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Analysis of biomarkers of caloric restriction in aging cells

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Summary

Caloric restriction (CR) has been extensively documented for its profound role in effectively extending maximum lifespan in many different species. However, the accurate mechanisms, especially at the cellular level, for CR-induced aging delay are still under intense investigation. An emerging technique, recently explored in our laboratory, provides precisely controllable caloric intake in a cultured cellular system that allows realtime observation and quantitative analysis of the impact of CR on the molecular cellular level during the aging processes. This *in vitro* method allows investigation of the molecular mechanisms pertaining to how CR influences aging processes leading to life extension in human cellular systems. It will provide important clinical implications for future preventive approaches for aging and aging-related degeneration diseases in humans. Hence, we will discuss the detailed procedures of this novel technique as well as analysis of relevant aging biomarkers and its broad application in the field.

Keywords

Caloric restriction; aging; cell senescence; β -Galactosidase assay

1. Introduction

Aging is inevitable biological processes associated with degenerative changes at both the cellular and organic levels that result in multiple degenerative diseases and even death in senior populations. Advances in scientific understanding of basic aging mechanisms increase the likelihood of postponing aging processes and increasing the lifespan of humans. Among many well-investigated factors that effectively reverse the progression of aging, nutritional intervention has shown promising prospects due in part to its obvious merits such as safety and availability (1).

Energy control such as caloric restriction (CR), as a major nutritional manipulator, is by far the most effective environmental intervention that can extend maximum lifespan in many

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different species (2, 3). Numerous studies have revealed the remarkable effects of CR on aging intervention and lifespan extension among diverse species including yeast, worms, flies, fish, and even mammals such as mice (4–6). In addition, studies in higher mammals such as non-human primates and humans revealed a profound influence of CR in delaying a wide range of aging-associated diseases such as cancer, diabetes, atherosclerosis, cardiovascular diseases and neurodegenerative diseases (6–9). However, besides the overwhelming effects of CR in experimental animal research, convincing outcomes between CR and aging delay/lifespan extension are limited in human studies primarily due to uncontrolled ethical limitations for long-term dietary control, especially lifelong CR studies, which can be much more easily controlled in animal studies. Potential mechanisms by which CR induces aging delay and prevents the onset of many aging-related degenerative diseases include reduction of oxidative stress or regulation of metabolic pathways during aging progression (10–12). However, the precise mechanisms of CR-induced longevity, especially in human systems, are not very well understood.

While acknowledging the maximal benefits of CR in experimental animals, it is of essential importance to apply this benefit to human health. A novel *in vitro* human cellular system explored in our laboratory could serve a research bridge to investigate the effect of CR in human systems that may benefit future clinical applications (13, 14). This *in vitro* system is designed to mimic CR-controlled longevity by reduction of glucose, the main caloric resource, in cell culture medium. This system allows more precise analysis of molecular mechanisms of CR and the effects of CR on human cellular lifespan, specifically at the cell molecular level. For example, a homogeneous cellular system provides an effective and consistent platform to monitor changes during aging progression which avoids fluctuant metabolic changes in organisms. Therefore, compared to aging research in experimental animals, this novel mammalian *in vitro* cellular system has numerous advantages and unique features that benefit sophisticated aging studies at the molecular level in human systems.

In this chapter, we will discuss detailed information on how to manipulate glucose concentrations and mimic CR effects in human cellular systems, population doubling (PD) calculations, aging biomarker observations during the aging processes and data validation. This new technology detects molecular mechanisms of CR and CR-associated aging delay and lifespan extension in human cellular systems, which provides an excellent *in vitro* model to investigate aging and its important modulator, CR, especially for application to future human studies.

2. Materials

2.1. Cell culture

2.1.1. Caloric restriction (CR) in cell culture

1. Normal human fetal lung fibroblasts WI-38 (ATCC)
2. Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/L glucose (Invitrogen) for control cells
3. Glucose- and sodium pyruvate-free DMEM medium (Invitrogen) for CR cells
4. Glucose solution (Cellgro)
5. Fetal bovine serum (Atlanta Biologicals)
6. Penicillin/streptomycin (Mediatech)
7. Glucose Assay kit (Biovision)
8. 100 × 20 mm tissue culture disks (BD Falcon)

2.1.2. Population doublings (PDs) assessment

1. Microscopy (Nikon)
2. Neubauer haemocytometer

2.2. Aging biomarkers analysis

2.2.1. Senescence-associated β -Galactosidase (SA- β -Gal) activity assay

1. Phosphate-buffered saline (PBS)
2. Senescence β -Galactosidase staining kit (Cell Signaling)
3. 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal) (Sigma)

2.2.2. Age-related gene analysis

- 1 RNA extraction and cDNA reverse transcription: RNeasy kit (Qiagen); 2-Mercaptoethanol; Superscript II kit (Invitrogen)
- 2 Protein extraction: RIPA lysis buffer (Upstate Biotechnology); Proteinase inhibitors
- 3 Related primers and specific antibodies
- 4 SYBR Green qPCR Mastermix (Invitrogen)
- 4 Roche LC480 thermocycler (Roche)
- 5 Western-blot electrophoresis apparatus

2.2.3. Age-related oxidative stress analysis

1. Reactive oxygen species (ROS) detection assay (Abcam)
2. Superoxide Dismutase (SOD) Assay (Cell Biolabs)
3. Nitric oxide (NO) assay (Abcam)
4. Fluorescence spectroscopy

2.2.4. Age-related DNA damage analysis

1. DNA purification kit (Promega)
2. DNA Double-Strand Break Assay (Cell Biolabs)
3. 8-hydroxydeoxyguanosine (8-OHdG) DNA damage assay (Cell Biolabs)
4. Microplate reader

2.2.5. Comparison of CR-related gene profiles

2.3. Data interpretation

3. Methods

3.1. Cell culture

3.1.1. Caloric restriction (CR) in cell culture—The method described below is suitable for analysis of CR effects on cultured human cells. Normal diploid human fibroblasts such as fetal lung fibroblasts, skin fibroblasts and vascular fibroblasts can be used for this study (*see* Note 1). We will use WI-38 human fetal lung fibroblasts as an example to describe this method (14). Since glucose is the primary energy resource in culture medium, we have developed an *in vitro* system to mimic CR by reducing glucose

concentration in cell growth medium. Control WI-38 cells grown in 100 mm culture plates are maintained in DMEM medium supplemented with regular glucose level at 4.5 g/L. To restrain glucose, cells are cultured in glucose- and pyruvate-free DMEM medium and different CR levels can be obtained by adding various concentrations of glucose in the medium depending on the relevant study design. All culture media are supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in the presence of 5% CO₂ at 37 °C. Culture media should be replaced once every other day. The actual glucose concentration in the glucose restriction medium will be assessed by the Glucose Assay kit.

3.1.2. PDs assessment and sample collection during aging progression

1. WI-38 cells are typically within the range of 15–20 PDs (young cells) upon initiation of the experiment (*see* Note 1). Parallel culture control and CR cells should be set up in corresponding mediums and the experiments need be initiated synchronously.
2. PDs calculation: To quantify replication, cells are passaged weekly at a seeding density of 10⁵ cells per plate and counted using a haemocytometer until cells reach 90–95% confluence. Cellular PDs are calculated based on the following formula: $\log[(\text{number of cells harvested})/(\text{number of cells seeded})]/\log 2$ (*see* Note 2) (15). The first confluent cells are designated as PD 0 when the experiments are initiated. The duration of cellular lifespan can be equally divided in three stages including early (young), intermediate (middle age) and late passages (aging or senescence).
3. Harvest glucose restriction treated- and untreated-WI-38 cells and extract total RNA, DNA and protein once a week during the cellular lifespan; carefully label and appropriately store samples at different PDs.
4. Determine cellular replicative senescence at the end of late passage in the cellular lifespan by morphological changes (*see* Note 3) and senescence-associated β -Galactosidase (SA- β -Gal) activity assay. Experiments are considered to terminate when cells stop dividing and cellular senescence occurs.

3.2. Aging biomarkers analysis

3.2.1. Senescence-associated β -Galactosidase (SA- β -Gal) activity assay

1. Treat the WI-38 normal human fetal lung fibroblasts with normal level of glucose and glucose restriction medium as described previously; measure SA- β -Gal activity in control and CR cells weekly with PDs calculation (14).
2. Wash cells twice in phosphate-buffered saline (PBS) and then fix in 0.5% glutaraldehyde for 10 min at room temperature.
3. Incubate cells at 37°C with fresh senescence-associated β -Gal (SA- β -Gal) stain solution containing 1 mg/ml of 5-bromo-4-chloro-3-indolyl-beta-D-galactoside (X-Gal) overnight according to the manufacturer's protocols (Cell Signaling).

¹Normal human diploid fibroblasts such as fetal lung fibroblasts, skin fibroblasts and vascular fibroblasts with a finite replicative lifespan *in vitro* are the most commonly used cell types to manifest aging (16, 17). Other types of human normal cells including human lymphocytes and certain epithelial cells can also be applied in specifically designed aging studies (18). To induce the CR effects in cell culture, glucose restriction treatment should be initiated at the young stage of cells to allow persistent stimulation and permanent changes that affect aging processes.

²PDs calculation is the most critical procedure in this method. Counting using a haemocytometer may lead to potentially inaccurate PD calculations. Therefore, an automated cell counter is encouraged for use in this study.

³Another important biomarker for aging is cellular morphology (Fig. 1). The steady loss of replicative potential is accompanied by a greater heterogeneity of cell sizes. Senescent cells tend to be much larger and irregular as compared to cells at their earlier PDs. An increase in nuclear size, nucleolar size, and in the number of cytoplasmic vacuoles can be observed in senescent cells by light microscopy.

4. Blue-dye precipitated cells are SA- β -Gal-positive cells and are considered to have undergone cellular senescence (Fig. 1) (*see* Note 4). Positive-stained cells are counted by using a Nikon digital camera at 40-fold magnification. Counts should be performed at five random locations and every field is counted three times for determination of the mean percentage of positively stained cells.
5. Compare senescence rate (SA- β -Gal-positive cells) in control and CR groups at different PDs during aging progression.

3.2.2. Age-related gene analysis

1. Harvest the control and CR-treated WI-38 cells weekly at different PDs during the cellular lifespan and extract RNA, reverse transcript cDNA and extract protein as described in **Subheading 3.1.2., step 3** followed by the routine protocols.
2. Analyze age-related gene expression such as *p16^{INK4a}* at RNA and protein levels by using the methods of realtime PCR and western-blot followed by the routine protocols. Detection of specific age-related genes should be based on specific cell type as well as study design (Table 1) (*see* Note 5).
3. Monitor and compare age-related gene expression changes in control and CR-treated WI-38 cells at different PDs during the cellular lifespan.

3.2.3. Age-related oxidative stress analysis

1. Harvest control and CR-treated WI-38 cells at different PDs during the cellular lifespan according to **Subheading 3.1.2., step 3**.
2. Analyze age-related oxidative stress parameters such as ROS, SOD and NO according to the manufacturer's protocols (*see* Note 6).
3. Compare alterations of these parameters in control and CR cells at different PDs during aging progression.

3.2.4. Age-related DNA damage analysis

1. Harvest the control and CR-treated WI-38 cells weekly at different PDs during the cellular lifespan and extract DNA according to **Subheading 3.1.2., step 3** followed by the routine protocols.
2. Analyze age-related DNA damage parameters such as 8-OHdG by using commercially available kits (*see* Note 7).

⁴The SA- β -gal assay is the most extensively utilized biomarker for aging that catalyzes the hydrolysis of β -galactosides into monosaccharides at pH 6.0 and develops blue staining only in senescent cells (19, 20). SA- β -gal activity is routinely detected cytochemically, manually discriminating negative from positive cells by light microscopy. Flow cytometry-based SA- β -gal assay yields better differentiation to quantitatively calculate positive-stained fibroblasts as compared to the routine cytochemical-based method (21).

⁵Senescent cells show striking changes in gene expression. These changes include activation of cell-cycle inhibitors, such as cyclin-dependent kinase inhibitors (CDKIs) *p21* and *p16* and repression of cellular proliferation-promoted genes such as *hTERT* (human telomerase reverse transcriptase), *c-FOS*, *cyclin A*, *cyclin B* and *PCNA* (proliferating cell nuclear antigen) (14, 22–25). A gene expression microarray assay will help to identify more differentiated expression genes between cells treated with normal level of glucose and glucose-restricted medium.

⁶In contrast to cells cultured in normal level of glucose medium, cells in glucose-restricted (CR) medium tend to have longer lifespan which may be attributed to reduced oxidative stress. Therefore, the oxidative stress parameters such as ROS, SOD and NO could serve as useful markers in this study (26). Other oxidative stress markers can be applied in this study including protein oxidation/nitration markers such as Protein Carbonyl Content (PCC) and lipid peroxidation marker such as 4-Hydroxynonenal (4-HNE).

⁷Stress-induced cellular senescence results in stress-related DNA damage, which can be detected by certain parameters such as 8-OHdG. Another obvious marker for senescent cells is the lack of DNA replication, which is typically detected by the incorporation of 5-bromodeoxyuridine or H³ thymidine. Some senescent cells can also be identified by the cytological markers of senescence-associated heterochromatin foci (SAHFs) and senescence-associated DNA-damage foci (SDFs) (22, 27, 28).

3. Compare alterations of these parameters in control and CR cells at different PDs during aging progression.

3.2.5. Comparison of CR-related gene profiles

1. Analyze CR-related gene expression such as *SIRT1* at the RNA and protein levels using realtime PCR and western-blot followed by the routine protocols as described in **Subheading 3.2.2.** (see Note 8 and Table 2).
2. Monitor and compare CR-related gene expression change in control and CR-treated WI-38 cells at different PDs during the cellular lifespan.

3.3. Data interpretation

Our *in vitro* cellular system mimics CR by reducing glucose concentration in cell growth medium. Following consecutive treatment and monitoring, extended lifespan or increased PDs can be observed in CR-treated WI-38 cells. Delayed cell senescence rate can also be determined by SA- β -Gal assay under CR treatment. In addition, CR treatment can prevent age-associated oxidative stress and DNA damage. Altered age- and CR-related gene expression profiles such as decreased expression of certain age-dependent genes and increased expression of CR-related genes can be observed in CR-treated cells. Therefore, by comparing these biomarkers, we can easily investigate the effects of CR on aging processes at the cellular and molecular levels in human systems (Fig. 2).

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⁸CR studies conducted in experimental animals have indicated that CR treatment induces overwhelming gene profile changes that may contribute to CR-induced aging delay and longevity. For example, up-regulation of *SIRT1*, a NAD-dependent histone deacetylase, has been confirmed to correlate with CR-induced longevity (14, 30). Other known CR-related genes can be detected to determine the efficacy of this *in vitro* CR system (29–33).

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Fig. 1. Cellular senescence is determined by the Senescence β -Galactosidase (SA- β -Gal) assay. Human WI-38 fibroblasts at a proliferating state (young, **A**) and senescence (old, **B**) are subjected to SA- β -Gal staining and photographed. Blue staining cells are SA- β -Gal-positive or senescent cells. Magnification, $\times 100$.

Fig. 2. Schematic representation of the procedure for aging biomarker analyses in the CR *in vitro* model.

Table 1

Selective age-related genes

Gene	Function in aging	Expression change during aging	Reference
<i>p16^{INK4a}</i>	Cell-cycle inhibitor; induce senescence growth arrest via activated p16–pRB pathway	Activation	13, 14, 22
<i>p21</i>	Cell-cycle inhibitor; induce senescence growth arrest via activated p21–p53 pathway	Activation	22, 23
<i>p53</i>	Tumor suppressor gene; induce senescence growth arrest via activated p21–p53 pathway	Activation	22, 23
<i>pRB</i>	Tumor suppressor gene; induce senescence growth arrest via activated p16–pRB pathway	Activation	22, 24
<i>hTERT</i>	Human telomerase reverse transcriptase; activate <i>hTERT</i> expression to elongate telomeres	Inactivation	13, 14, 22
<i>Ras</i>	Oncogene; cause oncogene-induced senescence	Activation	22, 25

Table 2

Selective CR-related genes

Gene	Function in CR	Expression change	Reference
<i>SIRT1</i>	NAD-dependent histone deacetylase; activation of <i>SIRT1</i> is seen in different animal organs affected by CR	Increased expression	14, 30
<i>Foxo</i>	Forkhead transcription factors; controls various biological functions; involves CR-related longevity	Decreased expression	30, 31
<i>PGC-1α</i>	Regulates mitochondrial function and glucose homeostasis during CR	Increased expression	30, 32
<i>PPAR-γ</i>	Insulin sensitizer; increase insulin sensitivity during CR	Increased expression	29, 33
<i>Glucose-6-phosphate</i>	Involve energy metabolism such as glycolysis	Increased expression	29
<i>Hsp105</i>	Heat shock protein; stress-related gene	Decreased expression	29