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Techniques for Analysis of Biological Aging

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Summary

The aging process encompasses changes at the molecular, cellular, and organismal levels that can be analyzed by a variety of methods. For several decades, a popular mode of studying biological aging has been the analysis of cells cultured in vitro that display cellular senescence. Current interest is also focused on models of senescence that include organismal aging such as aging of yeast and *Drosophila*. The number of techniques applied to biological aging has increased exponentially over the past decade. Although approaches to biological aging vary greatly, they can be generally grouped into basic techniques, intervention methods, and protocols for analyzing the many molecular and cellular changes seen in aging cells. Hence, this volume organizes the topics into these three categories.

Keywords

Aging; biological; methods; techniques; analysis; biogerontology

1. Introduction

The number of processes impacted by aging is so diverse that it can be a rather daunting task to organize the approaches to the study of biological aging. A major breakthrough in aging research occurred in the early 1960s when Leonard Hayflick showed that cells cultured in vitro have a limited life span, a process generally referred to as cellular senescence (1). Many alterations such as telomeric attrition, oxidative stress, DNA damage, and oncogenic activity can lead to cellular senescence, during which the cells undergo a number of morphological and metabolic changes and eventually cease to divide (2). Although numerous molecular processes have been associated with biological aging, the primary molecular aberrations appear to be related to genomic instability, genetic programs, and/or reactive oxygen species. A major factor leading to age-related genomic instability appears to be a shortening of the telomeres. Telomeric attrition occurs because telomerase, the enzyme that maintains the lengths of telomeres, is expressed at extremely low levels in most somatic cells (3). Support for a role for telomerase in cellular senescence has been significantly bolstered by the finding that many cells can remain young and viable and can proliferate indefinitely when supplemented with an exogenous source of telomerase (4). Genetic programs have also been shown in a number of studies to contribute to biological aging. For example, mutations in several genes such as *daf* and *age-1* have been shown to delay biological aging (5). Reactive oxygen species are free radicals generated during metabolic processes that lead to cumulative damage of various molecules that can contribute to aging (6). Additional alterations undoubtedly also occur during aging and the continued development of new tools to analyze biological aging will greatly facilitate our understanding of the many processes that contribute to aging and life span determination.

2. Contents of This Book

2.1. Basic Methods of the Aging Process

A major advance in biogerontology occurred with the development of techniques to serially propagate cells in culture to senescence. This technique revolutionized biological aging

research when it was first introduced in the 1960s. Chapter 2 details the protocols necessary for culturing and subcultivation of normal human diploid fibroblasts. This system appears to be only an approximate approach to understanding the aging process because a direct correlation between cellular senescence and biological aging has been a subject of debate. However, it is through methods such as these that we have learned the vast majority of the molecular processes that contribute to senescence. An important advancement in cellular senescence studies was the development of a reliable biomarker, the senescence associated β -galactosidase (SA- β -gal) assay. This method involves the histochemical staining of cells using the substrate X-gal and distinguishes senescent cells from quiescent cells, an important determination when assessing biological aging (*see* Chapter 3). The SA- β -gal technique reliably distinguishes senescent and nonsenescent cells. In part because of its ease of detection, this assay is now widely used in biological aging studies. Another approach to identifying aging cells is described in Chapter 4 and uses the method of cell sorting by flow cytometry that is based on the physical separation of young and old cells that have different characteristics. With the use of green fluorescent protein (GFP)-expressing reporter constructs for genes transcriptionally regulated by senescence, quantitative estimates of frequencies of senescent vs nonsenescent cells can be obtained. Although some heterogeneity in the insertion of the retrovirally transferred reporter can occur, this method is proving to be useful in separating young versus old cells which has many obvious advantages in cellular senescence research.

Telomeric shortening has been shown to be a major process associated with aging and Chapter 5 describes three major approaches for analyzing telomere lengths: (1) telomere restriction fragment (TRF) analysis; (2) quantitative fluorescence *in situ* hybridization (Q-FISH); and (3) flow fluorescence *in situ* hybridization (Flow-FISH). Although TRF analysis of telomere lengths has been a mainstay in aging research for many years, this technique has a number of drawbacks which led to the development of Q-FISH and Flow-FISH as well as other more contemporary approaches for telomere length assessment in aging systems. Epigenetic processes, mediated in part by the DNA methyltransferases, play a major role in aging as evidenced by the dramatic genomic hypomethylation that occurs during cellular senescence and in aging tissues (7). DNA methyltransferase (DNMT)1 is the major DNA methyltransferase in mammalian cells, and Chapter 6 describes a comprehensive method for detecting and evaluating its transcription in senescing cells. A related technique is discussed in Chapter 7, which details methods to study protein expression of the DNA methyltransferases in cells undergoing senescence.

Many studies on the basic aspects of aging have relied on yeast models and the chronological life span of yeast described in Chapter 8 has been successfully coordinated with several of the key pathways that are important in regulating the aging process. Using both the normal and calorie restriction paradigms, methods are delineated for determining the chronological life span of the unicellular *Saccharomyces cerevisiae*. Although additional basic methods have been applied to biological aging, those described in Chapters 2–8 should provide a basic set of tools for assessing cellular senescence and facilitate insights into the cellular biology, genetics, and molecular aspects of biological aging.

2.2. Techniques for Intervening in the Aging Process

Besides basic methods for analysis of biological aging, many techniques have been recently developed for intervening in the aging process as an approach to determining the major factors that regulate and mediate biological aging. Caloric restriction is probably the most widely accepted approach to modulate life span and its application to the budding yeast, *S. cerevisiae*, is described in Chapter 9. Methods are detailed for determining the replicative life span of single yeast cells as well as for measuring the recombination frequency of the rDNA locus and the production of extrachromosomal rDNA circles that are a primary cause for aging

in this yeast model. The fruit fly, *Drosophila*, is another popular model for studying age-related changes, and Chapter 10 describes nutrigenomic techniques that allow analysis of *Drosophila* larvae and adults fed control diets high in palmitic acid (a saturated fat), soy, or 95% lean ground beef. These methods incorporate microarray analyses that allow determination of alterations in mRNA expression associated with these diets in the aging flies. Chapter 11 is a review of the use of caloric mimetics as applied to aging research. Because caloric restriction of 30–40% in food intake, the level often showing effectiveness in retarding age-related effects in animal models, is not practical for most humans, alternative approaches are needed. Calorie-restriction mimetics target alterations in pathways involved in energy production to mimic the benefits of caloric restriction without the need to significantly reduce food intake. The utility of glycolytic inhibitors, antioxidants, and specific gene-modulators as caloric restriction mimetics are analyzed.

A completely different approach for intervening in the aging process is described in Chapter 12 and is based on the important role of telomerase in biological aging. Telomeres can be reconstituted by overexpression of hTERT, the catalytic subunit of telomerase, which frequently results in immortalization of the cells or an extension of their replicative life span. In the past decade, these methods have been widely applied to unraveling the molecular basis of immortalization and thereby revealing mechanisms that are likely involved in limiting the replication of aging cells. Utilization of hTERT may also eventually have important applications to tissue engineering. As we have gained insights into tumorigenesis through the use of carcinogens, we can also learn about cellular senescence through methods that can accelerate the aging of cells. For instance, Chapter 13 delineates methods whereby cellular senescence can be recapitulated by the introduction of defined genetic elements such as a dominant negative version of telomerase. Techniques are also described for the induction of premature cellular senescence through the overexpression of oncogenic *ras* or *p16*. Moreover, premature cellular senescence can be induced by various techniques such as oxidative stress through the administration of hydrogen peroxide as detailed in Chapter 14.

Because the nucleus contains the basic blueprint for the aging process, nuclear transfer methods could have far-reaching potential in intervening in the aging process. In Chapter 15, nuclear transfer methods to study aging are described at the germinal vesicle stage as well as nuclear transfer in zygotes at the pronuclear stage. The use of ribozymes represents yet another approach for intervening in the aging process as described in Chapter 16. The construction of conventional and hybrid hammerhead ribozymes are described since such gene knock-down experiments can affect the aging process and reveal key regulatory genes that modulate cellular senescence (8). A randomized approach is delineated that is very flexible, is not dependent on enzymes, requires no prior knowledge of the target sequence, and has high specificity. The development of anti-aging drugs also has obvious implications for intervention in biological aging and procedures for evaluating pharmacological drug effects on aging and life span in mice are described in Chapter 17. Ultimately, some level of control of biological aging is the goal of many studies in biogerontology and Chapters 9–17 provide many of the most promising and effective methods for modulating cellular senescence and/or life span.

2.3. Protocols for Analysis of Biological Aging

Besides basic biological tools and methods that allow us to modify the aging process, techniques for analyzing the many molecular and cellular changes that occur during aging are also of high importance. A high-throughput functional genomic screening system applied to *S. cerevisiae* (see Chapter 18) allows identification of the many genes that can prolong life span. In this model system for aging, mother cells accumulate bud scars on their surface as they age and these bud scars contain high levels of chitin. The chitin can be stained with wheat germ agglutinin and changes in the life span of aged yeast can be assessed using a bud-scar

based flow cytometry sorting system. Significant progress in microarray technology has led to the application of these techniques to biogerontology. In Chapter 19, two different protocols using oligonucleotides and cDNA microarray platforms are described. Both radioactive and non-radioactive (fluorescent) approaches may be used and these methods have proven to be powerful gene analysis tools that are high-throughput and are rendering a wealth of data not previously obtainable using more conventional approaches to biological aging. Somatic mutations have been associated with the aging process for many years and methods are now available for detecting a broad range of mutational events in aging cells (*see* Chapter 20). For instance, a mutational reporter system based on lacZ-containing plasmids integrated into the germline of model systems such as *Mus musculus* or *Drosophila melanogaster* can be recovered allowing identification of mutant lacZ genes. The mutations can then be characterized to create a detailed analysis of the many mutations that can accumulate in aging systems. In Chapter 21, methods are described for detection of differentially expressed genes during aging through the use of subtractive hybridization. This technique is powerful in that it enables researchers to compare two populations of mRNA and identify the differentially expressed genes and could be applied to cells at various levels of senescence.

In order to detect naturally occurring molecular polymorphisms that are responsible for variation in life span, a map of the quantitative trait gene can be obtained followed by linkage disequilibrium mapping (*see* Chapter 22). The latter process is performed on a large sample of alleles collected from a natural population allowing genome-wide recombination mapping for identifying quantitative trait loci (QTL) where the quantitative trait genes affecting the life span can be identified. An application of this process is described in the *Drosophila* model system using quantitative complementation tests and linkage disequilibrium mapping to identify genetic polymorphisms that determine variation in longevity. QTL analysis can also be applied to mammalian systems for studying aging (*see* Chapter 23). Recombinant inbred strains of mice can be used to map genetic loci associated with age-related processes such as thymic involution. This approach involves identification of the phenotypes of interest and identification of the age-related changes in phenotypes, analysis of the QTL associated with the defined phenotypes, and confirmation of the genetic effects of the loci. Although specific techniques are described as applied to thymic involution during aging of mice, these methods can be applied to any quantitative trait that can be measured at various ages within recombinant inbred strains of mice and that is characterized by a hereditary difference within the chosen recombinant inbred strains.

Of course, protein analysis is also of high importance in biogerontology, and Chapter 24 describes protocols for two-dimensional (2D) gel proteomics to study age-related differences in the abundance of protein or isoform complexity in aging samples. A number of protocols are detailed and include isoelectric focusing followed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), Sypro Ruby fluorescent dye staining of gels, 2D gel image analysis, peptide mass fingerprint analysis using matrix-assisted laser desorption/ionisation (MALDI)-time-of-flight (TOF) mass spectrometry (MS), liquid chromatography (LC)-tandem mass spectrometry (MS/MS), Western blot analysis of protein oxidation, and mass spectrometric mapping of sites of protein oxidation. These procedures are of great importance to studies of biological aging because oxidative stress and protein interaction alterations are a common occurrence in cellular senescence and age-related disease. Lastly, methods are delineated for metabolomic analysis in aging and caloric restriction systems (Chapter 25). The techniques involved in high-performance liquid chromatography (HPLC) separations coupled with coulometric electrode array detection are described to allow a comprehensive analysis of the changes in small molecules that occur in aging samples such as sera or plasma. The advantages of this system are its quantitative precision and high sensitivity, although drawbacks include limited scale-up capacity and scant structural information on the metabolites that are altered in aging systems. These techniques may play a role in a number of

areas of aging and caloric restriction research such as classification, hypothesis generation, and mechanism determination as well as clinical practice. Therefore, Chapters 18–25 provide a broad array of methods that can be used to analyze the many molecular changes that occur during biological aging in systems ranging from yeast to human plasma.

3. Conclusion

The most current and more established techniques for assessing the many changes associated with biological aging are provided in this volume. Procedures involving basic areas of aging such as cell culturing and telomere analysis, intervention in the aging process by methods such as the introduction of oxidative stress or caloric restriction, and analysis of the numerous molecular alterations in aging systems by approaches such as QTL, proteomics, and metabolomics, are presented. It is likely that many investigators will not want to limit their investigations to any one technique, but to use some of these protocols in conjunction. It is only through novel approaches that build on our extant tools that progress will continue to increase exponentially in biological aging research. This book is intended to provide many of the most useful, relevant, and powerful techniques for studying the myriad processes that control and are affected by the biological aging process.

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