. Complex II, which catalyzes electron transport from succinate to ubiquinone, is composed of SDH (a flavoprotein: Fp and iron-sulfur protein: Ip) and two other subunits (small and large subunits of cytochrome *b* as CYT-1).

The biochemical pathologies include elevated ROS present in *mev-1* mutants. Specifically, O_2^- levels in both intact mitochondrial and sub-mito-

chondrial particles are approximately two times greater in *mev-1* mutants than in the wild-type. The electron transport system is also the major endogenous source of ROS. It has been estimated that generation of the O_2^- and its dismutated product H_2O_2 may constitute as much as 1–2% of total electron flow, although others have placed this value at 0.1% [60]. It is known that oxygen is initially converted to the O_2^- by electrons leaked from complex I and mainly complex III [61–63]. This means that the *mev-1* mutation either exacerbates O_2^- production at this location or, in some indirect way, increases O_2^- production at another point in electron transport, perhaps even at complex II.

A second important biochemical pathology is that of reduced glutathione (GSH) concentration in *mev-1* animals. GSH is an important ROS scavenger, and its reduced levels in *mev-1* likely exacerbates the deleterious consequences of increased O_2^- production described immediately above. Third, a number of biochemical pathologies likely derive from the role played by SDH in the Krebs cycle. 1) The ratio of lactate to pyruvate is significantly higher in *mev-1* mutants, suggesting that a metabolic imbalance known as lactate acidosis occurs in these animals. 2) A number of Krebs cycle intermediates are present at abnormal concentrations in *mev-1* mutants. Finally, a loss of mitochondrial membrane potential ($\Delta\psi_m$) and alteration of CED-9 and CYT-1 protein levels under hyperoxia were observed [64]. As a result, ultra-structural abnormalities of mitochondria have been noted in *mev-1* animals, particularly those reared under hyperoxic conditions. The abnormality causes apoptosis or programmed cell-death, in *C. elegans*. Hyperoxia induces massive apoptosis in *mev-1* animals, particularly in embryos. Even under atmospheric oxygen apoptosis is significantly elevated in a *mev-1* genetic background. In all likelihood these cellular pathologies are precipitated under the increased oxidative stress, biochemically manifested as both increased ROS and reduced GSH as well as the various metabolic imbalances. Figure 2 shows the summary of the pleiotropic phenotypes of *mev-1*.

At the organismal level, *mev-1* mutants are hypersensitive to oxidative stress in terms of both development and aging. In addition, the developmental hypersensitivity and life span shortening are likely, at least in part, due to the supernumerary apoptosis. Finally, the observation that SOD/catalase mimetics can extend the life span of *mev-1* to wild-type duration provides further evidence of the important role

Fig. 2. Summary of the pleiotropic phenotypes caused by the *mev-1* mutation.

of ROS and their sequelae in determining organismal life span [65].

On the other hand, in addition of longer life span, mutations in *clk-1* exhibit pleiotropic phenotypes that have a temporal component, including in the cell cycle, embryonic development, postembryonic development, behavioral rhythms (e.g., swimming, pharyngeal pumping, and defecation) and fertility [66]. The *clk-1* gene has been characterized and found to be conserved among eukaryotes, including humans and rodents, and was identified as a homologue of the gene of *Saccharomyces cerevisiae* COQ7/CAT5 located in the inner membrane of yeast mitochondria [67]. The COQ7/CAT5 is necessary for the biosynthesis of CoQ in yeast. As CoQ acts in electron transport, fatty acid oxidation, and uridine synthesis, yeast *coq7/cat5* mutants, which lack CoQ₆, are unable to grow on nonfermentable carbon sources. In contrast to the situation in yeast, which is defective in respiratory growth, *C. elegans clk-1* mutants are able to respire almost normally. In fact, the metabolic capacities and the ATP levels of adult *clk-1* mutants are unchanged or even higher than those of the wild type [68], and the mitochondria of *clk-1* mutants exhibit succinate-cytochrome *c* reductase activity that is comparable with that of wild-type mitochondria [69]. These observations suggest that CLK-1 is not exclusively involved in CoQ biosynthesis in *C. elegans*. When *clk-1* mutants were cultured on an *E. coli* mutant that lacks CoQ, they displayed early developmental arrest from eggs, or sterility emerging from dauer stage. Provision of CoQ-replete *E. coli* rescued these defects [70]. It was found that *clk-1* mutants lack the nematode CoQ₉ isoform and instead contain a large amount of a metabolite that is slightly more polar than CoQ₉ and

have increased levels of CoQ₈, the *E. coli* isoform and reodoquinone-9. CoQ could be supplied from wild-type *E. coli* as a food. Recently, we found that: i) CoQ biosynthesis is dramatically altered in *clk-1* mutants of which the mitochondria do not possess the detectable levels of CoQ₉, and instead contain a CoQ biosynthesis intermediate, demethoxy coenzyme Q (DMCoQ₉); and ii) DMCoQ can functionally replace CoQ to maintain active respiration in *clk-1* mutant mitochondria, despite the absence of CoQ₉ [71].

How do these biochemical data translate to the organismal phenotype of longer life span? This is likely related to the fact that *clk-1* mutants also have an increased resistance to stress induced by UV-irradiation, heat, and oxidative stress [48]. This implies that the chemical properties of the Q semiquinone produced from DMCoQ₉ reduce O₂⁻ concentrations and consequently oxidative stress. This is supported by the recent observation by Stenmark *et al.* [72] that the *clk-1* gene encodes a DMCoQ hydroxylase.

Conclusion

There are many exogenous and endogenous factors that affect aging. In these, ROS is thought to be an extremely important factor. ROS are continuously produced in living organs as byproducts of normal energy metabolism. It is worth emphasizing that the majority of mutations in *C. elegans* that affect life span, including those that lengthen it, probably do so by modulating oxidative stress. We have presented evidence that both *gas-1* and *mev-1* mutants are hypersensitive to oxidative stress, which likely contributes to or is a primary cause of their precocious aging. Both mutants are characterized by O₂⁻ overproduction in mitochondria and hypersensitivity to oxidative stress. Conversely, most long-lived mutants appear significantly more resistant to oxidative stress than the wild type. Moreover, a strong inverse correlation has been demonstrated between life span and metabolic rate in long-lived mutants of *C. elegans* [73]. Thus, it appears that the mutations that lower metabolism confer longevity probably due to reducing oxidative stress. Given the central role of energy metabolism including mitochondrial functions in perpetrating oxidative stress, it seems clear that the mutants such as *age-1*, *daf-2*, and *mev-1* will continue to yield insightful views into the mechanism of aging.

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