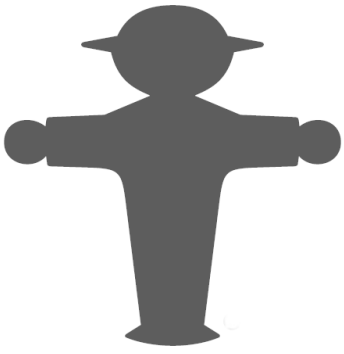
Chapter 1

General introduction



Aging theories

As we age, the organs that make up our bodies progressively lose their functionality, resulting in age-related diseases and ultimately death. Various theories have been put forward to explain why the process of aging should occur (1). Initially it was proposed that aging is programmed to prevent populations from becoming too large, but in the wild mortality is much more likely to occur as a result of extrinsic mortality (predation, infection, starvation, environmental conditions). According to the 'mutation accumulation theory', germ-line mutations which act late in life are not selected for by evolution since in the wild, organisms will be removed from the population before the mutations have any effect. This selection shadow is also important in the theory of 'antagonistic pleiotropy', proposing that genes with beneficial effects early in life can be deleterious later in life. In our modern world we can live up to and into the selection shadow, allowing pleiotropic genes to affect our bodies. In the 'disposable soma theory' the organism is thought to distribute a finite amount of energy between maintenance of the body and reproduction. The 'disposable soma theory' implicates that organisms are subject to damage. One of the first theories to explain this damage is the 'oxygen radical theory of aging' (2).

Sources and types of damage

The sources of cellular damage can be both extrinsic and intrinsic and mostly consist of or result in free radical molecules. Examples of extrinsic sources are sunlight (UV-A and UV-B), ionizing radiation (radioactivity), polluted air (NO-radicals), food (fat, charred products) and environmental chemicals (pesticides) (3). Whereas common sense can protect us from many extrinsic stressors, this is more difficult for intrinsic sources of stress. The very process that keeps us alive, that of cellular energy production, also results in the production of damaging reactive oxygen species (ROS)(4). Additionally, the fuel used to produce energy in the mitochondria, glucose, acts as a damaging agent, either directly damaging proteins by binding to them (nonenzymatic glycation or NEG) or by inducing ROS (5). The damaging nature of ROS has even been harnessed by evolution: leukocytes produce ROS and use them to attack infectious pathogens and there is increasing evidence that ROS serve as signaling molecules (6). When these processes are not regulated properly, excess ROS will

lead to damage, for example in chronic inflammations. Indeed, with increasing age the immune system gets progressively deregulated (7).

Cellular responses to stress-induced damage

Multicellular organisms have become increasingly complex during evolution and many have renewable tissues. The skin and gut are examples of organs which are continuously renewed by proliferative progenitor cells, but damage to tissues is also repaired by cells which have become proliferative temporarily. In a healthy organism, the loss of cells is balanced by the new formation of cells, a situation also known as 'proliferative homeostasis'. As described above, damage on the cellular level can deregulate vital cellular processes, resulting in cell death or the opposite: uncontrolled proliferation or cancer. Both will clearly disturb the proliferative homeostasis and affect organs and the body. Current thinking dictates that evolution has devised proteins to both protect cells (caretakers) and remove damaged cells from the pool of proliferative cells (gatekeepers) (8).

When cells contract damage, various mechanisms come into action to first arrest the cell cycle, and then gauge the extent of the damage and to finally react accordingly. If the damage is repairable, the cell will do so and continue proliferating afterwards. Autophagy, the process in which the cell degrades its own organelles in the lysosomes, is an important process for repair and the turnover of damaged cellular constituents and affects longevity (9).

If the damage is irreversible, cells can die in an orchestrated and controlled manner, a process called apoptosis, or stay alive in a permanent state of arrest termed cellular senescence. In case of extensive damage the regulation of apoptosis and senescence might also be compromised and cells will die in an uncontrolled fashion, a phenomenon called necrosis.

Apoptosis

Apoptosis is currently relatively well-understood. Though apoptosis is an important anti-cancer mechanism, it is not only essential for the removal of damaged cells but also plays an important role in the development and maintenance of the healthy organism (10). Morphological hallmarks of apoptosis are condensed chromatin, nuclear fragmentation, shrinkage of the cell and plasma membrane blebbing. The process can be initiated

intracellularly, in case of cellular damage, or by external signal molecules (e.g. TNF-family proteins). When stress has led to DNA damage, the tumor suppressor proteins p53 and Rb are activated and will activate a pathway leading to mitochondrial membrane permeabilization and release of cytochrome c which activates a number of proteases from the caspase-family. Despite being fragmented, the degradation of all cellular constituents happens in vesicles, preventing surrounding cells from being damaged by degrading enzymes.

Senescence

Already in 1956, Hayflick observed that human fibroblasts, though growing very well *in vitro*, had a limited lifespan of about 50±10 population doublings (PDs), the so-called Hayflick limit (11), after which fibroblasts stopped proliferating but could be kept alive for months and displayed an abnormal morphology when compared with proliferating fibroblasts. This state was called replicative senescence (RS). Later studies described more morphological and biochemical properties that are different in senescent cells: they contain higher levels of ROS and of ROS-induced damage products like lipofuscin and changed mass and functionality of mitochondria and lysosomes. Indeed, one of the most widely used markers, Senescence Associated-β-galactosidase (SA-β-gal) activity (12) is of lysosomal origin.

It was suggested that the aging of an organism was the result of proliferative cells having a maximum replicative capacity (13), implying the existence of a 'cellular clock'. When proliferating, cells are not capable to replicate their DNA all the way to the end of linear chromosomes. Non-coding G-rich repeats, called telomeres, function as buffer zones and shorten after every division. This led to the 'telomere-length mitotic clock hypothesis', but it soon became obvious that the maximum replicative capacity of cells was not only a matter of shortening telomeres. Some cells can actively elongate their telomeres with the enzyme telomerase. Indeed, artificial expression of telomerase dramatically extends replicative capacity of cells *in vitro*, even if the telomeres are shorter than those of control cells which do not express telomerase, suggesting that there is no simple relation between telomere length and maximum replicative capacity (14;15).

Replicative exhaustion is not the only cause of cellular senescence. It can also be induced prematurely by various types of stressors like signaling molecules (e.g. cytokines), ROS and

oncogenes (stress induced premature senescence or SIPS) (16;17). In most cases, senescent cells display upregulated activity of the tumor suppressor proteins p53 and Rb, which also play an important role in the DNA damage response (DDR) which has been shown to be an important initiator of cellular senescence. Telomeres are bound to a complex of proteins forming a protective 'cap'. Shortening of telomeres or damage-inducing factors like ROS can lead to 'uncapping' after which the exposed telomeres are considered as DNA damage by cells.

The cyclin-inhibitor p16 plays an important role in senescence. It is thought to activate Rb in a p53-independent manner, thus creating an extra level of protection against cancer. Indeed, in many human tumors p16 is inactivated. Not all senescent fibroblasts express p16 and those that do, generally do not express p21 or display the typical DNA damage foci at the telomeres. These findings suggest that there is a DNA damage induced route to senescence and a route activated by other stressors, leading to senescence via p16.

The involvement of p53 and Rb in both apoptosis and senescence indicates an interaction between the two pathways, but it is not well understood which factors determine if a cell goes into apoptosis or into senescence (8;10).

Models for studying aging

Animal models

Our knowledge of the mechanisms of aging is to a large extent the result of studying short-lived species (18). Much has been learned from knocking out or overexpressing single genes in these model organisms, but most age-related processes and diseases involve many genes in multiple signaling pathways and complex interactions on the systemic, tissue, cellular and genetic level. Although species ranging from worms to mammals seem to share many mechanisms, translation of results from experiments with these animal model systems to the human situation is very precarious (19). In Drosophila and C. Elegans, the p53 and Rb homologues also regulate apoptosis and senescence but not as an anti-cancer mechanism (8). Between mammals significant differences in signaling can be found. In mouse cells, either p53 or Rb inactivation prevents senescence, whereas in human cells inactivation of both p53 and Rb is required. In mouse cells, p16 inactivation in senescent cells does not

delay the onset of senescence, whereas it does in human cells (17). There are also many differences in signaling between the different tissues within an organism. Thus, cellular responses to stress and damage will be dependent on species, cell type and the type and extent of stress or damage (16).

In vivo versus in vitro

As already alluded to, manipulation of single genes in model organisms has given much insight in to the pathways governing the mechanisms of aging. Evidently, this genetic manipulation is not possible in humans for ethical reasons. It is, however, possible to experiment with cells isolated from humans. Analogous to the translation of results from animal models to humans, the translation of *in vitro* results to the *in vivo* situation is difficult for two main reasons. First, *in vitro* characteristics of cells depend on culture conditions like the type of medium, batch and concentration of added serum, oxygen concentration and the number of PDs undergone *in vitro*, making it difficult to compare studies (20). Second, cells *in vitro* have been removed from their natural environment, being deprived of many factors in the blood (e.g. cytokines and growth factors) and cell-cell and cell-matrix interactions (19). Despite these caveats, cells cultured *in vitro* reflect differences between the organisms they were derived from. Skin fibroblasts derived from longer living species are more resistant to toxic stress when compared with shorter living species (21). Even within species, skin fibroblasts from longer living mutant mice were more resistant to stress (22).

Aim of this thesis

Earlier we showed that nonagenarian siblings from families with the propensity for longevity displayed a 41% lower risk of mortality compared with sporadic nonagenarians (23) and that the offspring of these nonagenarian siblings displayed a significantly lower prevalence of myocardial infarction, hypertension and diabetes mellitus when compared with their partners (23). The general objective of this thesis is to study cellular processes responsible for the increased longevity in the offspring, using human dermal fibroblast strains. We aimed to first show differences in *in vitro* cellular phenotypes, comparing fibroblast strains from offspring and partners under non-stressed conditions and after oxidative stress. As a proof-of-principle we also compared fibroblast strains from chronologically young and old subjects. We

focussed on apoptosis and senescence since they are thought to play a major role in stress responses. Second, we were interested in the signaling pathways driving the differences in cellular phenotypes.

Study populations

The Leiden Longevity Study

This study was set up to investigate the contribution of genetic factors to healthy longevity by establishing a cohort enriched for familial longevity (24). From July 2002 to May 2006, 420 families were recruited consisting of 991 long-lived Caucasian siblings together with 1705 of their offspring and 760 of the partners thereof. There were no selection criteria on health or demographic characteristics. Compared with their partners, the offspring were shown to have a 30% lower mortality rate and a lower prevalence of cardio-metabolic diseases (23;24). A biobank was established from fibroblasts cultivated from skin biopsies from a subset of offspring-partner pairs.

The Leiden 85-plus Study

This study is a prospective population-based follow up study in which 599 inhabitants of Leiden, the Netherlands, aged 85 years took part (25). The study was set up to assess common chronic diseases and general impairments in the general oldest-old population. Information on common chronic diseases was obtained from records of subjects' general practitioners and pharmacies while general impairments were assessed with functional tests and standardized questions during face-to-face interviews. A biobank was established from fibroblasts cultivated from skin biopsies of surviving 90-year-old participants and from biopsies taken from 27 young subjects, serving as a control group.

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