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Honeybee trophocytes and fat cells as target cells for cellular senescence studies

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ABSTRACT

Trophocytes and fat cells are distributed around the abdominal segments in honeybees (*Apis mellifera*). Whether these cells are a good model for cellular senescence studies is unknown. Here we used histochemical, biochemical, and genetic techniques to investigate the fluctuation of age-related molecules in trophocytes and fat cells of newly emerged and old worker bees. Histochemical studies revealed that old worker trophocytes and fat cells exhibited more senescence-associated β-galactosidase, lipofuscin granules, and non-homogeneous cellular morphology compared to the same cells in newly emerged workers. Biochemical assays demonstrated that trophocytes and fat cells of old workers expressed more lipid peroxidation and protein oxidation than those of newly emerged workers. Molecular genetic analyses detected no difference in telomerase activity or telomere length in trophocytes and fat cells between newly emerged and old workers, showing that these cells do not divide in adulthood. These results suggest that the expression of age-related molecules in trophocytes and fat cells is associated with the senescence of the cells and that honeybee trophocytes and fat cells can serve as a mode for cellular senescence.

1. Introduction

Cellular senescence is a complex process of progressive deterioration that is usually associated with a decrease in cell proliferation. Accordingly, aging leads to a gradual decline in biological function and an increased incidence of age-associated diseases, such as cardiovascular disease, cancer, arthritis, cataract, osteoporosis, type 2 diabetes, and Alzheimer's disease (Chapman et al., 1989; Smith et al., 1996; Minamino et al., 2002). Understanding the biology of aging can help prevent, and possibly cure, age-associated diseases.

One of the most studied and widely accepted conjectures on possible aging mechanisms centers around the oxidative stress hypothesis (the free radical theory of aging) (Sohal and Weindruch, 1996; Finkel and Holbrook, 2000). Oxidative stress, an imbalance in the production and detoxification of reactive oxygen species (ROS), causes damage to lipids, proteins, DNA, and mitochondria, thereby impairing cellular integrity and functionality (Sohal and Weindruch, 1996; Finkel and Holbrook, 2000). The hypothesis states that aging is a result of ROS accumulation and its associated cellular damage (Terman and Brunk, 2006).

According to the free radical theory of aging, age-related molecules in cells have been used as indices of aging in many organisms. Many studies indicate that expression of age-related molecules in cells and tissues increases or decreases with advancing age. Furthermore, expression changes of age-related molecules may provide a means to assess aging rates in organisms (Mecocci et al., 1999; Hsu et al., 2008).

Senescence-associated β-galactosidase (SA-β-Gal) is a eukaryotic hydrolase that is localized in the lysosome (Kurz et al., 2000), and SA-β-Gal expression increases with age (Dimri et al., 1995; Kishi et al., 2003; Genade et al., 2005; Hsu et al., 2008). Lipofuscin is an intralysosomal polymeric material that originates from autophagocytosed cellular components oxidized outside or inside the lysosomal compartment (Terman and Brunk, 2004). Lipofuscin cannot be degraded by lysosomal hydrolases or exocytosed. Thus, accumulation of lipofuscin granules has been reported to increase with age (Reichel, 1968; Nakano et al., 1995; Brunk and Terman, 2002; Kishi et al., 2003; Genade et al., 2005; Hsu et al., 2008). Accumulation of both SA-β-Gal and lipofuscin are considered reliable indices of advancing age.

Lipids and proteins are damaged by ROS, resulting in lipid peroxidation and protein oxidation. Lipid peroxidation and protein oxidation increase with age and are also good indicators of aging (Oliver et al., 1987; Sohal et al., 1993; Welis-Knecht et al., 1993; Almeida et al., 1998; Hsu et al., 2008).

Telomerase is a ribonucleoprotein reverse transcriptase that synthesizes terminal telomeric repeats at the ends of chromosomes (Morin, 1989). Previous studies demonstrate that telomerase activity decreases with age in normal organisms (Davis et al., 2005; Wright et al., 1996). Telomeres are specialized DNA protein complexes consisting of simple repetitive DNA that serves to cap the ends of linear chromosomes. Telomeres become shorter with age, resulting in

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cellular senescence (Hastings et al., 2004; Nasir et al., 2001; Davis et al., 2005; Argyle et al., 2003; Lindsey et al., 1991; Hsu et al., 2008).

Honeybees (*Apis mellifera*) have been used to study aging (Remolina et al., 2007; Corona et al., 2007; Seehuus et al., 2006a,b; Amdam and Page, 2005; Amdam et al., 2004; Page and Peng, 2001; Tofilski, 2000; Weirich et al., 2002; Williams et al., 2008; Robertson and Gordon, 2006; Collins et al., 2004; Remolina and Hughes, 2008; Neukirch, 1982; Jemielity and Keller, 2007; Rueppell et al., 2007a,b). Honeybees are an excellent aging model because worker bees have a short lifespan, queen bees have a long longevity, they live together in a large colony population, they are easily manipulated and the honeybee genome is sequenced. Oxidative stress has been studied in honeybee spermathecae, muscle, ventriculi, hemolymph plasma, semen, and brain (Weirich et al., 2002; Seehuus et al., 2006a,b; Williams et al., 2008; Collins et al., 2004). We hypothesized that honeybee trophocytes and fat cells are useful for studying cellular senescence. Trophocytes, which are large and irregularly shaped, and fat cells, which are small and spherical, attach to one another to form a single layer of cells around each segment of the honeybee abdomen. Trophocytes and fat cells are immersed in body fluid and can be used to test anti-aging drugs when drugs are microinjected into the body fluid. In a sense, honeybees can be used as an animated biomicroincubator. In this study, we examined the expression of age-related molecules in the trophocytes as well as fat cells of newly emerged and old worker honeybees.

2. Materials and methods

2.1. Honeybees

Honeybees (*A. mellifera*) were bred in a breeding room with an open window for outdoor access at our institute in Taiwan. Sucrose and pollen grains were sometimes added to the hives as dietary supplements. Newly emerged workers were labeled with paint on the dorsal side of the thorax to determine their age. Newly emerged and old workers (50 days after eclosion) were selected from the same colony on the same dates for the same experiments during the summer.

2.2. Transmission electron microscopy (TEM)

Trophocytes from three newly emerged and old workers were fixed in 2.5% glutaraldehyde in a 0.1 M phosphate buffer containing 0.35 M sucrose at pH 7.4 for 30 min at 25 °C and postfixed in 1% osmium tetroxide in a 0.1 M phosphate buffer with 0.35 M sucrose at pH 7.4 for 2 h. Trophocytes were dehydrated via an ethanol series and embedded in Spurr's resin. Thin sections (60–90 nm thick) were cut with a diamond knife, stained with uranyl acetate and lead citrate, and then examined using a TEM (JEOL JEM-2000EXII, Tokyo, Japan), operating at an accelerated voltage of 100 kV (Hsu and Li, 1993; Hsu, 2004).

2.3. SA-β-Gal expression analysis

SA-β-Gal expression was measured as described previously (Dimri et al., 1995). Briefly, trophocytes and fat cells were isolated from a worker, immersed in SA-β-Gal staining solution at 37 °C, washed with PBS, mounted onto glass slides, and viewed under light microscope (Olympus BX-61, Tokyo, Japan). SA-β-Gal area was analyzed with Image J software (http://rsb.info.nih.gov/ij/).

2.4. Lipofuscin granule analysis

Lipofuscin granules were observed by confocal microscopy (Brunk and Terman, 2002). Trophocytes and fat cells were isolated from a worker, mounted onto glass slides, and viewed under a confocal microscope (Leica TCS SP2; Leica, Wetzlar, Germany). Lipofuscin granule area was determined by autofluorescence of lipofuscin granules under blue (450–490 nm) excitation laser, with 520-nm emission filters and analyzed by QWin image processing and analysis software (version 2.5, Leica, Wetzlar, Germany) (Hsu et al., 2008).

2.5. Lipid peroxidation analysis

Workers were freshly collected from hive and dissected at 4 °C. Trophocytes and fat cells from fifteen newly emerged or old workers were collected at 4 °C and homogenized using a polytron and sonicator in 1.0 ml 50 mM phosphate buffer (pH 7.5) containing protease inhibitors (leupeptin 0.5 μg/ml, aprotinin 0.5 μg/ml, pepstatin 0.7 μg/ml, and phenylmethylsurfonyl fluoride 40 μg/ml) at 4 °C. Cell extract were centrifuged at 5000 *g* for 10 min at 4 °C, and the resulting supernatant was immediately used in the following experiments. Protein concentration was determined using protein assay reagent (Bio-Rad, Hercules, CA, USA). Lipid peroxidation was evaluated using the thiobarbituric acid reactive substances procedure (Draper and Hadley, 1990). Briefly, 210 μl of fresh resulting supernatant was mixed with 210 μl of PBS, 420 μl of 28% trichloroacetic acid, and 630 μl of 1% thiobarbituric acid (TBA, pH<2). The solution was placed in boiling water for 15 min. After cooling, the absorbance of the solution was measured using a spectrophotometer at 532 nm. The concentration of malondialdehyde (MDA) was calculated using the absorbance coefficient of the MDA-TBA complex (absorbance coefficient $= 1.56 \times 10^5\ cm^{-1}\ M^{-1}$) and expressed as nanomoles per gram of protein (Okutan et al., 2005).

2.6. Protein oxidation analysis

The fresh supernatant was obtained as described in the lipid peroxidation analysis. Protein carbonyls in trophocytes and fat cells were assessed using a spectrometric 2,4-dinitrophenylhydrazones (DNPH) assay with minor modifications (Levine et al., 1990). Briefly, 300 μl of cell supernatant was treated with 300 μl of 10 mM DNPH in 2 M HCl (2 M HCl was used for control samples). Samples were then incubated for 1 h at room temperature, stirred every 10 min, precipitated with 10% trichloroacetic acid (final concentration), and centrifuged at 7800 *g* for 3 min. The pellet was washed with 1 ml of 1:1 ethanol/ethyl acetate and redissolved in 1 ml of 6 M guanidine in 10 mM phosphate buffer/trifluoroacetic acid (pH 2.3). Any trace insoluble material was removed by centrifugation at 7800 *g* for 3 min. The difference in absorbance between DNPH-treated and HCl-treated samples was determined at 366 nm, and the results were expressed as nanomoles of carbonyl groups per mg of protein, using the extinction coefficient of 22.0 $mM^{-1}\ cm^{-1}$ for aliphatic hydrazones (Sohal et al., 1993).

2.7. Telomerase activity analysis

Telomerase activity was measured using the telomere repeat amplification protocol (TRAP) by using the TRAPeze XL Telomerase Detection Kit (Chemicon, Temecula, CA, USA; S7707). Briefly, 0.2 g of trophocytes and fat cells isolated from five workers was treated with 1.5 ml ice-cold lysis buffer (0.5% 3-([3-cholamidopropyl]-dimethylammonio)-1-propane sulfonate (CHAPS), 10 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 5 mM β-mercaptoethanol and 1 mM PMSF), then incubated on ice for 30 min. After centrifugation at 15,000 *g* for 30 min at 4 °C, DNA concentration in the supernatant was measured by UV absorbance before it was stored at −80 °C. Telomerase extension products (2 μg/μl) were amplified by a three-step PCR (94 °C for 30 s, 59 °C for 30 s, 72 °C for 60 s) for 38 PCR cycles with TS primer (5′-AATCCGTCGAGCAGAGTT-3′) and CX primers (5′-CCCGGGCCTAACC-TAACCTAA-3′ for bee and 5′-GCGCGGCTAACCCTAACCCTAACCCTACCC-3′ for mammal), and resolved in 10% polyacrylamide (Kim and Wu,