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Colostrinin delays the onset of proliferative senescence of diploid murine fibroblast cells

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Abstract

Colostrinin™ (CLN), a uniform mixture of low-molecular weight, proline-rich polypeptides, induces neurite outgrowth of pheochromocytoma cells and inhibits beta amyloid-induced apoptosis. Moreover, its administration to patients with Alzheimer's disease resulted in improved cognitive functions. In this study, we investigated the impact of CLN on the lifespan of murine diploid fibroblast cells (MDF), an in vitro model for cellular aging. Here, we show that CLN significantly decelerates the senescence of cultured MDF and increases their population doubling levels. This action of CLN is associated with a decrease in the intracellular levels of reactive oxygen species, which may be due to senescence-associated mitochondrial dysfunction. These data suggest that CLN may delay the development of cellular aging at the level of the organism. Thus, CLN may be used in the prevention and/or therapy of diseases associated with aging processes.
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Keywords: Colostrinin™; Oxidative stress; Lifespan

1. Introduction

Colostrinin™ (CLN) is isolated from colostrum by various chromatographic steps, including ion exchange and affinity, and molecular sieving, combined with ammonium sulfate precipitation (Janusz and Lisowski, 1993; Janusz et al., 1974; Kruzel et al., 2001). It has been shown that CLN is an important immune-modulator, which induces maturation and differentiation of murine thymocytes (Janusz and Lisowski, 1993; Zimecki et al., 1984), promotes peripheral blood leukocyte proliferation, and induces various cytokines (Janusz and Lisowski, 1993; Stanton et al., 2001). We have recently shown that CLN decreases intracellular oxidative stress levels, reduces 4-hydroxynonenal (4HNE)-mediated cellular damage and suppresses 4HNE-induced cellular signaling in cultured cells (Boldogh et al., 2001, 2003a). Most importantly, CLN induces delicate cassettes of signaling pathways common to cell proliferation and differentiation, and mediates activities that are similar to those of hormones and neurotrophins, leading to neurite outgrowth (Bacsi et al., 2005). CLN protects neuroblastoma cells from beta amyloid-induced apoptosis by inhibiting amyloid aggregation (Schuster et al., 2005). In a recent study, its administration to

Abbreviations: CLN, Colostrinin™; DhEt, dihydroethidium; cH_2D-CF-DA, carboxy-2′,7′-dichlorodihydrofluorescein diacetate; DCF, dichlorofluorescein; MDF^{SP}, murine diploid fibroblast senescence prone; MDF^{R}, murine diploid fibroblast senescence resistant; PDLs, population doubling levels; $O_2^{\cdot-}$, superoxide anion; ROS, reactive oxygen species.

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one-day-old domestic chicks significantly enhanced long-term memory retention in a passive avoidance model (Stewart and Banks, 2006). Remarkably, its administration to Alzheimer's patients resulted in improvement in cognitive functions and instrumental activities in daily living (Bilikiewicz and Gaus, 2004; Leszek et al., 1999).

Human and rodent diploid fibroblast cells exhibit limited proliferative potential, undergo a limited number of population doublings, and enter a state of permanent growth arrest, so-called "replicative senescence" or "cellular aging", in which they remain alive and metabolically active but are completely refractory to mitogenic stimuli (Campisi, 1996; Campisi et al., 2001). These cells provide a model for studying the processes associated with senescence and unfolding events at the molecular level involved in aging processes and age-associated diseases. Due to CLN's neurotrophin-, hormone-like activities on pheochromocytoma cells and its ability to modulate cellular redox status, we hypothesized that CLN may delay the onset of cellular senescence. To test this hypothesis, we selected murine diploid fibroblast (MDF) cells isolated from senescence-accelerated and senescence-resistant mice (Fujisawa et al., 1998; Hosokawa et al., 1997). MDF cultures from senescence-accelerated mice show an early appearance of higher levels of lipid peroxides compared to cultured cells from senescence-resistant mice (Fujisawa et al., 1998, 1999). Thus the MDF model provides an excellent system to study CLN's effect on senescence-associated processes, such as reactive oxygen species (ROS) generation and mitochondrial dysfunction.

In this study, we show for the first time that treatment of cultured fibroblast cells with CLN delayed the onset of replicative senescence and increased their lifespan. This novel effect of CLN was mediated by its ability to decrease intracellular ROS levels. Our data underline CLN's utility in prevention and/or treatment of age-associated diseases, in which oxidative stress has an etiological role.

2. Materials and methods

2.1. Cell cultures and replicative lifespan

Mouse diploid fibroblast (MDF) cells were isolated from the dorsal dermis of newborn littermates from a senescence-prone strain (SAMP1) or senescence-resistant strain (SAMR), as previously described (Hosokawa et al., 1994). MDF cells from SAMP1 (MDF^{SP}) and from SAMR mice (MDF^{R}) were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), glutamine (292 mg l^{-1}), streptomycin (100 μg ml^{-1}) and penicillin (100 U ml^{-1}) at 37 °C in a 5% CO_2 atmosphere. Lifespan studies were terminated when population doublings were less than 1.4. The replicative lifespan of cells was determined as described previously (Szczesny et al., 2003). Briefly, 1.5×10^5 (6×10^3 per cm^2) cells were transferred to 25-cm^2 flasks (Corning, Acton, MA) with 4 ml medium (as above) ± CLN. In parallel experiments, a medical grade gas mixture containing 3% (or 8.6%) O_2 and 5% CO_2, balanced with N_2 (IWECO, Houston, TX) was used. By filling flasks (Corning) with these gas mixtures ~3.7% and 10% O_2 concentrations were achieved, respectively. The formula for calculation of gas concentrations in the atmosphere of sealed culture flasks was described previously (Saito et al., 1993). The proliferating cultures were incubated at 37 °C until they reached 90% confluence. At this confluence, the cells were removed by trypsinization, counted and subcultured, and population doubling levels (PDLs) were calculated (PDL = log(number of cells obtained at subculture per 1.5×10^5)/log 2) (Szczesny et al., 2003).

2.2. Assays of ROS levels

Cells were harvested, sedimented by low-speed centrifugation (600*g*, for 5 min) and suspended in DMEM containing 1% FBS and 2.5 μM carboxy-2′,7′-dichlorodihydrofluorescein diacetate (cH_2DCF-DA), which was added as we previously described (Boldogh et al., 2003a,b). After 15 min incubation at room temperature, changes in intracellular fluorescence intensities were assessed by flow cytometry (Becton–Dickinson FACScan flow cytometer).

Concentrations of hydrogen peroxide (H_2O_2) released from cells were determined by Amplex® Red (10-acetyl-3,7-dihydroxyphenoxazine) assay (Bacsi et al., 2006b; Votyakova and Reynolds, 2001). MDF^{SP} and MDF^{R} cells were grown in six-well plates to a confluence of 80%. Culture media were replaced with HBSS, and H_2O_2 measurements were carried out according to the manufacturer's instructions (Molecular Probes, Eugene, OR). Fluorescence was measured at 10-min intervals for up to 30 min using a microplate reader (SpectraMass M2, Molecular Devices, Sunnyvale, CA) at excitation and emission wavelengths of 530 and 590 nm, respectively. The addition of catalase (400 U ml^{-1}) decreased H_2O_2 levels by ~90%. A calibration curve was prepared for increasing dilutions (0–400 pM) of H_2O_2.

2.3. Microscopy

The intracellular site of ROS generation was identified by fluorescence microscopy (Bacsi et al., 2006b; Zhao et al., 2003). Dihydroethidium (DhEt)-loaded MDF^{SP}, and MDF^{R} cells were placed in a thermo-controlled microscopic chamber, and fluorescent images