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Long-Lived Cells and Long-Lived Proteins in the Human Body

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1.1 What Constitutes a Long-Lived Cell and a Long-Lived Protein?

It is sometimes stated in textbooks that all proteins in the body are being continuously degraded and resynthesized. It will become clear after reading this chapter that this statement is untrue.

Although one subject of this book is long-lived proteins (LLPs), the definition of what constitutes an LLP is somewhat arbitrary. Clearly, it does not apply to a polypeptide whose half-life is measured in minutes or a few hours; however beyond that, the importance of half-life in terms of its effect on biology is intimately linked to the susceptibility of a particular protein to modification.

In some cases, LLPs have been subdivided into categories, for example, by creating a subgroup of extremely long-lived proteins [1]. For reasons of simplicity in this book, LLPs will be regarded as one category. As a guide, a half-life of greater than 48 hours can be regarded as a yard stick for the classification of an LLP. In most cases, half-lives will be considerably longer than this, and in some cases, there is no turnover during a lifetime. The members of this latter category will be referred to as lifelong proteins.

In this context, one year of life, which we typically consider to be a very short period of time within a normal life span, corresponds to incubation of a lifelong protein at 37°C for 8760 hours. We can infer that in many cases, a period of several weeks may be sufficient to lead to significant LLP breakdown and that this may have major consequences. Predominantly, this conclusion arises from studies in which the main enzyme involved in protection from protein deterioration, protein isoaspartate methyl transferase (PIMT), was deleted. In the absence of PIMT, mice underwent seizures and died 42 days after birth [2]. Over this time period, progressive damage to cytosolic proteins was detected in the brain, heart, liver, and blood cells. Because this protein damage is due to spontaneous reactions, the extent of damage is likely to be very similar in mice and men. Information on the sites of LLPs in the body has been outlined previously [3] and will be summarized in this chapter.

Similar considerations with regard to definition apply to long-lived cells (LLCs). Typically, LLCs are present in the body for a period of many weeks and often may be present for years. Long-lived plasma cells within the bone marrow are responsible for the fact that serum antibodies arising from childhood vaccinations against smallpox are still present in adults, despite the disease being eradicated more than 30 years ago [4]. It should be noted that the focus of this chapter will be human studies.

1.2 Aim of the Chapter

The purpose of this chapter is not to provide a comprehensive review of particular sections that are highlighted but rather, within the confines of word limits, to provide an overview of the field of LLPs and LLCs with a particular reference to their locations within the body.

Where information is available, an attempt will be made to link age-related decline in function of the tissue to the presence of LLPs or LLCs. This is a nascent field and it will evolve rapidly.

1.3 Aging

Although aging is obviously intimately related to the degradation of LLPs, and this is encapsulated in the title of this book, it is not the purpose of this chapter to review aging. Aging is a large, complex subject with a number of theories. Broadly, it can be stated that there are two main camps: researchers who believe that aging is genetically regulated and the other group who believe that aging is primarily the result of molecular degradation. In the latter group, DNA deterioration, particularly as it concerns telomeres, has been a prime focus.

There are numerous phenotypic changes that accompany human aging, among which are wrinkling of skin, increase in abdominal fat, decrease in bone density, loss of cartilage and muscle mass, and, in late stages, a compilation of adverse features that can be termed frailty.

Partridge et al. [5] subdivided the “hallmarks of aging” into nine categories: genomic instability, telomere attrition, epigenetic alterations, stem cell exhaustion, loss of proteostasis, deregulated nutrient sensing, altered intercellular communication, mitochondrial dysfunction, and cellular senescence.

It may seem surprising that LLPs and their age-dependent deterioration were not incorporated as one of the hallmarks of aging in this article [5]. This omission probably reflects the current lack of awareness of the existence of LLPs and will no doubt be rectified in subsequent reviews on the mechanisms of aging. The author predicts that it will rise to become recognized as one of the most important factors responsible for aging of humans and the associated age-related decline in organ, tissue, and overall bodily function. The detailed role of LLP breakdown in determining human life span may remain more elusive; however, a framework for this was published a decade ago [6]. As the name implies, aging is the major risk factor for many age-related diseases including cataract and neurological diseases such as dementia.

1.4 Location of LLPs Within the Body

Many LLPs serve a structural function. LLPs will be considered in two classes: those that lie within the cell and those that are extracellular. It is important to recognize that these are not two independent classes.

It is now known that the extracellular matrix (ECM) is a complex structure composed of many different macromolecules whose structural integrity is crucial for maintaining tissue function. Abnormalities in ECM biosynthesis and catabolism are responsible for a number of inherited and acquired diseases, but it is unknown whether insidious age-related modifications to LLPs within the ECM affect the functions of tissues. For a more detailed discussion of ECM, the reader should refer to Ref. [7].

1.4.1 ECM and Tissue Function

The ECM has a profound effect on cellular function. Four broad types of interactions regulate the growth, development, and function of cells: growth factors/cytokines, cell-to-cell contact, hormones/vitamins, and ECM. Each of these operates via specific cell surface or sometimes via cytoplasmic receptors. This is further complicated by the knowledge that many growth factors and cytokines are bound specifically by matrix components and also that ECM can modulate the expression of cellular receptors for growth factors. Thus, there is an intimate reciprocal relationship between cellular function and the ECM. These complex interactions should be borne in mind when evaluating the aging of individual components within the ECM.

1.5 Extracellular LLPs

1.5.1 Several ECM Components Are Long Lived

The LLPs of the ECM can be subdivided into four groups: *elastin*, *structural glycoproteins*, *proteoglycans*, and *collagens*. These will be considered individually with particular attention to data that pertain to longevity. In a recent study in mice using stable isotope labeling, ECM proteins were among the longest lived of any of the ~3500 proteins analyzed [1].

1.5.1.1 Elastin

Elastic fibers and sheets confer strength, distensibility, and flexibility to tissues. Unlike other ECM family members, elastin is present in DNA as a single-copy gene. This codes for the soluble precursor tropoelastin. Elastic fibers are insoluble, which is a consequence of the high proportion of hydrophobic amino acids and the presence of unique intermolecular covalent cross-links: desmosine and isodesmosine [8]. Elastin has a similar amino acid composition to collagen, in that approximately one-third is composed of glycine. Alanine and valine are also prominent with substantial amounts of proline. The sulfur-containing amino acids, cysteine, and methionine are absent.

Other macromolecules such as lysyl oxidase, glycoproteins, and fibrillin occur together with elastin in the elastic fibers. Elastin plays a vital role in tissues such as the aorta where,

as in other large arteries, it functions in pressure wave propagation and pulse dampening. It is also a major component of the lung, skin, ligaments, oesophagus, cartilage, and the bladder.

It is clear that there are major changes to tissues with aging. Cutaneous aging is the most obvious because its consequences are clearly visible. In the skin, most of the elastin is located in the dermis, which is the spongy middle layer [9]. Degeneration of the elastic fiber network coupled with loss/modification of collagen and a decrease in hydration are linked to visible changes in the dermis with age.

The expression of the tropoelastin gene mainly occurs in the first years of life when the cells of elastic tissues produce the elastin required for the body. After that time, gene expression is reduced significantly and less elastin is made, such that by middle age, only a trace of elastin is produced and we rely on the elastin that was deposited before and during childhood [10, 11]. Therefore, our elastic connective tissues depend on the persistence of elastin. To this end, elastin has been shown to have a half-life of about 74 years [12], and aging of elastin has been proposed to limit human life expectancy [13]. During our life span, the elastin D-Asp content increases to reach ~15% by the age of 60 years. Interestingly, the rate of increase in D-Asp was most rapid in the years up to the age of 20 years and then became linear [10] (Figure 1.3). This mirrors the accumulation of D-amino acids in the lens proteins; however, the reason for this biphasic graph is not known.

1.5.1.2 Structural Glycoproteins and Proteoglycans

It appears that both elastin and the non-elastin components of the elastic fiber are long-lived. This was the conclusion of a study by Shapiro et al. [12] who compared the nuclear weapon-derived ¹⁴C content and the D-Asp levels of elastin.

The content of D-Asp in elastin from aged human lung tissues amounts to approximately 17% by the eighth decade [12]. Such a large extent of racemization would be expected to be accompanied by changes to the properties of the elastin fibres. This is especially so, given that racemization of Asp is but one of many age-related post-translational modifications (PTMs). Very few studies have examined the properties of isolated elastin itself, but there are a number of studies in which the properties of the tissues that depend on elasticity have been documented as a function of age. For example, in the case of lung elasticity, one study measured the age-dependent change in the aerobic capacity of healthy adults [15]. Age-related decline in peak oxygen consumption (VO_2) was pronounced and the decline was not linear. The rate of decline was found to accelerate from 3% to 6% per decade in the 20s and 30s to more than 20% per decade in the 70s.

1.5.1.3 Collagens

Collagens are the most abundant animal proteins accounting for approximately one-third of total body protein by dry weight. The term collagen encompasses more than 30 gene products with approximately 20 different types of collagens. For more information, the reader is referred to detailed reviews, e.g. Ref. [16].

Collagens are predominantly extracellular. The primary gene products form triple helices, and the polypeptide chains undergo extensive PTMs, in particular hydroxylations involving the formation of hydroxyproline (hydroxyPro) and hydroxylysine residues. A common repeating sequence in collagen is Gly-X-Y, with X and Y being Pro or hydroxyPro.

The other unusual amino acid, hydroxylysine, acts as a site of covalent cross-linking, as well as attachment of carbohydrate. Some collagens form sheets, others form fibrils, and yet others form filaments.

The presence of repeated amino acid sequences renders the collagens difficult to analyze using proteomics, although four collagen proteins were present in the list of LLPs identified by Toyama et al. [17]. Most data on the effect of aging on collagens have been derived from the measurement of D-Asp.

A mean collagen half-life of 197 years was calculated for the superficial digital flexor tendon (SDFT) in horses, which was significantly higher than that for the common digital extensor tendon (34 years). By comparison, the half-life of noncollagenous proteins was two years in the SDFT [18]. In these tissues, the D-Asp levels correlated with those of pentosidine. Pentosidine is an advanced glycation end (AGE) product and is a marker of carbohydrate modification. Evidence suggests that the turnover of collagen in patellar tendons may be similar to that from more metabolically active tissues such as skeletal muscle [19]. In biological terms, turnover appears minor and tendon collagen can be considered relatively inert.

Collagen half-life has been calculated in other tissues with Sivan et al. [20] reporting a half-life of 95–215 years in intervertebral disc collagen, whereas Verzijl et al. [21] reported 117 years in articular (knee) cartilage. By comparison, when a noncollagenous protein half-life was calculated from the same tissues, values of 3–25 years were reported for aggrecan fractions in articular cartilage [21] and 6–26 years for intervertebral disc aggrecan [21]. Thus, collagen may be largely stable over our lifetimes in tendons and many other cartilage-containing samples, whereas the other protein components of these same tissues are also LLPs, but with significantly shorter half-lives.

In collagen isolated from both articular tissue and skin, AGE concentrations increased linearly in parallel with D-Asp. The half-life of human skin collagen was calculated to be approximately 15 years [22].

1.5.1.3.1 Aging Skin and Collagen Cutaneous aging occurs through two processes that can be categorized as intrinsic and extrinsic aging [23].

In the human dermis, intrinsic aging is characterized by atrophy because of loss of collagen, degeneration in the elastic fiber network, and loss of hydration. Extrinsic aging is due to environmental factors with a principal cause being ultraviolet (UV) exposure, although tobacco use can also contribute.

Intrinsic aging takes place over time, regardless of outside environmental influences. After the age of 20, a person produces about 1% less collagen in the skin each year, and the skin becomes thinner and more fragile. Less elastin and glycosaminoglycan (implicated in hydration) formation also contributes to skin aging, together with diminished functioning of the sweat and oil glands. Some of the age-related changes to the mechanical properties of dermal connective tissue can be correlated with covalent cross-linking [24]. Accumulation of advanced glycation cross-links and other adducts is covered in Chapter 8. It should be noted that the processes underpinning collagen modification are still subject to revision. For example, it was long accepted that cross-linking on the lysine aldehyde pathway, which is the major one in skin and cornea, involved histidine residues. It has recently been demonstrated that this cross-link, histidinohydroxylysinonorleucine, is in fact a laboratory artifact [25].

Some wrinkle formation as a result of intrinsic aging is inevitable. In purified elastin from skin, the increase in D-Asp was highly correlated with age. Racemization rates were found to be higher in elastin from skin than from lung parenchyma and from aorta [26].

Because of the multiple events taking place in collagens, it is difficult to highlight one degradation or cross-linking process as being responsible for the changes in physical properties associated with aging. It is very likely that multiple chemical and biochemical events are responsible. Aging of elastic fibers and its influence on the elasticity of tissues has been reviewed [27].

1.6 Intracellular LLPs and LLCs

Several questions need to be addressed before the role of LLPs and LLCs can be properly evaluated in terms of the impact of the longevity of macromolecules and their deterioration on overall human health and age-related diseases.

Firstly, how many tissues/organs in the body contain components that are long-lived? Once such sites have been examined, which of the cells within them are old, and which of the macromolecules within these LLCs are also old? Lastly, is there evidence of LLP breakdown and what is known about the consequences?

Before discussing these aspects, it should be recognized that, at the moment, little is known about many issues, partly because LLPs/LLCs is a newly recognized field. It is also worth noting in terms of background that in mice the abundance of the vast majority of proteins in organs and tissues remains unchanged with age [28]; however, some alterations in protein abundance between the livers and brains of young and old rats has been detected [29].

1.6.1 LLCs and LLPs in the Organs of the Body

Individual organs and tissues will be discussed where information about LLPs or LLCs is known. At the end of the discussion of each tissue, a section on aging and possible age-related consequences will be discussed.

A very brief summation of the main function(s) of the particular organ is provided at the beginning of each section. Under each heading, some of the main conditions or diseases associated with aging of that particular organ will be described. Within the scope of this book, it is not possible to cover these comprehensively.

Following this, evidence will be provided for the existence of LLCs or LLPs within the organ.

Before this general description of cells and organs within the body, a recent comprehensive program to examine the lifetimes of brain proteins has revealed some remarkable results [1]. Neurofilament proteins and transmembrane proteins were found to be stable. The cytoskeleton was composed of protein components with varied lifetimes. For example, neurofilaments, tubulin, and intermediate filament proteins were found to be LLPs, whereas the lifetimes of actins approximated that of the average proteome. A picture is emerging in which the actin cytoskeletal network within a cell may be dynamic, whereas the microtubule-based cytoskeleton is more stable. Generally, individual protein lifetimes were conserved across the organs examined. Mitochondrial proteins tend to be long-lived,

although there was some heterogeneity [1]. It is important to recognize that proteomic investigations despite being very elegant cannot provide data on all proteins in a sample because of issues of solubility, amino acid sequence repeats, PTM, and low copy numbers.

1.7 Organs and Tissues that Contain LLCs or LLPs

1.7.1 Long-Lived Cells

The following organs and tissues will be discussed in relation to their content of LLCs and LLPs: the eye, oocytes, kidney, adipose tissue, brain, heart, lung, skeleton, teeth, hair, joints, liver, pancreas, and intestine (see Figure 1.1).

1.7.1.1 Eye

The eye is composed of the lens, cornea, aqueous humor, vitreous humor, and the retina. Tissues will be discussed separately.

1.7.1.1.1 Lens The lens focuses and filters external light onto the retina. The lens is known to be made up of many LLCs. This may be a direct consequence of the growth pattern of the lens. During our lives, epithelial cells are continuously added on the outside of a lens that was present at birth. These epithelial cells differentiate into very long fiber cells that are packed with crystallin proteins but lack nuclei, mitochondria, and other cellular organelles. Lens proteins are known to be LLPs [30].

The human lens has proven to be an ideal tissue for studying the age-related decomposition of LLPs (see also Chapters 3–5). Partly, this is a consequence of there being only a few major proteins, so proteomic investigations are made easier. Another factor is the absence of active enzymes in the center of the adult lens, i.e. the lens that was present at the time of birth. This is due to the fact that all proteins in this region have been exposed to an elevated

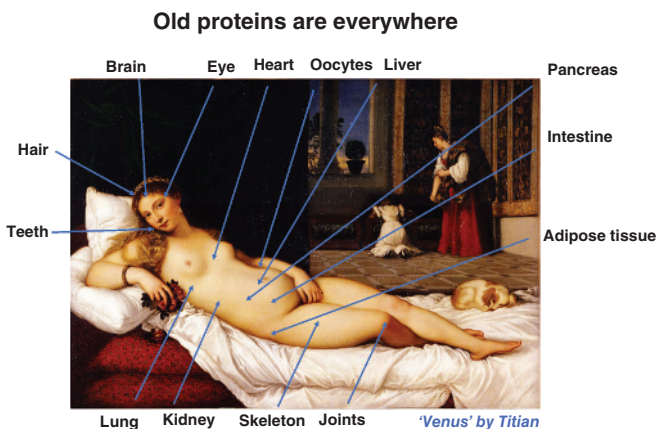


Figure 1.1. “Venus” by Titian is used to illustrate the many sites within the human body where long-lived proteins and long-lived cells are present. Each site is described in greater detail in this chapter. Mark Twain in “A Tramp Abroad” described this masterpiece as the “the foulest, the vilest, the obscenest picture the world possesses.” Source: Adam eastland/Alamy Stock Photo.

temperature ($\sim 35^{\circ}\text{C}$) for 175 000 hours by age 20. Thermal denaturation of the enzymes has taken place.

The lack of enzyme activity indicates that any changes to LLPs in the lens have come about as a result of spontaneous processes. The interior of the adult lens is thus the realm of chemistry and not biochemistry.

This fact has been enormously useful for researchers because it indicates that events such as protein truncation, if found, can be attributed not to enzyme activity but to other reactions such as spontaneous peptide bond cleavage [31, 32]. Similarly, when protein methylation was detected in the nuclear (central part of the lens) researchers recognized that this could arise only from non-enzymatic reactions. This led to the discovery that exposure to *S*-adenosyl methionine alone was sufficient to cause methylation of cysteine residues [33]. As an aside, it should be noted that methylation of DNA bases (as well as of histone Lys and Arg residues) is one of the primary means by which epigenetic processes are mediated. It is not known if non-enzymatic methylation might also be involved in the nuclei of cells or if this might increase with age.

Indeed, the human lens has proven to be a gold mine for elucidating the processes responsible for age-related degradation of LLPs. These various modifications, including racemization, deamidation, truncation, and cross-linking, will be documented elsewhere. The degree of modification is great and increases with age. To illustrate this point, by the age of 60 years, every single lens polypeptide contains, on average, two to three D-amino acids.

Age-Related Changes to the Lens: Presbyopia and Cataract Presbyopia, the inability to focus on nearby objects, affects almost all individuals in the fifth decade. This results primarily from a massive increase in the stiffness of the lens [34]. By this stage in life, all free α -crystallin has been consumed by binding to other lens proteins as they denatured [35]. At this time, an internal barrier to diffusion arises within the lens at a position that corresponds approximately to the lens at birth. The result is that essential antioxidants such as glutathione, which are synthesized or re-reduced only in the outer region of the lens, cannot readily access the central part of the lens. Ultimately, this increasingly oxidative environment leads to the widespread oxidation of lens crystallin proteins in the lens center (nucleus). This oxidation is accompanied by insolubility and coloration [36]. Age-related nuclear cataract is the outcome.

1.7.1.1.2 Cornea Major changes in the cornea with age include thickening of both the epithelial and the endothelial basement membranes. One change in the cornea with age is the decrease in the density of the single layer of corneal endothelial cells, which line the posterior surface of the cornea. Corneal endothelial cells are not known to proliferate [37], so may be LLCs.

1.7.1.1.3 Vitreous Humor The vitreous humor is a transparent gel situated between the lens and the retina, occupying most of the eye's volume. It is composed of hyaluronic acid interspersed in an aqueous network of collagen type II and collagen type IX fibrils [38]. The properties of the vitreous humor change with age, becoming more like a two-phase system. More than half of the vitreous humor becomes liquified in 25% of individuals aged

40–49 years, and this increases to 62% of individuals aged 80–89 years [39]. Although the volume of liquid vitreous increases with age due to phase separation, the gel component becomes stiffer [40].

Age-Related Changes to the Vitreous Humor: Retinal Detachment The most common age-related pathology of the vitreous humor is posterior vitreous detachment (PVD). This involves separation of the posterior vitreous cortex from the lamina of the retina. The incidence of PVD rises from 53% in people older than 50 years to approximately 65% over the age of 65 years.

Aggregation of collagen fibrils may cause vitreous liquefaction in older people, which predisposes them to PVD. The total amount of collagen in the vitreous humor does not change throughout life, so the concentration decreases as the eye grows. It is thought that there is no, or very little, postnatal synthesis of vitreous collagen [41]. Thus, vitreous collagen is probably an LLP that undergoes typical age-related degradation; however, these have not been documented. It is not known if this possible change in the structure and properties of collagen in aged eyes is a factor that predisposes the elderly to PVD; however, this proposition is worthy of investigation.

The most common reason for retinal detachment is age-related shrinkage of the vitreous gel, described above, which can lead to tearing at a weak point in the retina. Once such a tear, or a hole, develops, fluid can collect beneath it and reduce the adhesion of the retina to the choroid, resulting in retinal detachment.

1.7.1.1.4 Retina The retina is the innermost, light-sensitive part of the eye. Photons from the outside world are converted into a neural signal that is transported to the brain via the optic nerve. The structure of the vertebrate retina is complex, consisting of 10 layers. In addition to three types of glial cells [42], five types of neurons are present in the retina: amacrine cells, ganglion cells, photoreceptors, horizontal cells, and bipolar cells. Within retinal ganglion cell neurons, and other neurons, a stable neurofilament network is present, which is composed of proteins that self-assemble, and such structures tend to remain intact [43]. While these may be LLPs, the vast majority of labeled actin is cleared within seven months of its synthesis [44].

Age-Related Changes to the Retina Like other neurons in the central nervous system (CNS), there may be little or no turnover of nerve cells after childhood years. This includes the optic nerve. Photoreceptors are renewed continuously in a process termed disc shedding. Equatorial cones and retinal pigment epithelial (RPE) cells decrease at uniform rates from the second to the ninth decade [45]. Interestingly, the rates of rod and ganglion cell loss were faster between the second and fourth decades.

The RPE is a single layer of cells located in the outermost part of the retina, which nurtures adjacent photoreceptor cells and helps in the renewal of photoreceptor outer segment membranes. The age-related pigment, lipofuscin, accumulates in the human RPE and by the age of 40, approximately 8% of the cytoplasmic volume of macular RPE cells is occupied by lipofuscin granules, whereas by the eighth decade of life, lipofuscin content reaches an extraordinary 19% of the cytoplasmic volume [46, 47]. Such an observation implies, but

does not necessarily mean, that the RPE cells are LLCs because in this case, the accumulation of lipofuscin may be influenced by exposure to light [48].

Age-Related Conditions Affecting the Retina A number of diseases affect the retina, and there is insufficient space to describe them all.

Age-related macular degeneration (AMD) is one of the most common. In AMD, the macula (the central part of the retina responsible for high-resolution color vision) is degraded, which means that vision is distorted. Thus, reading, for example, becomes difficult. The cause of AMD is not known.

Retinitis pigmentosa is characterized by photoreceptor degeneration and progressive blindness. The molecular mechanisms involved in photoreceptor death are also not understood.

Glaucoma is caused by damage to the optic nerve. In glaucoma, the ganglion cells, as well as other cells within the optic nerve, die. One reason is excessive fluid pressure within the eye. Because regeneration of the nerve does not take place, damage is irreversible.

Lamin B1 and lamin B2 are known LLPs that have distinct functions in retinal homeostasis and are present in both rod and cone photoreceptors [49]. In the absence of Lamin B1, cone photoreceptor survival is decreased and synaptogenesis is impaired; however, it is not known if the age-related modification of lamin B1 and lamin B2 affects cell function.

1.7.1.2 Oocytes

Every woman is born with all the eggs already inside her ovaries, and human egg cells are huge (~100µm diameter). A female will release one of these eggs during every menstrual cycle throughout her fertile lifetime. This remarkable sequence of events means that a mother carries egg cells, one of which may one day be fertilized and grow into her own grandchild. The consequence of this process is that eggs are decades old and are thus LLCs. Despite this longevity, once ovulation occurs, the egg cell deteriorates very quickly and dies after 12–24 hours.

1.7.1.2.1 Age-Related Changes to Oocytes Fertilization and pregnancy rates of unfertilized oocytes were measured in three groups of women based on age (group 1, ≤34 years; group 2, between 35 and 39 years; and group 3, ≥40 years). [50] Under the same conditions, fertilization rates in the three groups were indistinguishable; however, pregnancy rates dropped by one-third (group 1, 43.2%; and group 3, 14.3%). The authors concluded that the age-related decline in fertility was due to degeneration in the oocytes. On this basis, “women in the older age group have a higher chance of achieving pregnancy from ovum-donation programs than by persisting in using their own aged oocytes.”

Less is known about the biochemical changes that take place within the body in the oocyte at any stage. A protein involved in chromosome separation in eggs may be implicated in the age-related decline in fertility. The many functional changes associated with oocyte aging have been reviewed [51].

Because of the importance of oocyte health for assisted reproduction, much has been published on the influence of culture conditions and a variety of additives, e.g. [51]. As illustrated by the substantial increase in the rate of trisomy (2–3% for women in their 20s to ~30% for women in their 40s), it is clear that chromosomal errors, particularly relating to

segregation, are a feature of oocytes from older women [52]. It is not easy to tease out the relative impact of DNA degradation and LLP deterioration in these various age-related phenomena. Expression of *BRCA1* and other DNA repair proteins decreases with age, but it is not known if such tumor suppressor proteins are LLPs.

Acetylation of lysine 14 on histone H3 and lysines 8 and 12 on histone H4 in mouse oocytes gradually increased during aging, and it is known that acetylation of nuclear histones can play an important role in various cellular functions [53]. As noted elsewhere, histones H4 and H3 are LLPs, and if they are modified significantly as a result of aging, this may contribute to the alterations in the functional properties of the oocytes.

1.7.1.3 Kidneys

The kidneys maintain fluid balance and excrete waste products produced by metabolism. The functional unit of the kidney is the nephron, which filters the blood supplied to it. The end result is the reuptake of fluid and ions and production of urine.

1.7.1.3.1 Age-Related Changes to the Kidney There is a linear decrease in renal function with age [54], and this is associated with a decrease in the mass of the organ. A number of other physiological functions correlate with age, for example, glomerular size increases. There are approximately 1 million functional nephrons per kidney; however, this number progressively decreases over time [55]. All these changes lead to an overall decrease in glomerular filtration rate.

The kidney is widely thought of as being unable to repair itself once damaged, and it would appear that this organ is composed largely of LLCs [56]. More specifically, the adult kidney possesses some ability to repair the existing nephrons but cannot replace nephrons lost with age with new ones [57].

1.7.1.4 Adipose Tissue

Adipose tissue is present in two types. One type exists primarily for the storage of lipid, whereas brown adipose tissue produces heat by thermogenesis and functions in thermoregulation. Brown adipocytes contain numerous small lipid droplets and many mitochondria, which gives the tissue its color. For a review, see Ref. [58].

1.7.1.4.1 Age-Related Changes to Adipose Tissue Brown adipose tissue is abundant in newborns (~5% body weight), where it has an important role in providing resistance to hypothermia; however, the amount decreases with age. Graja and Schulz evaluated a number of mechanisms for the age-related loss of brown adipocytes. A decline in brown adipogenic stem/progenitor cell function was one [59].

The number of adipocytes is set during childhood. Using bomb-pulse data to calculate cell turnover, it was revealed that neither adipocyte death nor generation rate was altered in early onset obesity and that only 10% of fat cells were renewed annually [60].

1.7.1.5 Brain

It is well known that neurons are LLCs [61]. With regard to proteins within the brain, the vast majority of total proteins have lifetimes between 3 and 13 days [1], although some are much longer. Within this latter group, the proteins of myelin have been the most

thoroughly studied by proteomics, with the evidence strongly supporting the case that the proteins in myelin are LLPs [17] and are probably lifelong. Myelin proteins such as MBP deteriorate with age and to a greater degree in multiple sclerosis patients [62]. The degradation pattern of MBP closely resembles that of lens crystallins, supporting the fact that MBP and presumably the other proteins that compose myelin are LLPs.

Other intracellular proteins are also long-lived. The axons of peripheral nerves contain a large population of very stable microtubules [63, 64] composed of tubulin. Recent pulse-labeling studies have also confirmed the long lifetime of neurofilament proteins [1].

It is also clear that the regions of the brain may differ in terms of protein stability. The lifetimes of some histones, septins, cell adhesion molecules, and exo- and endocytosis cofactors differed significantly in cortex compared with cerebellum [1]. Comparison of whole mouse 13-C Lys labeling with cell culture studies [65, 66] uncovered a greater range of protein lifetimes [1] that more likely reflects the true nature of these within the brain.

1.7.1.5.1 Age-Related Changes to the Brain There are many diseases associated with aging of the brain and CNS. Alzheimer's disease (AD), Parkinson's, motor neurone disease (ALS) are some of the more common diseases. Due to an increase in the longevity of the population, these age-related neurological diseases are becoming more prevalent and are already consuming a huge proportion of the health budget. Despite an enormous research effort, it could be argued that there has been little progress made in understanding the fundamental basis of this group of diseases. At the time of writing, major pharmaceutical companies have scaled down, or exited AD research, because of the failure of many clinical trials. To some degree, this author believes that the lack of progress is due to the widespread use of animal models, which do not adequately replicate the human afflictions, as well as an overall lack of appreciation of the role of LLPs and LLCs in these diseases.

With regard to the cell types other than neurons, it appears that the oligodendrocytes that are responsible for myelination of axons in the CNS are also LLCs [67]. In mice, most oligodendrocytes are formed in the first six weeks of life, and in the corpus callosum, more than 90% of labeled cells survived for more than 1.5 years and as the authors stated "probably outlive the mouse." Despite the vast majority being LLCs, a very small number may be made after one year [68].

With regard to nerves and other cells outside of the CNS, less is known. Myelination is complete in the peripheral nervous system by 20–40 months. In one study, Schwann cells showed very little turnover in the adult nerve, with not a single instance of myelinating Schwann cell division observed over a 70-day period in mice. Despite this quiescence, cells could proliferate within days of an injury [69].

Elastin within cerebral arteries from older people loses its functionality [70], with large differences apparent by confocal microscopy, and this contributes to stiffening of the arterial walls with implications outlined elsewhere in this chapter.

1.7.1.5.2 Protein Isoaspartate Methyltransferase Is Crucial for Brain Function Age-dependent breakdown of LLPs takes place in the normal brain, and ongoing repair is essential for normal function. This is clearly illustrated by studies where PIMT has been deleted [2]. If PIMT is absent, the restitution of Asp (and to a more limited degree, Asn)

residues that have broken down via spontaneous processes is therefore prevented. Although this is just one pathway of LLP deterioration, it is apparently one that must be prevented to preserve functionality.

Analyses of tissues from PIMT knockout mice revealed a striking accumulation of protein substrates for this enzyme, none more so than in the brain. PIMT activity is highest in this tissue [2]. To give some idea of the rate of accumulation, the amount of Asp/Asn-damaged protein in the brain doubled in just 20 days. Many different proteins in the cytosol suffered damage with the authors calculating that 6% of all brain cytosolic proteins were substrates for PIMT.

In relation to the susceptibility of human brain proteins to spontaneous age-related damage, it should be noted that approximately 30% of eukaryote proteins are either completely disordered or partially unstructured [71] with many brain proteins being intrinsically disordered, e.g. Ref. [72], or having flexible regions. This conformational feature renders such polypeptides particularly susceptible to the sorts of spontaneous processes outlined in this book.

1.7.1.6 Heart

The heart is responsible for pumping blood throughout the body. It is composed largely of cardiac muscle cells, also known as cardiomyocytes. A smaller number of pacemaker cells involved in the beating of the heart are also present, as well as endothelial, connective tissue, and mesenchymal cells.

1.7.1.6.1 Age-Related Changes to the Heart Growing older is linked to a decline in maximum heart rate. A decrease in exercise tolerance is evident from the progressive decline in maximal oxygen uptake ($V_{O_2\max}$), starting in the third decade of life and falling by approximately 10% per decade [73].

Over a 70 year lifetime, elastic fibers in the heart and aorta must maintain the ability to recoil an astonishing 3×10^9 times! Heart failure is primarily a disease of the elderly. Half of all heart failure diagnoses and 90% of all heart failure deaths occur in people above 70 years of age [73], and the various age-related changes in human heart structure and function are detailed in this review. Among the many changes documented, the heart fills with blood more slowly in older, healthy individuals.

The turnover of cells in the human heart was studied using the 14-C bomb pulse approach [74]. The findings of this investigation demonstrated that cardiomyocytes are made prenatally and the numbers remain unchanged over the human life span. By contrast, endothelial and mesenchymal cells displayed turnover rates of ~15% per year and <4% per year, respectively. A related study reached similar conclusions [75].

In accord with this recognition of cardiomyocytes as LLCs, the amount of lipofuscin gradually increases with age [76]. Despite the maintenance of the number of cardiomyocytes, there is a pronounced decline in the number of pacemaker cells after the age of 60 years. By the age of 75 years, fewer than 10% of the number present in young adults remains.

1.7.1.7 Lung

The lung enables tissues to be oxygenated and CO_2 to be released.

1.7.1.7.1 Age-Related Changes to the Lung Progressive loss of recoil in the aging lung leads to decreased arterial PO_2 . Elastic recoil refers to the rebound of the lungs after having been stretched by inhalation and occurs principally because of the elastin in the elastic fibers in the connective tissue of the lungs.

Muscular strength of the respiratory apparatus declines and the chest wall stiffens with age. Less oxygen is delivered to the tissues. Loss of the functional capacity of the respiratory system with age increases the risk of respiratory failure and leaves elderly subjects more susceptible to stress [77]. Pioneering studies by Shapiro et al. [12] using measurement of D-Asp, in conjunction with nuclear weapons related 14-C, demonstrated the persistence of parenchymal elastic fibers in adult lungs across the human life span. Their data indicated that D-Asp levels reach approximately 15% by the age of 60 years, so it would not be surprising if such a large degree of modification (presumably together with the other age-related PTMs) may change the properties of aged elastin.

1.7.1.8 Skeleton

Bone is a dynamic tissue, and throughout life, new bone is added to the skeleton by osteoblasts and older bone is resorbed by osteoclasts. During childhood and teenage years, bones become larger and denser because new bone is added faster than old bone is removed. Maximum density and strength are reached around the age of 30 years.

1.7.1.8.1 Age-Related Changes to Bone Osteoporosis, which is characterized by weak and brittle bones, is associated with decreased bone calcium, and this leads to an increased risk of fracture. Osteoporosis develops when bone resorption occurs too quickly, or when replacement occurs too slowly. For women, bone loss begins after menopause. Women can lose 30% of their bone mass over their lifetime, whereas men lose about half of this amount. It is thought that osteoporosis results largely from an imbalance in the activity of osteoclasts and osteoblasts. The average life span of human osteoclasts is about two weeks while that of osteoblasts is three months [78].

One key type of LLC present in bone marrow is plasma cells, which play an important role in the long-term production of antibodies. As an example, smallpox antibodies are maintained in the serum for more than 70 years [4]. Labeling experiments have revealed that most plasma cells in the bone marrow are long-lived [79].

1.7.1.9 Teeth

Each of us gets two sets of teeth in a lifetime, so the teeth of an adult are clearly long-lived. Some age-related changes have been documented [80]. Dentin becomes more yellow with age, and aging is associated with a linear increase in D-Asp in tooth enamel such that it amounts to ~8% of Asp by the age of 60 years [81]. These processes could cause teeth in older individuals to become more brittle. Collagen in dentine is an LLP, and the sequencing of this group of proteins from a 160,000-year old jaw of a Denisovan [82] and other hominids has allowed scientists to construct evolutionary linkages to modern humans.

1.7.1.10 Hair

Hair is quite clearly a LLP, but it is unusual in that it is mostly external to the body, and it is exposed to varying environments, which can include detergents, bleach, dyes, oils, and

UV light. The distance from the root is proportional to the time since formation because each strand grows from the follicle at approximately 15 cm per year. Hair is predominantly made up of keratins, many of which are deamidated, together with some histones [83]. With age, melanocytes within the follicle gradually become less active, fewer molecules of melanin are incorporated into the growing hair strand, and hair becomes gray. Associated with this, hydrogen peroxide can build up to mM levels within the follicles effectively bleaching the hair [84].

1.7.1.11 Joints

Joints occur where two bones are linked together. There are more than 300 in the human body. A major component of joints is cartilage, with articular cartilage being responsible for the mechanical distribution of load. The majority of its structure is controlled by chondrocytes, which are the only cells detected in healthy cartilage. Chondrocytes make the collagen and proteoglycan of which the cartilage is composed [85] and also supply nutrients to the tissue.

1.7.1.11.1 Age-Related Changes to Joints The properties of cartilage change with age [86]. Cartilage is avascular and chondrocytes rely on diffusion of oxygen and metabolites. There is evidence that chondrocyte numbers decrease progressively in articular cartilage as a function of age [87]. Although solid data are lacking, the chondrocytes present in the articular cartilage of an elderly person may be the same cells as those present in a 20-year-old. The half-life of collagen in articular cartilage is estimated to be of the order of 120 years [88] and that of cartilage aggrecan approximately 25 years [89]. Aging is associated with an increase in D-Asp content but, as with other instances, it is unclear to what extent this change in macromolecular structure is responsible for changes to the biophysical properties of the tissue.

1.7.1.11.2 Rheumatoid Arthritis Rheumatoid arthritis (RA) is one of the most common and highly studied autoimmune diseases. It is associated with swollen painful joints. This is discussed in greater detail in Chapter 6.

1.7.1.12 Pancreas

The pancreas functions to aid digestion and to regulate blood sugar. It contains both endocrine and exocrine cells. Some cells of the pancreas synthesize digestive enzymes, whereas others secrete insulin and glucagon for maintenance of glucose levels.

1.7.1.12.1 Age-Related Changes to the Pancreas Numerous morphological changes take place with age [90]. Imaging mass spectrometry revealed that the pancreas is composed of cells with very different lifespans. Some pancreatic beta cells proliferated during adulthood, whereas alpha, delta, and stellate cells together with other beta cells are LLCs and may be lifelong [91] (Figure 1.2). Endothelial cells were also found to be LLCs. The conclusion regarding overall cellular life span for the pancreas is supported by separate studies on lipofuscin accumulation [92].

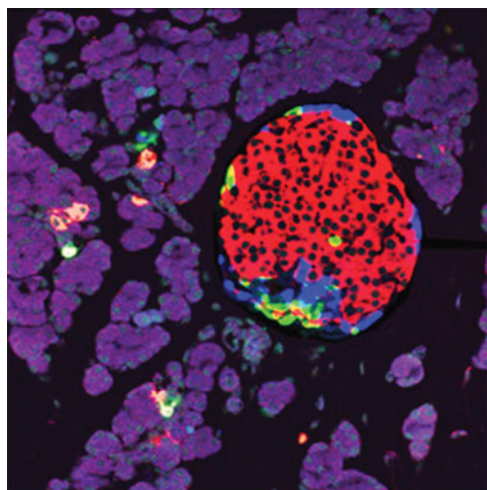


Figure 1.2. *Different cells within the pancreas.* An image illustrating the different types of cells that make up the adult pancreas. Some exocrine acinar cells (purple), whose function is to secrete digestive enzymes, appear to be LLCs, whereas others are renewed. Most endocrine alpha cells (blue) and beta cells (red) are LLCs and appear not to proliferate over a lifetime, whereas other beta cells do proliferate. This phenomenon has been referred to as mosaicism. Data suggest that endothelial cells within the blood vessels may display a similar mosaic distribution. All delta cells (green), as well as most duct cells, resemble neurons in showing no cell turn over. Alpha cells secrete glucagon; beta cells, insulin, and delta cells secrete somatostatin. LLC turnover information from Arroyo e Drigo et al. [91]. This immunofluorescent image was kindly provided by Dr Guoquiang Gu from Vanderbilt University. Source: Provided by Dr. Guoquiang Gu from Vanderbilt University.

1.7.1.13 Liver

The liver is the site of hundreds of different metabolic reactions, and it is generally thought that this organ has a high regenerative capacity. It was surprising therefore to discover that most hepatocytes are as old as neurons [91].

1.7.1.13.1 Age-Related Changes to the Liver Liver function deteriorates with age [93]. Of clinical importance, the oxidative capacity of the liver decreases in older people and therefore medications such as benzodiazepines, which require oxidative degradation, are more likely to accumulate to toxic levels.

Cholangiocytes (epithelial cells of the bile duct) and most hepatocytes are as old as neurons [91]. Endothelial cells within the portal and central veins also did not appear to divide. This was in contrast to sinusoidal capillary endothelial cells and stellate cells [91].

1.7.1.14 Intestine

The intestine is responsible for absorption of nutrients and water.

Plasma cells, which are derived from B cells in the gut, produce antibodies that provide protection against certain microbiota. Some of these antibody-producing plasma cells in the human intestine were found by 14-C birth dating to have a median age of 22 years [94]. This is in stark contrast to epithelial cells of the small intestine, which have a lifetime of a few days.

1.7.1.15 Dividing Cells and LLPs

It may not be surprising that postmitotic cells contain LLPs; however LLPs can also be present in dividing cells undergoing repeated asymmetric divisions in an aging organism [95]. In yeast, for example, the number of cell divisions from a single cell is finite (25–30 cell divisions), and similar asymmetric cell division can lead to dysfunctional stem cells [96]. Using yeast as a model system for dividing stem cells, LLPs were retained in the “mother cell” after cell division [95] in two forms. One was as full-length LLPs and others as fragmented versions. It is not known if fragmentation of the LLPs was due to enzymatic or the spontaneous peptide bond cleavage processes outlined herein.

1.7.2 Sensory Tissues

As well as the lessening of vision, some aspects of which are documented above, our ability to hear, taste, and smell also decreases with age. Some changes take place early in life, with a decreased ability to hear frequencies above 20 kHz noticeable in teenagers [97].

1.7.2.1 Hearing

Inner ear hair cells in mammalian inner ears are fragile and barely proliferate [98]. In humans, approximately one-third of hair cells are lost in subjects over 60 years, with even greater losses in those cells involved in hearing low-frequency and high-frequency sounds [99], consistent with them being LLCs. Interestingly, in this study, neural loss in the inner ear exceeded hair cell loss.

In a lovely isotope mass spectrometry imaging study, the turnover of hair-cell stereocilia from several animal species were examined [100]. One conclusion from this work was that “stereocilia appear to develop once, elaborating an intricate staircase of heights that persists for the life of an animal.” Only the tips of stereocilia showed evidence of rapid turnover.

1.7.2.2 Smell

Decreased olfactory function is common in the elderly, being present in over half of people between 65 and 80 years of age and in over three quarters of those over the age of 80 years [101]. A loss in the ability to smell can also be an early sign of neurodegenerative diseases such as Alzheimer’s and Parkinson’s disease [102].

The loss of the olfactory nerves continues throughout life at approximately 1% per year; however, loss was most evident after the fifth decade of life [103]. This age-related loss has been corroborated using magnetic resonance imaging of the brain in human subjects exposed to particular odors [103] that accord with age-related changes to the olfactory epithelium.

1.8 Protein Changes and DNA Changes with Age

Before the identification of LLPs, and their ubiquity and degradation was recognized, loss of cell and tissue function with age was generally ascribed to damaged DNA. DNA damage is certainly implicated in a number of progerias (accelerated aging), and humans have many enzymes to detect DNA damage. In mitotic cells, telomere shortening with

successive divisions may also be implicated and telomere shortening may influence life span of species [104]. Unlike protein repair, DNA repair involves a highly regulated series of pathways and is overseen by scores of enzymes. For a review, see Scully et al. [105]. The ability to repair DNA also is age dependent. Humans appear to be able to repair DNA damage effectively until around the age of 55 years, after which there is a noticeable decline [106].

In some cases, it may not be necessary to clearly differentiate these two phenomena because DNA and LLP damage may be inextricably linked. For example, aging of long-lived histones may lead to errors in transcription, although this has yet to be demonstrated. Deterioration of nuclear pore LLPs may impact upon the communication between nuclear DNA and mitochondrial DNA and their mRNA products, as well as transcription factors.

It is probable that both protein and DNA deterioration have significant impacts on human aging.

It needs to be emphasized that it is not proposed in this chapter that all defects associated with aging are the result of LLC and/or LLP breakdown, rather that LLPs should be considered as part of an overall age-related degradative scenario that involves both DNA and protein.

There are many ways in which DNA and LLPs could interact in the aging process, and some have already been mentioned. As noted earlier, several progerias are linked directly with defective DNA repair [107] with each recapitulating certain aspects of aging. DNA repair is performed by enzymes; however, it is not known if any are LLPs or if they are susceptible to age-related protein modification. A significant increase in 8-oxo-2-deoxyguanosine levels has been observed in nuclear DNA with age in all tissues [108], and it appears that DNA repair pathways may become less efficient with age [109].

In organs and tissues where LLPs are synthesized and under normal circumstances do not turnover, it is unlikely that age-related DNA degradation will have a direct impact. Structural integrity and function at such sites will mostly depend on the ability of the LLPs to resist protein deterioration.

1.9 Processes Responsible for the Breakdown of LLPs

It is apparent from the foregoing summary that the human body contains many organs and tissues where cells are long-lived (see Figure 1.1). As illustrated for several organs, not all the cells within them have the same turnover rates. This has been referred to as mosaicism [91]. It is likely that the activity of an organ will be compromised, even if only some of the cells within it suffer age-associated damage.

Major reactions responsible for the age-related decomposition of LLPs can be divided into three categories: modification by reactive chemicals, spontaneous reactions, and enzymatic processes. These categories have been reviewed [110] and see also Chapters 4 and 8.

Possibly, the degree to which one of the three categories is dominant may depend on the protein and its site within the body, although insufficient information currently exists to properly evaluate this. In the lens, the most abundant reactions are due to spontaneous reactions arising from the chemistry of amino acid side chain and alpha amino groups. They can be summarized as truncation, deamidation, racemization, and cross-linking.

Reactive chemical modification in the lens is primarily due to exposure to sugars/ascorbate (Chapter 8) and *S*-adenosyl methionine. A surprising finding is that lens crystallins are exposed constantly to mM concentrations of ascorbate and glucose, yet the levels of protein modification appear to be very low [110].

Enzymatic modification to the lifelong proteins in the lens center is negligible, but this is probably a reflection of the fact that active enzymes are absent because of thermal denaturation as described earlier. One illustration of this is the absence of citrulline in lens proteins. By contrast, in myelin basic protein from adult brain, many Arg residues have been converted to citrulline, presumably because of the action of arginine deiminase.

Two prominent causative agents for human LLP denaturation are simply heat and time [111]. It is possible that chaperones may play an important role in modulating the spontaneous breakdown processes by binding to LLPs and preventing conformations that may otherwise lead to modification. They can also sequester LLPs when they unfold, and this is discussed in more detail in Chapter 3.

1.10 Oxidation: Methionine Sulfoxide Reductases and the Glutathione System

One category of age-related decomposition of LLPs by reactive chemicals deserves special mention: oxidation. Although there is only one enzyme (PIMT) to protect against general racemization reactions to which LLPs are prone, several enzymes are present to minimize oxidative stress.

Until the spontaneous chemical reactions of proteins were appreciated, if age-related protein damage was encountered, oxidation was typically invoked. In some cases, there does appear to be a relationship between oxidized proteins and age [112]. In the normal human lens, this is not the case, even up to the age of 80 years [113], despite there being massive oxidation of protein thiols and methionine once age-related nuclear cataract commences. Analysis of protein carbonyls as an indicator of age-related oxidative damage is fraught with methodological difficulties, see Ref. [114].

Even if oxidation does increase with age, as Stadman has noted [115], the accumulation of oxidized protein in one case might reflect an increase in the rate of reactive oxygen species (ROS) formation, whereas accumulation in another could reflect a loss of antioxidant activity.

Cysteine and methionine residues of proteins are the most sensitive to oxidation by the kinds of ROS generated within the body. Cysteine residues oxidize to form protein disulfides and can be repaired by disulfide exchange reactions carried out by thiol transferases that catalyze reactions that involve glutathione or thioredoxin to regenerate the protein sulfhydryl groups. Enzymes such as glutathione reductase, superoxide dismutase, and catalase contribute to protection of macromolecules by maintaining a reducing environment within the cell and scavenging ROS.

Methionine sulfoxide reductase is present in mammalian cells in two forms that differ in location and substrate specificity [116]. These enzymes help protect cells from oxidative stress by converting Met sulfoxide residues back to Met, and knocking out one form (MsrA) in mice is associated with a greater accumulation of aggregated proteins in the hippocampus [117].

In summary, ROS-mediated oxidation represents another panoply of reactions to which LLPs are exposed. One result is that damaged LLPs build up within aged cells. Although we have a number of antioxidants and enzyme systems to protect us from our dependence on oxygen, these cannot be 100% effective. Therefore, over time, ROS-generated damage may act in concert with the spontaneous chemical pathways detailed elsewhere to diminish the viability of aged cells.

1.11 Consequences of LLP Decomposition

Based on PIMT knockout data, a significant percentage of cytosolic proteins, in a variety of organs, undergoes spontaneous breakdown on a time scale measured in days [2]. If PIMT is present, many Asp/Asn sites will be repaired to some extent and presumably polypeptides damaged at other sites will undergo recycling as part of proteostasis. Therefore, to some degree, this is a dynamic system.

It needs to be emphasized that we have a repair system for only one type (Asp/Asn) of age-related protein decomposition and that even this single defense is imperfect. Asn residues that undergo spontaneous deamidation induce a charge change at that location and cannot be returned to their initial state. Deamidation even at just a single site in a protein can lead to major changes in structure and its polymerization [118]. There is some evidence that PIMT activity may decrease with age based on transcriptional data [119].

If spontaneous chemical processes involve amino acids other than Asn or Asp, enzymatic defenses apparently do not exist. Inevitably, the levels of these PTMs will increase over time, and this will be especially important for postmitotic cells. As noted elsewhere, PIMT is a cellular enzyme, so extracellular LLPs do not have even this level of protection.

1.11.1 Protein Modification and Cellular Processing

Breakdown of damaged LLPs is also compromised because the proteolytic enzyme systems used by the cell for protein recycling are unable to adequately handle some age-related PTMs. This is particularly true for racemized and cross-linked species [120, 121]. This may be one major source of lipofuscin (age pigment), whose appearance tracks that of cell age [122]. Lysosomes progressively accumulate lipofuscin, an autofluorescent, polymeric material of an ill-defined composition that is made up of degraded lipid and protein. Its accumulation has been linked with the appearance of abnormal mitochondria [123]. For more details on this aspect, readers are referred to Chapter 7.

1.11.2 Lifelong Proteins and the Consequences

The considerations discussed above for the recycling of such LLPs do not apply for proteins that are lifelong. There is no prospect of them being replaced by new versions.

The consequences of such a scenario are most clearly evident in the lens. The outcomes of such insidious protein breakdown processes eventually become apparent in terms of tissue function. For the lens, this is presbyopia and cataract.

While the outcome of having LLPs in the lens may be intelligible, there appear to be many other LLPs that are lifelong; however, the consequences of their degradation are less well known. Decomposition of such polypeptides will also have major consequences. Among such lifelong proteins are elastin in the heart and lung, collagen at several sites including the eye and cartilage, histones, some nuclear pore proteins, and the proteins of myelin. This is not a comprehensive list because additional members of the lifelong protein family will be identified as more information is garnered in the future. Information is now available about the sites and types of modification of lens proteins as a function of age; however, in every other case of a lifelong protein, with the few exceptions being elastin [12] and myelin basic protein [62], very little is known about the sites, extent, or time frame of decomposition.

It should be noted that some of the lifelong proteins listed above are extracellular. Because PIMT is cytosolic, for extracellular proteins, even this single protein repair pathway is unavailable. One factor that may limit the age-related carnage is that many extracellular LLPs are insoluble or poorly soluble. Although it has not been examined experimentally, it is likely that spontaneous decomposition processes that are dependent on intrinsic flexibility [124, 125] will take place to a smaller degree in insoluble proteins, as will the addition of reactive small molecules or enzymatic modification.

1.12 LLPs and Age-Related Disorders

If LLPs do play an important role in age-related diseases, how might this occur?

It would be expected that problems would arise mostly in postmitotic cells or at least those that are LLCs.

1.12.1 Modified LLPs Acting as Novel Antigens: Autoimmune Diseases

The role of age-altered LLPs as “nonself-antigens” and thus stimulators of the body’s immune system and potentially the subsequent induction of autoimmune diseases is covered in Chapter 6.

1.12.2 Defects in Cytosol/Nuclear Communication

All proteins of the cell nucleus are imported from the cytoplasm. mRNA, tRNA, and rRNA that are synthesized in the nucleus need to be exported to the cytoplasm. This process can involve multiple import and export events [126]. For example, for the biogenesis of ribosomes, the ribosomal proteins that are synthesized in the cytoplasm from exported nuclear mRNA must then be imported back into the nucleolus, where they combine with rRNAs and then finally are transported via the pores to the cytoplasm.

The nuclei of aged LLCs contain pores where some of the scaffolding LLPs are defective [17], and this may compromise transport into and out of the nucleus. Because the majority of components of the mitochondria are coded for inside the cell nucleus, this could eventually interfere with mitogenesis. It may also lead to mitochondrial mutations. One reason for this is that mitochondrial DNA is repaired and replicated using polymerase, which is encoded by the nucleus [127]. Errors caused by this impaired access to replication

machinery, as well as a failure to repair errors, would result in mitochondrial DNA mutations. Clonal expansion of such mutations could result in mitochondrial dysfunction. Defective mitochondria and an increase in ROS arising from the electron transport chain have long been proposed to have a role in aging.

Transport defects may become affected and noticeable only under some conditions. For example, a G protein pathway suppressor protein moves from the mitochondrion to the cell nucleus in response to stress [128].

In aged cells that contain damaged nuclear pore components, such as nucleoporins, some cytoplasmic proteins may be able to diffuse into the nucleus in an unregulated manner. An example of this was observed in the case of a tubulin, which is normally found only in the cytosol, but was detected in the nuclei of some aged rat cells that were also permeable to a 70 kDa dextran [129].

It should be noted that these particular scenarios link age-related defects in LLPs with possible age-related dysfunction in nuclei and mitochondria. Defective LLPs may also alter nuclear DNA transcription in other ways as discussed briefly in Section 1.12.3.

1.12.3 Defects in Nuclear Transcription

Age-related changes to some histones may well compromise transcription in older cells. Many histones show low turnover [130]. In particular, histones H4 and H3 are LLPs and are tightly bound to DNA in the nucleosome [131].

1.12.4 Breakdown of Abundant Macromolecules

If LLPs within the body are abundant, then incremental changes over time may end up having large ramifications for the function of tissues/organs and therefore the health of the individual. Aspects of this have already been covered in relation to the lung, heart, joints, etc.

1.12.5 Elastin

One such example is elastin. As the name suggests, this macromolecule is responsible for tissue elasticity, and with age, tissue elasticity decreases. Pressure surges in blood flow have been implicated in AD [132], and aortic stiffness is significantly associated with an increased risk of dementia [133]. As illustrated in Section 1.16.6, decreased elasticity of the lung may lead to diminished oxygenation of tissues, predisposing them to damage and also infection. The consequences of age-related changes to elastin are considered in more detail later in this chapter.

1.12.6 Collagen

Collagen is an LLP and is the most abundant protein in the body. Its age-related decomposition at numerous sites is likely to have major implications.

Limited metabolism of collagen has been detected at some sites, for example, in the Achilles tendon [134], although other investigators found very low, or negligible, turnover

in this tissue using 14-C bomb-pulse experiments [135]. In other sites, such as joints, there may be little or no turnover. Osteoarthritis is present in at least one joint in over 80% of older adults [136]. Damage to the meniscus is commonly observed in osteoarthritis of the knee, and radiocarbon dating has clearly demonstrated that there is essentially no turnover of collagen in human articular cartilage [137].

1.13 Neurological Diseases Where LLPs May be Implicated

1.13.1 Multiple Sclerosis

Axons within the CNS are myelinated, and it is probable that all of the proteins within myelin are LLPs. Certainly, the breakdown of MBP is consistent with this. It has been proposed that myelin protein degradation is the primary cause of MS [138].

1.13.2 Motor Neuron Disease (MND)/Amyotrophic Lateral Sclerosis (ALS)

Motor neuron disease (MND) is a neurodegenerative disease that affects the motor neurons that control muscle fibers. It is characterized by the presence of ubiquitinated aggregates composed of selected proteins, such as FUS and TDP43 [139].

The same proteins are detected in aggregates associated with other neurodegenerative disorders, suggesting that they may have a more widespread role in neurodegeneration.

The axons of motor neurons are very long, and the transport of molecules, such as proteins, from the site of synthesis in the cell body to the synaptic terminal can take hours to weeks [140]. If those proteins being transported contain unstructured regions, and this is true for FUS and TDP43, then this period of time may be sufficient to cause significant modification, unfolding, and aggregation. Along with an age-associated decrease in PIMT, such a sequence of events may be a root cause of MND, although this novel hypothesis needs to be tested.

1.13.3 Alzheimer Disease (AD)

A comprehensive stable-isotope labeling study of over 3000 brain proteins revealed the unusually long lifetime of neurofilaments [1]. Related to this, there are a number of studies which suggest that the proteins involved in AD; Tau, and A β , are also long-lived. Incubation of recombinant Tau, a protein involved in microtubule assembly, replicated the nonenzymatic fragmentation at Asn residues, deamidation, and smearing of Tau observed in human AD brain samples [141]. This is consistent with Tau, at least in AD, being a LLP.

Similarly, the spontaneous fragmentation of A β coupled with β isomerization of Asp residues, and some Ser residues [142], demonstrate that A β is a LLP. Such changes can be reproduced in the test tube [143, 144]. Does this high degree of age-related modification indicate that A β in human AD plaque has been present for many years? Imaging has established that amyloid plaque is present in normal aged brain, and this observation is consistent with the components being very long-lived. It is likely that the many PTMs present in plaque A β impede its clearance. Several groups have reported that cleavage of A β ,

as well as racemisation/isomerisation of Asp and Ser residues, can increase the neurotoxicity and aggregation propensity of the modified peptides (see Ref. [145]). Another key question is whether, and to what extent, any of these PTMs are present within the amyloid precursor protein before its enzymatic hydrolysis to A β .

1.14 Aging DNA and LLPs

It should be emphasized that it is not proposed in this chapter that all defects associated with aging are necessarily the result of LLP breakdown, rather that LLPs should be considered as part of an overall age-related degradative scenario that involves both DNA and protein.

It is now apparent that LLCs and LLPs are found within many organs of the human body. Over time, long-lived macromolecules decompose, and it is likely that such degradative processes play a role in aging as well as a number of age-related diseases, in particular neurological diseases. Once this phenomenon is recognized, novel experimental avenues for investigation become apparent. As outlined in a separate chapter, LLP decomposition could be responsible for autoimmune diseases.

On the other hand, some experimental approaches commonly employed to gain information on human aging, for example, those that involve the use of short-lived animals, may become less relevant.

There are many ways in which DNA and LLPs could interact in the aging process, and some have already been mentioned. Several progerias (diseases where the rate of aging is increased) are linked directly with defective DNA repair, e.g. [107] with each one recapitulating certain aspects of aging. DNA repair is performed by enzymes; however, little is known about their longevity or susceptibility to age-related protein modification. A significant increase in 8-*oxo*-2-deoxyguanosine levels has been observed in nuclear DNA with age in all tissues [108], and it appears that DNA repair pathways may become less efficient with age [109].

1.15 How Can the Role of LLPs in Aging and Disease Be Investigated? What Can Be Done

Animal Models

As I have discussed elsewhere, the tools available to study the role of LLP degradation in age-related disease, and aging itself, are limited [3]. To a large degree, it is not possible to use laboratory animals that have until now yielded such a treasure trove of data on biochemical pathways and diseases. There are several reasons for this. The most obvious reason is that the common organisms used in laboratories throughout the world, such as rodents, fruit flies, and nematodes, simply do not live long enough. For example, the mean life span of nematode worms (*Caenorhabditis elegans*) is ~17 days [146]; fruit flies (*Drosophila melanogaster*), ~50 days [147]; mice (*Mus musculus*), 1.3–3 years, and zebrafish (*Danio rerio*) ~3.5 years [148]. Although this statement relating to the lack of utility of laboratory animals is generally true, it should be recognized that in some specialized cases, such as PIMT KO animals, valuable data can be obtained, particularly on the identification

of susceptible polypeptides and sites of Asp/Asn modification. Using this KO model, some aspects of age-related protein degradation in the brain can be replicated.

Another feature that should be considered is that even members within the class *mammalia* can vary significantly. Thus, extrapolation of data from rodents to humans cannot be undertaken without considerable caution. One simple example serves to illustrate this. The lens of the eye in both rats and man acts to transmit visible light to the retina; however, lens shape, flexibility, protein content, compaction, phospholipid content, the presence of UV filters, and some metabolic pathways differ significantly in humans and rodents [149].

1.15.1 Heterogeneity of Aged LLPs: A Large Hurdle to Overcome

In addition, aged LLPs are very heterogeneous and are thus almost impossible to create artificially, for example, using molecular biology. This can be readily illustrated using a hypothetical example, where the outcome of decomposition at one Asn site in one LLP is examined. Spontaneous reactions of this amino acid over time will lead to deamidation and the introduction of a negative charge. Typically, deamidation will only be partial, and some of the Asn side chains at this site will remain unchanged. In the subsection of proteins where deamidation has taken place at that site, there will be a mixture of four Asp isomers (see Scheme 4.4) in ratios that may depend on the particular Asn and its local environment. In addition to this, there is the possibility of spontaneous peptide bond cleavage [150] and covalent cross-linking [150] at this Asn site.

The heterogeneity illustrated above exists for just one site in an old protein. Based on lens protein data, it is rarely the case that an aged LLP is modified at just a single position in the polypeptide chain. An example is gamma S crystallin, where in adult human lenses, Asn deamidation is present at several sites [151], and this is accompanied by Gln deamidation [124], covalent cross-linking [152], oxidation of thiol groups [153, 154], and peptide bond cleavage [155].

It is unquestionably a difficult, and in some ways a wicked, problem. To a large degree, it is my opinion that at present, in the absence of suitable models, there is no option other than to accept correlation as an outcome, a conclusion that may be problematic for many scientists to embrace. Unfortunately, despite its knotty nature, there is little doubt that it is one that needs thorough investigation if scientists are to truly understand the processes responsible for human aging and especially those implicated in human age-related diseases. LLP degradation may be a key piece of the puzzle that has been missing thus far, and recognition of this feature will assist in tackling the etiology of human neurological diseases that have thus far proved to be largely intractable.

1.16 We Will Not Live Forever

As elaborated in the earlier part of this chapter, the identification of ubiquitous LLPs has profound consequences, fundamentally because they decompose in a time-dependent manner. LLP decomposition will adversely affect the properties of tissues and organs, which contain them such that fitness of the individual decreases. As the process continues, multiple organs will be less able to function as they did in youth. Because this process takes

place at numerous sites, overall well-being, as well as the ability to fight disease diminishes. Ultimately, the body will succumb.

1.16.1 LLP Degradation and Tissue Function: Is There a Threshold for Decay?

One hypothesis is that organs and tissues may be able to cope with a certain percentage of LLP modification without significant alteration in properties. Beyond this level, cell and tissue damage, with measurable functional consequences, will ensue. An illustration of such a phenomenon is the human lens, where marked modification of crystallins can be tracked from the time of birth, yet functional decline is not evident until the fifth decade, when presbyopia occurs. As indicated earlier, this age is when all of the chaperone, α -crystallin, has been utilized in binding to other proteins as they unfolded in the lens because of the many events listed in this book. Now, in the absence of intracellular chaperones, events not observed in the early years of life can be detected – the most obvious biochemical one being the strong association of protein aggregates with cell membranes. Nuclear cataract is the eventual outcome. It is conspicuous that at every age, the extent of overall racemization of proteins from cataract lenses is significantly greater than in people with clear lenses [14]. It was proposed that once a certain threshold level of racemization is exceeded, that cataract may be inevitable.

In what other organs and tissues in the body might such processes be evident? Data are only preliminary, and there is much research that needs to be done. Each organ will no doubt differ in terms of the impact of age, but we can formulate some hypotheses based on the existing information.

1.16.2 Lifelong Proteins May Degrade at Similar Rates

Lifelong proteins appear to decompose over time at similar rates. This is illustrated in Figure 1.3. The graphs demonstrate that soluble intracellular proteins, crystallins, undergo racemization in a time-dependent way. Racemization occurs more rapidly in the earliest years of life for reasons that are still unclear.

There is a striking correlation of this lens crystallin graph with that of another lifelong protein, lung elastin. Elastin is an insoluble extracellular protein.

What makes this high level of similarity even more remarkable is not due to their different functions, nor to the fact that one LLP is from the eye and one from the lung, but rather that the two proteins have very different amino acid compositions and structures. The best approximation of the structure of elastin is that of a twisted rope [156], whereas lens crystallins have a globular conformation made up of beta sheets. Crystallin structures are illustrated in detail in Chapter 3.

The discovery that two such fundamentally different proteins decay at almost identical rates could not have been predicted. At this point, care must be taken not to over extrapolate because racemization is simply one measure of LLP deterioration, albeit probably the most abundant one. In addition, general conclusions should not be drawn from a comparison of just two proteins. In relation to this latter point, another lifelong protein, myelin basic protein, obtained from the human cerebellum has also been found to degrade in a

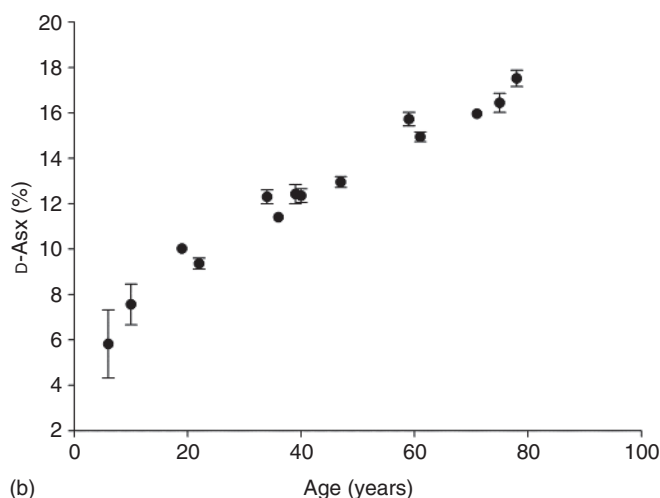
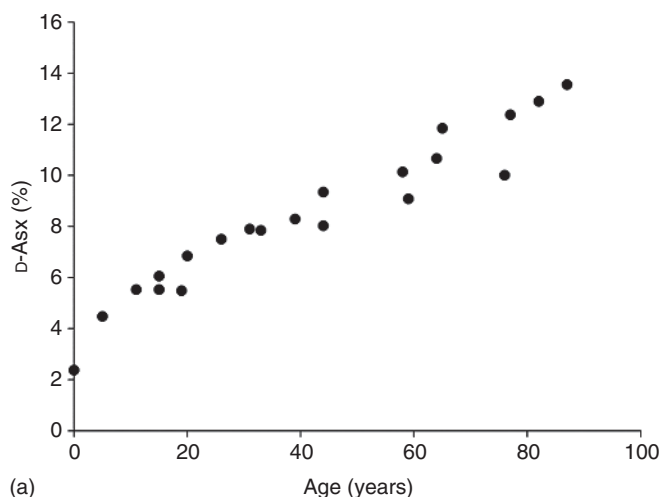


Figure 1.3. *Lifelong proteins breakdown at similar rates.* The two graphs show the progressive increase in the D-aspartate (D-Asx) content within lens proteins from the human eye (a) [14] and elastin from lung (b) [12] as a function of age. All proteins are synthesized in the body with L-Asp or L-Asn. Over time, these L-amino acids undergo spontaneous reactions that cause them to convert to the D-form (see Chapter 4). This racemization can have major consequences for the structures of the proteins. Source: (a) Modified from Hooi and Truscott [14]; (b) Reproduced from Shapiro et al. [12]. Copyright 1991, American Society for Clinical Investigation.

similar manner [62]. Myelin basic protein is an LLP in a completely different environment; it is embedded within a lipid membrane. Despite this lipophilic milieu and the fact that it is unusual in being among the most highly positively charged proteins known, the absolute amount of racemization [62] of myelin basic protein was found to be similar over time to that of elastin and crystallins. Clearly, the time course of degradation of other lifelong proteins such as nucleoporins, once known, would add considerably to our knowledge of this field and the apparent commonality in terms of rate of lifelong protein degradation.

1.16.3 Decay in Tissue Function with Age and Its Effect on Fitness, Health, and Mortality

Aging is the largest risk factor for most human diseases, and it has been calculated that ~90% of people die from age-related causes in industrialized nations, which is in stark contrast to the situation in low- and middle-income countries [157].

If it is true that the function of tissues is impacted by large-scale modification of abundant structural proteins, then some predictions can be formulated. Firstly, where might age-dependent decreases in tissue and organ function be observed?

If deterioration of the amino acids in elastin affects its properties, the performance of both heart and lung, where function depends on this elasticity, should diminish in an age-dependent manner. This has been observed. In addition, if the major functional unit, elastin, is compromised, then because elasticity of the major blood vessels diminish, greater pulses will be generated within the cardiovascular system.

Collagen and elastin in many tissues such as joints will be less functional. Muscle mass (in which elastin is present) and strength decline significantly after the third decade [158], so once diminished heart and lung capacity are added to this sorry framework, it is perhaps not surprising that sportspeople reach maximal performance in their 20s and early 30s. (NB golfers are not included in this category.) We do not know the extent to which LLP degradation is responsible, or the degree to which other factors might also contribute.

The cover of this book has been chosen to represent in graphic terms, the inevitable decline in body function, which follows the second decade of life, and an individual's response to this rude awakening.

1.16.4 LLPs and Life Span

The potential impact of LLPs on life span has been reviewed previously [6], but one aspect that relates tissue elasticity to aging, health, and life span is discussed in more detail below in a number of organs.

1.16.5 Heart

Cardiovascular disease is the leading cause of mortality and is responsible for approximately 30% of deaths worldwide [159]. Many studies have found that blood pressure increases with age, and this is mostly related to the increase in the stiffness of the large arteries, e.g. Ref. [160]. Blood pressure is strongly associated with mortality rates from stroke, ischemic heart disease, as well as other vascular diseases [161]. The Framingham Heart Study monitored people for 30 years and found that systolic blood pressure increased from age 30 to 84 years, whereas diastolic blood pressure slowly decreased after the age of 50–60 years [162]. There was a steady rise in pulse pressure beginning around the age of 40 years, which the authors indicated was consistent with “increased large artery stiffness.” It has been hypothesized that this factor may initiate microbleeds in the brain and could lead to Alzheimer's disease [163].

1.16.6 Lung

Pulmonary function is inversely correlated with death from all causes, and poor lung function, as measured by forced expiratory volume, is a well-established predictor of mortality. Numerous factors have been investigated in an attempt to explain why poor lung function is associated with mortality [164]. Forced expiratory volume declines in an age-dependent manner, and the rate of decline may increase with age [165]. Factors other than diminished elasticity, such as respiratory muscle strength, may also be responsible for some age-dependent loss of lung function.

With regard to the role of degraded LLPs and life span, what is currently lacking for both heart and lung is a clear demonstration that diminished elasticity is due to alterations in the properties of aged elastin itself. If this is confirmed using tissue samples, the specific involvement of racemization and other age-related PTMs of elastin in affecting its properties would need to be established. Such studies are feasible.

1.16.7 Nerves and Brain

In terms of our life span, and longevity, it is within nerves and brain where age-related damage to LLPs may have another huge impact. The heart, lungs, and joints can now be replaced surgically, but there is no prospect of this for the brain. Even if it becomes possible to replace a brain in the future, who would be the recipient?

1.17 Conclusion

If indeed degradation of the ubiquitous macromolecules, proteins and DNA, over a period of years is largely responsible for human aging, and if this is also the basis for numerous age-related diseases, then the ramifications are profound. Recognition, however, is accompanied by a sense of inevitability. There can be no fountain of youth, nor will it be possible to turn back the clock. Life becomes an inexorable march toward the grave. The best we can hope for in the future is that regulatory biochemical pathways are discovered, which respond to drugs and diets, so that it becomes possible for us to extend the young adult phase of life. Our slow decline would thus be ameliorated. It will be feasible to replace some organs and tissues with artificial ones, but this will never be possible for the brain.

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