

Cholesterol-producing transgenic *Caenorhabditis elegans* lives longer due to newly acquired enhanced stress resistance

Eun-Young Lee^a, Yhong-Hee Shim^b, David J. Chitwood^c, Soon Baek Hwang^d, Junho Lee^d, Young-Ki Paik^{a,*}

^a Department of Biochemistry, Bioproducts Research Center and Yonsei Proteome Research Center, Yonsei University, 134 Shinchon-dong, Sudaemoon-ku, Seoul 120-749, Republic of Korea

^b Department of Biological Science and BioMolecular Informatics Center, Konkuk University, 1 Hwayang-dong, Gwangjin-ku, Seoul 143-701, Republic of Korea

^c Nematology Laboratory, USDA, ARS, Bldg. 011A, Room 165B, BARC-West, Beltsville, MD 20705, USA

^d Department of Biological Sciences, Seoul National University, 56 Silim-dong, Kwanak-Ku, Seoul 151-742, Republic of Korea

Received 31 December 2004

Available online 25 January 2005

Abstract

Because *Caenorhabditis elegans* lacks several components of the de novo sterol biosynthetic pathway, it requires sterol as an essential nutrient. Supplemented cholesterol undergoes extensive enzymatic modification in *C. elegans* to form other sterols of unknown function. 7-Dehydrocholesterol reductase (DHCR) catalyzes the reduction of the Δ^7 double bond of sterols and is suspected to be defective in *C. elegans*, in which the major endogenous sterol is 7-dehydrocholesterol (7DHC). We microinjected a human DHCR expression vector into *C. elegans*, which was then incorporated into chromosome by γ -radiation. This transgenic *C. elegans* was named *cholegans*, i.e., cholesterol-producing *C. elegans*, because it was able to convert 7DHC into cholesterol. We investigated the effects of changes in sterol composition on longevity and stress resistance by examining brood size, mean life span, UV resistance, and thermotolerance. *Cholegans* contained 80% more cholesterol than the wild-type control. The brood size of *cholegans* was reduced by 40% compared to the wild-type control, although the growth rate was not significantly changed. The mean life span of *cholegans* was increased up to 131% in sterol-deficient medium as compared to wild-type. The biochemical basis for life span extension of *cholegans* appears to partly result from its acquired resistance against both UV irradiation and thermal stress.

© 2005 Elsevier Inc. All rights reserved.

Keywords: Aging; 7-Dehydrocholesterol reductase; Life span; Longevity; Oxidative stress; Cholegans

Sterols are important constituents of the plasma membranes of eukaryotic cells. Cholesterol, which is found predominantly in animal cells, influences membrane permeability and the fluidity of the lipid bilayers. Data accumulated in recent years demonstrate that cholesterol is not only a membrane structural component but is also actively involved in modulation of cell signaling [1]. Fungi, plants, and mammals have complex sterol

biosynthetic pathways, with more than 39 enzymatically catalyzed reactions. In contrast, nematodes such as *Caenorhabditis elegans* cannot synthesize sterols de novo [2]. *C. elegans* does express homologues of the enzymes up to the point of farnesyl pyrophosphate biosynthesis, a major branching point for the biosynthesis of other major isoprenoids [3]. Therefore, in order to culture *C. elegans* in the laboratory, agar plates must be supplemented with cholesterol or other sterols, as nematodes can modify many externally added sterols by transforming them into sterols permissible for nematode growth.

* Corresponding author. Fax: +82 2 393 6589.

E-mail address: paiky@yonsei.ac.kr (Y.-K. Paik).

Only the final products of the plant and mammalian biosynthetic pathways such as ergosterol, sitosterol, cholesterol or closely related sterols or their precursors can fully support worm growth. However, when one of the steps converting these sterols (e.g., sitosterol) to cholesterol is blocked by an inhibitor (e.g., azacoprostane), there are serious defects in egg laying, development, and growth [4]. A mammalian counterpart to this phenomenon occurs in the case of the enzyme 7-dehydrocholesterol reductase (DHCR), which biosynthesizes cholesterol from its immediate precursor, 7-dehydrocholesterol (7DHC). The accumulation of 7DHC in human fetuses deficient in DHCR leads to a morphological disorder termed the Smith–Lemli–Opitz syndrome. Thus, 7DHC does not seem to fulfill the cholesterol requirement of the developing embryos in humans and exerts an additional embryotoxic effect, probably via oxidized byproducts [5]. In contrast to mammals where cholesterol is the major sterol, *C. elegans* contains 7DHC as its major sterol [2].

Caenorhabditis elegans has proven to be an excellent experimental system for dissection of the aging process, which is modulated by complex genetic regulation (e.g., *age-1*, *daf-2* genes). Aging is associated with an accumulation of molecular and cellular damage that correlates with a decline in normal physiological function. Where tested, all known *C. elegans* longevity mutants exhibit increased resistance to environmental stress such as oxidative stress (e.g., hydrogen peroxide, paraquat), heat shock, UV irradiation, and heavy metal stress, which result in the decline of cellular homeostasis [6,7]. It is not known whether enhanced resistance to stress is required for extended life span in *C. elegans*, but various interventions that enhance stress tolerance result in longevity.

Despite the possible importance of sterols in stress tolerance in *C. elegans*, either as biophysical modulators of membrane fluidity or as precursors to bioregulatory steroids, physiological studies on the role of cholesterol in the aging of *C. elegans* have not been reported. Therefore, we attempted to create a transgenic cholesterol-producing *C. elegans*, termed *cholegans*, and examined changes in behavior, reproductive activity, and longevity. Our results with this transgenic strain indicate that cholesterol plays an important role in *C. elegans* longevity apparently by imparting stress resistance.

Materials and methods

Nematode strains and culture. Wild-type *C. elegans* (Bristol strain, N2) was provided by the *Caenorhabditis* Genetics Center. Worms were routinely grown at 20 or 25 °C on 50-mm-diameter nematode growth medium (NGM) agar plates containing *Escherichia coli* (OP50) as a food source [8]. For aging experiments, worms were grown in three different media: cholesterol (5 µg/ml) supplemented medium (CSM), 7DHC supplemented medium (DSM), and sterol depletion medium (SDM), prepared as previously described [9].

DNA transformation in *C. elegans*. DNA transformation in wild-type was achieved by inserting plasmid DNA into the distal arm of the hermaphrodite gonad as described [10]. The 1.5 kb *myc*-tagged human DHCR (hDHCR) cDNA fragment [11] was directionally cloned into expression vector pPD49.78, which has the *hsp* 16.41 small heat shock promoter and no enhancer (Fig. 1A). The sequence of the resulting cloned plasmid was confirmed by automated dye terminator, dideoxy sequencing (ABI Prism 377 Sequencer, Applied Biosystems, Foster City, CA). The hDHCR expression construct and the plasmid pRF4 containing a dominant genetic marker were microinjected into the gonads of wild-type at a final concentration of 100 ng/µl. Integration of the extrachromosomal arrays was achieved by γ -irradiation of transformants with γ -rays of 4000 Rad (15 R/s) from a ^{60}Co source. Two strains had stably integrated chromosomal transgenes: these worms were backcrossed with wild-type males to exclude the rare possibility of random mutation.

Northern blot analysis. Total RNA extracted from *cholegans* was separated on a 1.0% (w/v) agarose gel in 30% (v/v) formaldehyde and transferred to nylon membranes followed by immobilization with UV-cross-linking. hDHCR cDNA probe was labeled with [α - ^{32}P]dCTP by random-priming. After hybridization (42 °C, 50% (v/v) formamide), the membranes were washed with 0.1× SSC containing 0.1% SDS at 65 °C. Hybridization signals were visualized by autoradiography using

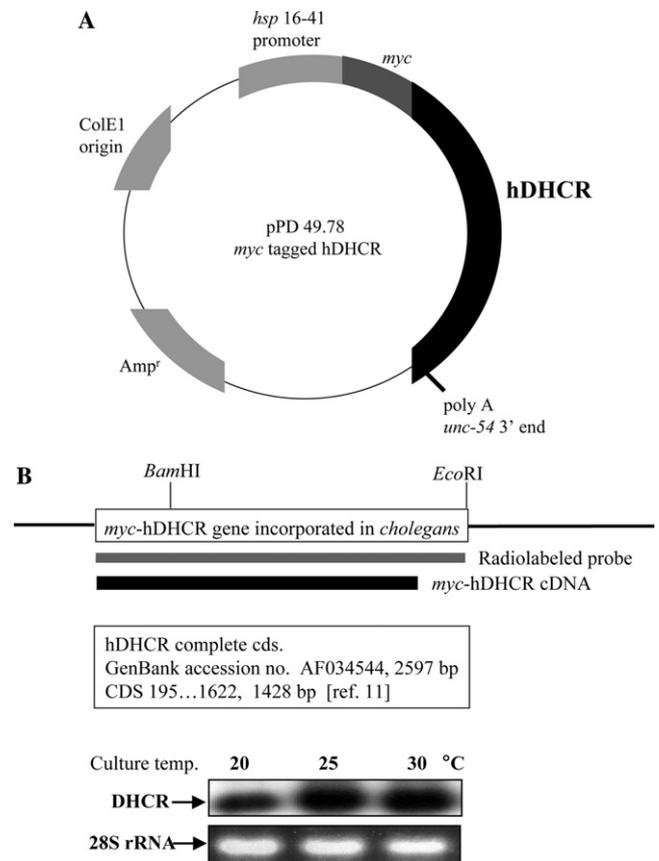


Fig. 1. (A) Expression vector of the recombinant *myc*-tagged hDHCR. The 1.5 kb *myc*-tagged human DHCR cDNA fragment was directionally cloned into the expression vector pPD 49.78 (3.8 kb), which has *hsp* 16.41 small heat shock promoter and no enhancer. (B) The radiolabeled hDHCR cDNA was hybridized to total RNA extracted from *cholegans*. After incubation at 20 or 25 °C daily, or 30 °C for 2–6 h, total RNA was prepared from the worms, and 20 µg of RNA was subjected to Northern blot analysis. hDHCR mRNA levels in this figure were normalized to the quantity of 28S rRNA expression.

Hyper X-ray film with intensifying screens at -70°C and normalized to the quantity of 28S rRNA expression.

Measurement of life span. For synchronous culture, eggs were collected by using sodium hypochlorite and allowed to hatch by incubation overnight at 20°C in S medium (0.05 M potassium phosphate buffer, pH 6.0, 0.1 M NaCl, and $5\ \mu\text{g}/\text{ml}$ cholesterol). Newly hatched L1 larvae were cultured on NGM agar plates. To prevent progeny production, 5-fluoro-2'-deoxyuridine (FuDR, Sigma, St. Louis, MO) was added at a final concentration of $40\ \mu\text{M}$ after worms had reached adulthood [13]. To measure survival and mean life span (MLS), worms were counted every day by eliminating dead ones. Death was recognized as a loss of spontaneous movement and lack of response to touch with a pick.

Brood size assays. The effects of cholesterol production on brood sizes and early larval arrest were examined at 20°C . Gravid adults ($n = 10\text{--}20$) were allowed to lay eggs for 1 h and then were removed. The resulting synchronous population was raised until the late L4 stage. Ten animals were placed singly on NGM plates with or without cholesterol, and these P_0 animals were transferred to a new plate every 24 h until the end of the reproductive period. Each brood plate was examined daily to follow development to a terminal phenotype. Any adult or L4 larva was counted and removed. Brood sizes of F3 progeny were assayed by the same procedure as described [14].

UV resistance. About 20 adult hermaphrodites were picked 3–6 days after hatching and were irradiated on NGM agar without *E. coli* using a germicidal bulb (254 nm) at $20\ \text{J}/\text{m}^2/\text{min}$ in a UV Stratalinker apparatus (Stratagene, California, USA), followed by transfer to NGM plates with *E. coli*. Liquid medium was not used in order to avoid the absorption and reflection by the liquid that would have affected the dosage. All UV-resistance assays were performed at 20°C and replicated blindly. An adult was scored as dead when it did not respond to a mechanical stimulus [6].

Thermotolerance. Four-day-old adult hermaphrodites were placed on small prewarmed (35°C) NGM plates and incubated at $35 \pm 0.5^{\circ}\text{C}$. At the end of incubation times ranging from 1 to 9 h, plates were removed and worms were examined for mobility, response to provoked movement, and pharyngeal pumping. Worms failing to display any of these traits were scored as dead. The mean survival rate (MSR, time of 50% survival) was determined [17]. As a control, adult hermaphrodites were incubated at 35°C until all worms were dead.

Catalase and superoxide dismutase activity. Catalase activity was determined spectrophotometrically by monitoring the disappearance of hydrogen peroxide at 240 nm. Superoxide dismutase (SOD) activity was determined by monitoring the inhibition of autooxidation of 6-hydroxydopamine [18]. Protein concentration was determined by the Bio-Rad assay using bovine serum albumin as a protein standard.

Preparation of membrane fractions. Worms were harvested from liquid culture and washed repeatedly in S medium to remove bacteria. Washed worms were collected by centrifugation at $1500g$ for 10 min. The pellets were resuspended in two volumes of reaction buffer (0.1 M potassium phosphate buffer, pH 7.4, 1 mM reduced glutathione, 0.5 mM EDTA, and 20% glycerol v/v) containing a proteinase inhibitor cocktail tablet (Boehringer Mannheim GmbH, Penzberg, Germany) and were disrupted with a Teflon homogenizer. The homogenate was centrifuged at $1500g$ for 10 min at 4°C to remove cuticles and large debris. The supernatant (whole homogenate: WH) was then spun at $105,000g$ for 1 h. The supernatant (cytosol) at $105,000g$, the pellets (microsomes) containing the membrane fraction and mitochondrial fraction, resuspended in reaction buffer, were used as the hDHCR enzymic source [19]. To obtain an intact mitochondrial fraction, we used the Mitochondria Isolation Kit (Sigma).

DHCR enzyme assays and sterol analysis by gas-liquid chromatography (GLC). Ergosterol or lathosterol (dissolved in Tween 80 at a weight ratio of 70:1, detergent:sterol) was added at a final concentration of $300\ \mu\text{M}$ to 1 ml of 20% (w/v) glycerol, 0.1 M Tris-HCl, pH 7.4, 1 mM glutathione, 0.5 mM EDTA, 2 mM NADPH, 140 mM glucose, and 20 U glucose oxidase that had been preincubated under N_2

atmosphere for 4 min [9]. Incubation at 20 or 37°C with 0.5–2.0 mg of microsomal protein of *C. elegans* was carried out anaerobically in sealed glass flasks and terminated by the addition of 1 ml methanolic KOH followed by heating under reflux for 10 min [20]. Sterols were extracted and processed for analysis as described [21]. Enzymatic activity was calculated from the relative amounts of substrate and product in incubated samples compared with unincubated controls.

Western blotting of DHCR in C. elegans. Protein (20 μg) from WH or microsomal fractions from worms expressing the *myc*-tagged DHCR cDNA was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and then electrophoretically transferred to a polyvinylidene difluoride membrane. The blot was immunostained with anti-*c-myc* antibody and analyzed with the ECL Western blotting detection system (Amersham–Pharmacia Biotech, Uppsala, Sweden) using a horseradish peroxidase-conjugated secondary antibody [22].

Results

Creation and biochemical validation of transgenic cholegans

To create a transgenic *C. elegans* that can produce cholesterol (*cholegans*), first we obtained a transient line containing the transgene hDHCR on extrachromosomal arrays, termed *cholegans*-transient, which was identified by observation of the roller phenotype. To prepare a stable line containing hDHCR integrated into the host chromosome, the *cholegans*-transient line was irradiated with γ -rays, and a *cholegans*-stable (thereafter referred to simply as *cholegans*) line was selected after the 6th generation of progeny and used for our experiments. The stable line exhibited 100% transmission of both roller markers and the presence of the chromosomal-localized hDHCR gene [12], as confirmed by Southern blotting, suggesting that the *myc*-hDHCR transgene was successfully integrated into *cholegans* (data not shown). Because hDHCR construct had an *hsp* 16.41 promoter, we were concerned about whether hDHCR was expressed well at 20°C . Our Northern blot data (Fig. 2B) demonstrated that hDHCR mRNA is expressed in a temperature-dependent manner, confirming the function of *hsp* 16.41 promoter in the expression vector. Transgenic worms expressing hDHCR (e.g., *cholegans*, *age-1* (*hx546*)) showed similar properties as those of wild-type in many aspects: body size, dauer formation rate, and time required for larval development at each stage (data not shown).

To validate the expression of recombinant *myc*-tagged hDHCR in *cholegans*, whole cell lysates of the transgenic lines carrying hDHCR were analyzed for expression of the transgene by Western blotting, enzymic assays, and GLC analysis. First, to determine the steady state level of exogenously introduced hDHCR expressed in *cholegans*, Western blot analysis was carried out using cell homogenates obtained from *cholegans* that contain integrated *myc*-tagged hDHCR cDNA in

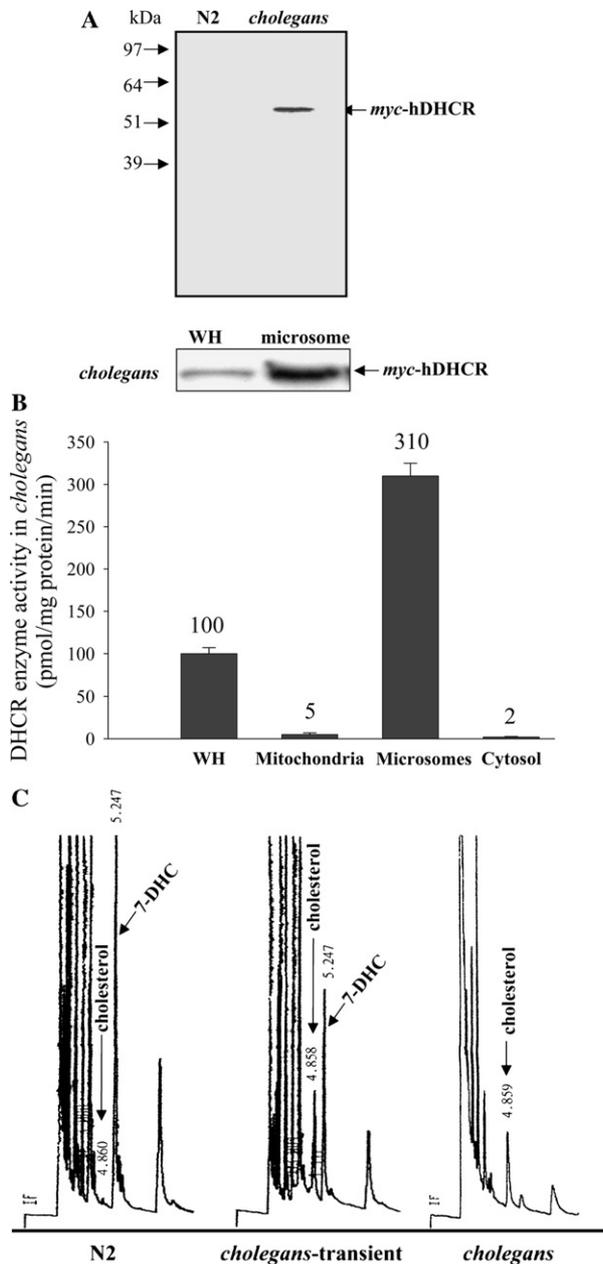


Fig. 2. Validation of the recombinant *myc*-tagged human 7DHCR expressed in *cholegans*. (A) Western blot analysis of recombinant hDHCR expression. About 20 μ g of whole homogenate (WH) or microsomal proteins from *cholegans* was blotted and then immunostained with c-myc antibody, which was analyzed with the ECL Western blotting detection system using a horseradish peroxidase-conjugated secondary antibody. (B) Subcellular distributions of DHCR. Enzyme activity was located almost exclusively in microsomes. The lower levels of activity in WH and mitochondria probably reflect small amounts of microsomes in these fractions. Little DHCR activity was detected in the cytosolic fraction. Each value represents the mean of triplicate determinations (\pm SD) obtained from two separate experiments. (C) Comparison of typical GLC chromatograms of the major endogenous sterols present in wild-type and transgenic *C. elegans*. Wild-type, *cholegans*-transient, and *cholegans* were grown at 20°C on 100-mm NGM agar plate with 5 μ g/ml cholesterol and analyzed by GLC. Experiments were replicated at least three times. Because of the very small amount of sterols in worm membranes, over 2 g worms were required for each GLC analysis.

the host chromosome. As anticipated, a 55 kDa recombinant protein was detected by an antibody against the *myc* protein in Western blotting, confirming the correct expression of the *myc*-tagged hDHCR protein (Fig. 2A). Second, having successfully established the DHCR expression system in *cholegans*, we examined the subcellular distribution of DHCR by analyzing the enzyme activity of whole homogenates (WH), mitochondria, microsomes, and cytosol (Fig. 2B). As in mammals [19], DHCR activity was mainly present in the microsomal fractions, although low levels of activity were also found in WH, indicating that exogenous hDHCR is localized in membranous organelles. There is a difference in DHCR specific activities between rat liver and *cholegans* microsomes; the former containing usually 450–500 pmol/mg/min while the latter contained 100–300 pmol/mg/min. Third, to examine whether transgenic worms can convert endogenous 7DHC to cholesterol, we compared the presence of endogenous cholesterol in wild-type and *cholegans* (Fig. 2C). The GLC chromatograms clearly demonstrated that *cholegans* converted 7DHC (rt. = 5.247 min, rrt. to cholesterol = 1.08, m/z = 384) to cholesterol (rt. = 4.858 min, m/z = 386) efficiently. Note that there was a huge difference between *cholegans*-transient (>40%) and *cholegans* (>80%) in terms of cholesterol conversion (Fig. 2C). The major sterol of *cholegans* detected by GLC was found to be cholesterol whether the worms were grown in the presence of 7DHC or cholesterol in the agar plate. Taken together, these results indicate that hDHCR cDNA was successfully integrated in *cholegans* and active enough to convert most of the detectable endogenous 7DHC to cholesterol.

Biological properties and characteristics of *cholegans*

The first observation we made from *cholegans* was its extended life span that was dependent on the sterol-supplemented condition. When we calculated mean life span (MLS) values from survival curves of nematodes grown in sterol-supplemented vs. sterol-depleted medium, the MLS of wild-type, *cholegans*, and *age-1* in cholesterol supplemented medium (CSM) were 21, 24, and 34 days, respectively (Table 1). However, when worms were grown on sterol depletion medium (SDM), *cholegans* lived at least 31% longer than the control wild-type animals ($n = 20$) (21 days vs. 16 days), and even 10.5% longer than the *age-1* mutant (21 days vs. 19 days). When grown in the 7DHC plate (5 μ g/ml) ($n = 30$), MLS of F2 *cholegans* was 19.6 ± 2.8 days, 24% longer than that of wild-type (19.6 days vs. 15.8 days) (data not shown), indicating that extended life span of *cholegans* in 7DHC supplemented medium (DSM) is essentially similar to that in SDM.

Second, we found differences in average brood size of progeny among the three strains ($n = 20$): 252 ± 22

Table 1
Changes in physiological characteristics of transgenic *C. elegans* in response to sterols

Strain	NGM (\pm)cholesterol 5 μ g/ml	Mean life span ^a (\pm SD, days) $n = 20$	Brood size ^b (mean \pm SD) $n = 20$	Adult number (mean \pm SD) $n = 20$	Percent development ^c (egg \rightarrow adult)
N2	+	21 \pm 5.1	252 \pm 22	229 \pm 14	90.8 \pm 5.5
	–	16 \pm 1.2	119 \pm 10	76 \pm 6	63.9 \pm 5.0
<i>Cholegans</i>	+	24 \pm 3.6	187 \pm 19	172 \pm 13	92.1 \pm 6.9
	–	21 \pm 4.1	108 \pm 7	93 \pm 9	86.4 \pm 8.3
<i>Age-1</i> (hx546)	+	34 \pm 2.5	153 \pm 17	140 \pm 13	91.5 \pm 8.5
	–	19 \pm 2.0	82 \pm 9	73 \pm 5	89.1 \pm 6.1

Each mean \pm SD was derived from three separate determinations.

^a To measure survival and mean life span (MLS) of F1 progeny in cholesterol supplemented medium (CSM) or sterol depletion medium (SDM), worms were counted every day by eliminating dead ones.

^b Brood sizes of F3 progeny in CSM or SDM plates are the total number of non-hatched and hatched eggs of each hermaphrodite, expressed as mean \pm SD ($n = 20$).

^c Percent development is the number of adults divided by the total number of eggs.

(wild-type), 187 \pm 19 (*cholegans*), and 153 \pm 17 (*age-1*) (Table 1). The reduction of brood size (\sim 40%) was also seen in the case of *age-1*, a long-lived mutant. Interestingly, percentage reductions in brood size were very similar (43–53%) among these strains when the growth conditions were shifted from CSM to SDM. However, in SDM there was also a clear difference in the percent development from eggs to adult among the three strains (wild-type: 90.8% [CSM] to 63.9% [SDM], *cholegans*: 92.1–86.4%, and *age-1*: 92–89.1%). The long-lived mutant *age-1* and *cholegans* showed similar percent development to adult (89.1% vs. 86.4%) in SDM, suggesting that other factors might exist besides sterol presence for development of eggs to adults. When worms were grown in SDM, *cholegans* had a substantially higher rate ($>$ 35.2%) of adult development from eggs (86.4 \pm 8.3%) as compared to wild-type (63.9 \pm 5.0%). This result suggests that both *cholegans* and *age-1* exhibit a clear advantage over wild-type in growth rate and longevity when faced with a harsh nutritional environment (e.g., SDM).

To examine whether extended life span in *cholegans* might correlate with stress resistance, we first determined mean survival rate (MSR) after the initial exposure of worms to increasing intensity of UV light with worms grown in CSM plates. We measured MSR of worms grown only in CSM, to exclude the possibility of change in MSR due to sterol composition in the media, which was already well established as described above. The worms of all three strains, wild-type N2, *cholegans*, and *age-1*, showed the same pumping rate per min during the measurement of MSR. The MSR of *cholegans* was very similar to that of *age-1* (4.17 days). The results from the UV-resistance assay at 20 °C showed that the MSR of both *cholegans* and *age-1* was almost twice that of wild-type (4.17 days [*cholegans*, *age-1*] vs. 2.38 days [wild-type]) (Fig. 3A). The *age-1* observation is consistent with that previously published [16]. Further examination of stress resistance was performed by measuring the thermotolerance of

wild-type, *cholegans*, and *age-1* against heat (35 °C) in CSM plates. *Cholegans* showed about an 18% higher MSR compared to wild-type [497 h vs. 420 h]. The longevity mutant *age-1* displayed a higher thermal tolerance that could not be calculated from the survival curve (Fig. 3B) [16,17]. Therefore, we tentatively conclude that production of cholesterol by DHCR might account for the observed decrease in lethality upon exposure of these transgenic worms to UV and elevated temperature.

Subsequently, we determined the relative activities of two representative antioxidant defense enzymes, catalase and SOD. The relative activities of each enzyme in *cholegans* were similar to those of wild-type regardless of developmental stages and nutritional conditions (e.g., for SOD in young adults, 4.7 \pm 0.5 U [wild-type] vs. 6.5 \pm 0.3 U [*cholegans*]), suggesting negligible influence of cholesterol production on these defense enzymes (data not shown).

Discussion

In this report, we demonstrate the first example of transgenic *C. elegans* that produces cholesterol, a line that we have named *cholegans*. It is important to note that *cholegans* and wild-type are both incapable of de novo sterol biosynthesis; *cholegans*, however, possesses the enzyme that converts 7DHC (typically the major sterol of *C. elegans*) to cholesterol. Interestingly, *cholegans* acquired an ability to resist stress, perhaps caused by the change of endogenous sterol composition from 7DHC to cholesterol. Despite the lack of difference between wild-type and *cholegans* in general behaviors and morphology, the change in sterol composition appeared to affect growth, development, reproduction, and longevity of *C. elegans*. Although the phenotypic differences are about 30% between the wild type and *cholegans*, it is a statistically meaningful and reproducible difference in longevity extension. Among the transgenic lines made

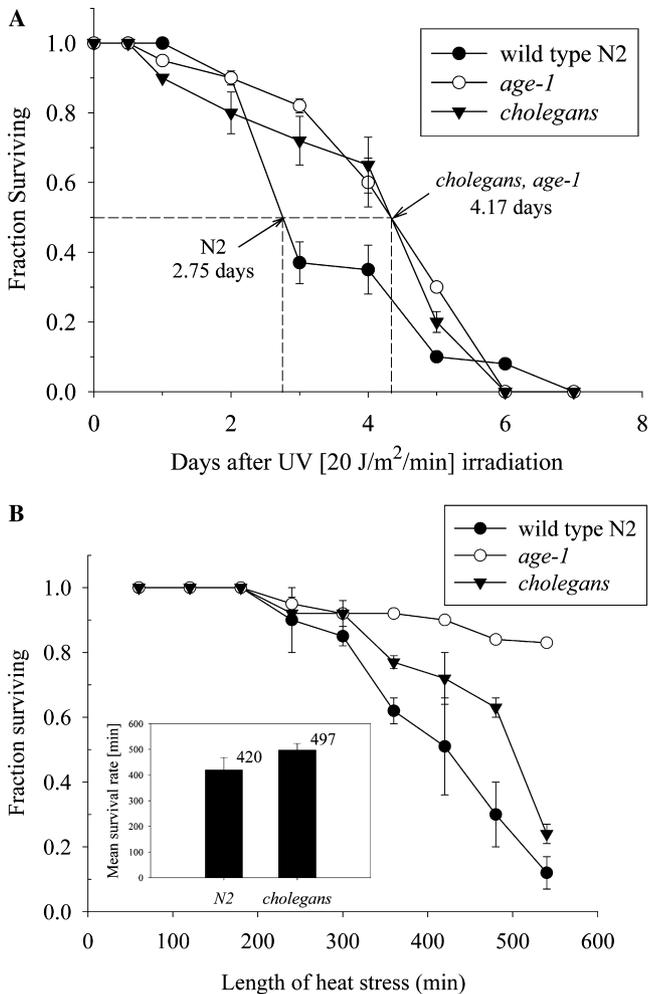


Fig. 3. Stress resistance of *cholegans*. (A) Increased UV resistance of *cholegans* after UV irradiation. Survival of the *cholegans* and *age-1* strains was significantly longer than wild-type. Survival immediately after UV irradiation at 20 J/m²/min did not differ significantly between *cholegans* and wild-type. Each value represents the mean of triplicate determinations (\pm SD) with 20 adult hermaphrodites of each strain. (B) Thermotolerance of *cholegans*. The fraction of surviving worms was scored during 35 °C thermal stress of six plates containing 10 animals of each strain. Each data point represents the mean fraction of live worms from three plates at each time point. Inset; the survival time of each strain is shown in mean \pm SD: wild-type, 420 \pm 48 min; *cholegans*, 497 \pm 26 min.

by γ -radiation, we found that some of the lines were not able to convert 7-dehydrocholesterol to cholesterol, suggesting that γ -radiation may create random integration of DHCR gene into the host chromosomes. The transgenic lines we selected were clearly stable strains which consistently express DHCR activity when tested.

In particular, the increase in MLS of *cholegans* may be due to enhanced stress resistance when the worms are faced with adverse environments such as UV and heat. Resistance to thermal stress has previously been shown to be related to longevity in *C. elegans* [23]. We speculated that production of cholesterol may lead to

protect *cholegans* against damage by UV or heat, perhaps by promoting the formation of oxidation products of cholesterol less toxic than the primary free radicals formed by oxidative stresses, as seen in mammals [24].

As a step toward understanding the specific biological roles of cholesterol in *C. elegans*, we investigated a mechanistic connection between stress resistance and longevity. Our survival experiments demonstrate that DHCR protects transgenic worms from damage generated by UV and heat. Apparently, SOD and catalase are not much involved in the DHCR-mediated stress resistance in *cholegans*. In the process of stress resistance, the presence or absence of sterol in the media affects significantly the changes in the MLS of worms. For example, F1 *cholegans* in sterol depletion medium (SDM) showed a 20–30% longer MLS than wild-type, but this difference was only 14% when worms were grown in cholesterol plates. This increased MLS of *cholegans* in SDM was even more prominent as the generations passed. That is, in the F4 generation, MLS of *cholegans* was almost double of wild-type 2 (10 days [wild-type] vs. 18 days [*cholegans*]). Interestingly, the MLS of wild-type grown in cholesterol was longer than wild-type grown in 7DHC in subsequent generations (e.g., F4). For UV and thermotolerance studies, it was very difficult to measure MSR because wild-type did not tolerate these kinds of stresses well when grown in SDM. We also wanted to eliminate the possible cause of changes in MSR by switching the media because differential effects of sterol composition on nematode biological processes have already been demonstrated.

The specific mechanism through which *age-1* enhances UV resistance is unknown. In the case of *cholegans*, we can only note that cholesterol may protect against UV-derived mitochondrial DNA damage, as previously reported in mammalian brains [24]. The brood sizes of all strains tested were reduced in SDM, which may result from the fact that embryogenesis did not progress well under sterol deficiency, regardless of the ability to produce cholesterol from 7DHC. One possible explanation for the thermotolerance of *cholegans* is that when worms were exposed to heat, the *hsp* promoter certainly directed the expression of DHCR cDNA, mostly in the L1 and L2 larval stages (data not shown), which then caused an increase in cholesterol production from 7DHC. As a result, membrane rigidity in localized microdomains may have also increased, which may be advantageous to survive under heat stress. Alternatively, a cholesterol-derived steroid of unknown identity could be involved in other responses to heat.

When sterol metabolism-related gene sequences were searched in the *C. elegans* genomic database, only two mRNA clones, GenBank Accession Nos. NM063363 and NM063362, were found as members of the sterol desaturase family; they are expressed at low levels. The sterol 5-desaturase family also contains sterol 4-methyl

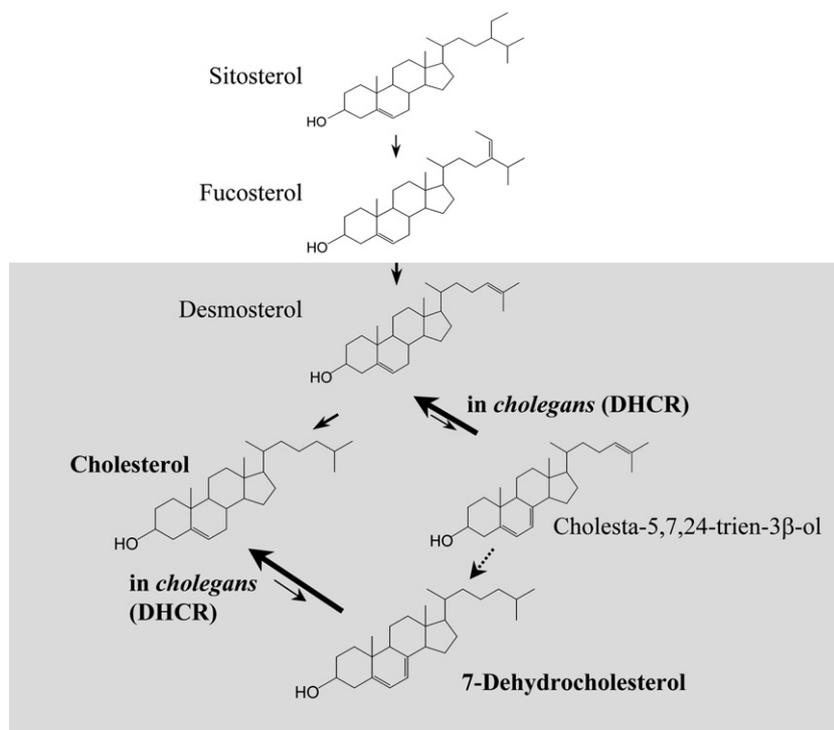


Fig. 4. Proposed pathway of sterol transformation in *cholegans* with acquired DHCR activity. Despite the lack of enzymes for de novo sterol synthesis, nematodes can modify externally added yeast, plant or mammalian sterols by introducing double bonds, reducing or isomerizing them, and dealkylating the side chain [15]. Arrows long and bold shape indicate that endogenous sterols in *cholegans* are converted from 7DHC to cholesterol.

oxidases. Although we expected that sterol 7-desaturase activity would be very prominent in wild-type in order to convert exogenous or endogenous cholesterol to 7DHC, we were not able to detect any activity by enzyme assays. In mammals, 7DHC and its oxidized products are embryotoxic [5] and perhaps adversely interact with proteins or enzymes containing a sterol-sensing domain [25]. In *cholegans*, we believe that the transgenic DHCR produces cholesterol from 7DHC, consequently overriding the activity of the existing sterol 7-desaturase (Fig. 4), but conclusive evidence remains to be established.

There seems to be a correlation between sterol structure and the MLS of *cholegans*. For example, when worms were grown in sitosterol or stigmasterol, both having an ethyl group at C-24, *cholegans* showed a 10% longer MLS in the F2 generation than lanosterol-supplemented worms. A similar MLS was observed in the worms grown in either 7DHC or ergosterol, as compared to wild-type grown in cholesterol (data not shown).

Cholegans and wild-type worms grown in lanosterol showed the same MLS pattern as worms grown under SDM conditions, suggesting that *C. elegans* does not uptake or metabolize lanosterol for further use. The three bulky methyl groups at C-4 α , 4 β , and 14 α might result in poor uptake or subsequent metabolism. For example, when worms were grown in the presence of miconazole

(0.1, 1.0, and 10 mM) plus lanosterol or azacoprostane plus sitosterol [4], the MLS of wild-type and *cholegans* was similar to that of wild-type and *cholegans* grown in SDM (data not shown), suggesting that *C. elegans* lacks sterol 4- or 14-demethylase.

In summary, the biochemical basis for the extended life span of *cholegans* may be due to two factors: first, the readily available cholesterol possesses a protective function against extreme stresses, either by a biophysical interaction in membranes or by the production of cholesterol metabolites that interact with other molecules. Second, DHCR overexpression in *cholegans* may stimulate uptake of sterol that is present in extremely low amounts in SDM and *E. coli*, or promote translocation of sterol within the nematode. Both aspects of the cholesterol-associated enhancement of longevity in *C. elegans* warrant further investigation.

Acknowledgments

This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea (03-PJ10-PG6-GP01-002 to Y.K.P.), and by a grant of the Basic Research program of the Korea Science and Engineering Foundation (R04-2002-000-20096-0 (2004) to Y.H.S.), Seoul, Korea. Nematode strains used in this work were provided by

the *Caenorhabditis* Genetics Center, which is funded by the National Institutes of Health Center for Research Resources.

References

- [1] P.M. Lewis, M.P. Dunn, J.A. McMahon, M. Logan, J.F. Martin, B. St-Jacques, A.P. McMahon, Cholesterol modification of sonic hedgehog is required for long-range signaling activity and effective modulation of signaling by Ptcl, *Cell* 105 (2001) 599–612.
- [2] D.J. Chitwood, W.R. Lusby, R. Lozano, M.J. Thompson, J.A. Svoboda, Novel nuclear methylation of sterols by the nematode *Caenorhabditis elegans*, *Steroids* 42 (1983) 311–319.
- [3] T.V. Kurzchalia, S. Ward, Why do worms need cholesterol?, *Nat. Cell Biol.* 8 (2003) 684–688.
- [4] B.K. Choi, D.J. Chitwood, Y.K. Paik, Proteomic changes during disturbance of cholesterol metabolism by azacoprostan treatment in *Caenorhabditis elegans*, *Mol. Cell Proteomics* 2 (2003) 1086–1095.
- [5] W. Gaoua, F. Chevy, C. Roux, C. Wolf, Oxidized derivatives of 7-dehydrocholesterol induce growth retardation in cultured rat embryos: a model for antenatal growth retardation in the Smith–Lemli–Opitz syndrome, *J. Lipid Res.* 40 (1999) 456–463.
- [6] Y. Honda, S. Honda, Oxidative stress and life span determination in the nematode *Caenorhabditis elegans*, *Ann. N.Y. Acad. Sci.* 959 (2002) 466–474.
- [7] S. Melov, J. Ravenscroft, S. Malik, M.S. Gill, D.W. Walker, P.E. Clayton, D.C. Wallace, B. Malfroy, S.R. Doctrow, G.J. Lithgow, Extension of life-span with superoxide dismutase/catalase mimetics, *Science* 289 (2000) 1567–1569.
- [8] S. Brenner, The genetics of *Caenorhabditis elegans*, *Genetics* 77 (1974) 71–94.
- [9] Y.H. Shim, J.H. Chun, E.Y. Lee, Y.K. Paik, Role of cholesterol in germ-line development of *Caenorhabditis elegans*, *Mol. Reprod. Dev.* 61 (2002) 358–366.
- [10] C.C. Mello, J.M. Kramer, D. Stinchcomb, V. Ambros, Efficient gene transfer in *C. elegans*: Extrachromosomal maintenance and integration of transforming sequences, *EMBO. J.* 12 (1991) 3959–3970.
- [11] B.U. Fitzky, M. Witsch-Baumgartner, M. Erdel, J.N. Lee, Y.K. Paik, H. Glossmann, G. Utermann, F.F. Moebius, Mutations in the delta 7-sterol reductase gene in patients with the Smith–Lemli–Opitz syndrome, *Proc. Natl. Acad. Sci. USA* 95 (1998) 8181–8186.
- [12] C.I. Bargmann, E. Hartweg, H.R. Horvitz, Odorant-selective genes and neurons mediate olfaction in *C. elegans*, *Cell* 74 (1993) 515–527.
- [13] H. Adachi, Y. Fujiwara, N. Ishii, Effects of oxygen on protein carbonyl and aging in *Caenorhabditis elegans* mutants with long (*age-1*) and short (*mev-1*) life spans, *J. Gerontol.* 53 (1998) 240–244.
- [14] D. Gems, A.J. Sutton, M.L. Sundermeyer, P.S. Albert, K.V. King, M.L. Edgley, P.L. Larsen, D.L. Riddle, Two pleiotropic classes of *daf-2* mutation affect larval arrest, adult behavior, reproduction and longevity in *Caenorhabditis elegans*, *Genetics* 150 (1998) 129–155.
- [15] D.J. Chitwood, Biochemistry and function of nematode steroids, *Crit. Rev. Biochem. Mol. Biol.* 34 (1999) 273–284.
- [16] S. Murakami, T.E. Johnson, A genetic pathway conferring life extension and resistance to UV stress in *Caenorhabditis elegans*, *Genetics* 143 (1996) 1207–1218.
- [17] G.J. Lithgow, T.M. White, S. Melov, T.E. Johnson, Thermotolerance and extended life-span conferred by single-gene mutations and induced by thermal stress, *Proc. Natl. Acad. Sci. USA* 92 (1995) 7540–7544.
- [18] V.D. Longo, E.B. Gralla, J.S. Valentine, Superoxide dismutase activity is essential for stationary phase survival in *Saccharomyces cerevisiae*. Mitochondrial production of toxic oxygen species in vivo, *J. Biol. Chem.* 271 (1996) 12275–12280.
- [19] J.N. Lee, Y.K. Paik, Cholesterol biosynthesis from lanosterol: development of a novel assay method, characterization, and solubilization of rat hepatic microsomal sterol delta7-reductase, *J. Biochem. Mol. Biol.* 30 (1997) 370–377.
- [20] Y.K. Paik, J.M. Trzaskos, A. Shafiee, J.L. Gaylor, Microsomal enzymes of cholesterol biosynthesis from lanosterol. Characterization, solubilization, and partial purification of NADPH-dependent delta 8,14-steroid 14-reductase, *J. Biol. Chem.* 259 (1984) 13413–13423.
- [21] S.H. Bae, Y.K. Paik, Cholesterol biosynthesis from lanosterol: development of a novel assay method and characterization of rat liver microsomal lanosterol delta 24-reductase, *Biochem. J.* 326 (1997) 609–616.
- [22] R.A. Bowman, G.L. O'Neill, T.V. Riley, Non-radioactive restriction fragment length polymorphism (RFLP) typing of *Clostridium difficile*, *FEMS Microbiol. Lett.* 63 (1991) 269–272.
- [23] G.A. Walker, D.W. Walker, G.J. Lithgow, A relationship between thermotolerance and longevity in *Caenorhabditis elegans*, *J. Invest. Dermatol. Symp. Proc.* 1 (1998) 6–10.
- [24] J.A. Joseph, R. Villalobos-Molinas, N.A. Denisova, S. Erat, J. Strain, Cholesterol: A two-edged sword in brain aging, *Free. Radic. Biol. Med.* 22 (1997) 455–462.
- [25] S.H. Bae, J.N. Lee, B.U. Fitzky, J. Seong, Y.K. Paik, Cholesterol biosynthesis from lanosterol. Molecular cloning, tissue distribution, expression, chromosomal localization, and regulation of rat 7-dehydrocholesterol reductase, a Smith–Lemli–Opitz syndrome-related protein, *J. Biol. Chem.* 274 (1999) 14624–14631.