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Review article

J. KNAPOWSKI, K. WIECZOROWSKA-TOBIS, J. WITOWSKI

PATHOPHYSIOLOGY OF AGEING

Department of Pathophysiology, University of Medical Sciences, Poznań, Poland

Ageing is characterized by a gradual decline in organ functional reserves which reduces the ability to maintain homeostasis under conditions of stress. Introduction of cell culture and molecular biology techniques has provided new experimental tools for the analysis of ageing at the molecular level. During ageing progressive degeneration of cells and loss of regenerative capacity are enhanced and with time the alterations caused by them ultimately lead to death. In this paper the current knowledge of the mechanisms of ageing is summarized.

Key words: *mechanisms of ageing, functional parameters, cellular ageing*

The past few years have seen an unparalleled increase in biomedical research aiming at defining molecular basis of ageing. This worldwide interest was dictated to a large extent by alarming demographic data showing that both the number and the percentage of elderly people in societies had been growing steadily. It is difficult to answer a question of how old one must be to be treated as an elderly man. For practical reasons this criterion has been set for 65 years of age. However, it is necessary to emphasize early in this review that the calendar age itself is an imperfect indicator of ageing. From the clinical point of view, the two people of the same calendar age are likely to differ far more when elderly than when at any other stage of life. This is because their physical condition is related not only to ageing, but also psychological and social status, and – importantly – the presence of chronic diseases.

Functional changes in ageing

For centuries people have been trying to find a miraculous remedy which could prevent or delay ageing. As these efforts have proved unsuccessful, ageing was put into the category of incurable diseases becoming a synonym of disabili-

ty, frailty and worthlessness. Thus, the elderly have been classified as hopeless patients (1). However, this miserable approach to ageing changed in the 1950s, when it became clear that ageing was nothing but a specific aspect of physiology, part of a continuum of developmental processes. Experimental methods available at that time, now referred to as classical, made possible to study the mechanisms of ageing at a tissue and/or organ level. These methods sufficed to demonstrate quickly enough that there were considerable differences in many bodily functions between young and old individuals (*Fig. 1*). For example, it had been found that

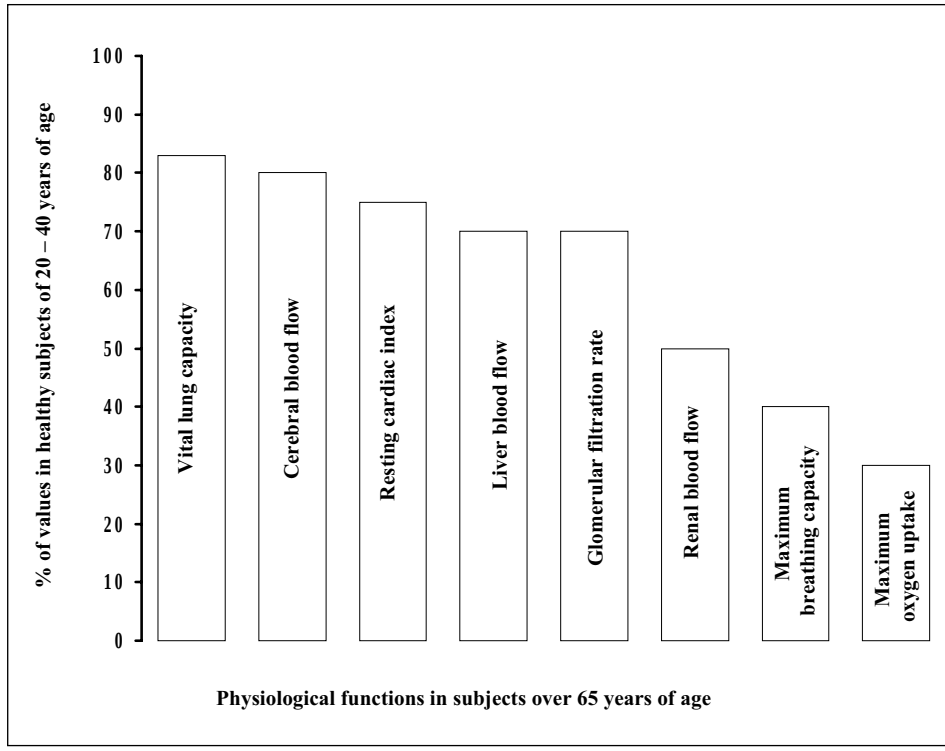

Fig. 1. Relationship between age and selected functional parameters (based on ref: (42-44)).

ageing is associated with decreased perception of environmental stimuli and impaired activity of both motor and sensory pathways. Higher functions of the central nervous system are also affected as typified by the loss of memory. The most frequent alterations in the cardiovascular system include decreased cardiac output, sclerosis of cardiac valves, decreased heart rate, and arrhythmias. Many abnormalities are observed in the endocrine (decreased levels of triiodothyronine and free testosterone, but increased those of insulin and parathormone), respiratory (reduced vital capacity and maximal oxygen uptake) and gastrointestinal (decreased secretion and absorption, slow motility) systems. Typical changes in the kidney comprise the decrease in glomerular filtration rate and renal blood

flow, and the reduced ability to excrete hydrogen and maintain the water balance. The list of alterations extents to the musculoskeletal system (atrophy of skeletal muscles, bone loss and fragility), the skin (wrinkling) and the immune system (increased incidence of autoimmune and neoplastic diseases). However, it is now well recognized that both the presence and the rate of progression of the above changes differ significantly among individuals and the calendar age is only one of the contributing factors.

Ageing is characterized by a gradual decline in organ functional reserves which reduces the ability to maintain homeostasis, especially under conditions of stress. It is believed that in many organ systems the loss of function begins as early as at the age of 30-40 years and then proceeds at an approximate rate of 1% annually. Although this process appears to be continuous and irreversible, ageing itself does not mean pathology. In the absence of additional pathogenic stimuli it will not lead to overt disease. However, age-related changes pave the way for disease. For example, decreased cardiac output observed in the elderly may easily progress to heart failure in the presence of precipitating factors such as hypertension or infection. Therefore, ageing in the absence of diseases is often referred to as normal or physiologic. In contrast, ageing associated with disease is called abnormal or pathologic. However, it may be very difficult to separate alterations related to ageing and disease respectively. Therefore, it is of paramount importance for studies focusing on ageing to select properly the population examined. Many previous studies performed with hospitalized patients might have been biased with respect to the pace at which ageing develops. For example, it is commonly believed that the kidney function deteriorates with age. However, it has been reported that in approximately 1/3 of individuals examined the kidney function did not decrease within a 20-year follow-up (2). More recent, well-controlled study has confirmed that the decline in glomerular filtration rate and renal blood flow was much less pronounced than suggested by earlier reports (3).

Although classical studies carefully delineated specific aspects of physiology of the aged, they dealt with consequences rather than causes of ageing. It soon became clear that these causes had to be looked for at the cellular level. Indeed, new experimental methods allowed the scientists to establish a link between intracellular processes and organ abnormalities. For example, several changes in the nervous system could be explained by impaired signal transduction through axons and synapses. Decreased cardiac output could be related to impaired electromechanical coupling and contractility in cardiac myocytes. It has also been demonstrated that changes in the endocrine system could be attributed to the progressive dysfunction of cells forming either endocrine glands or target tissues. Furthermore, musculoskeletal alterations arise from changes in the bone-forming cells, skeletal myocytes, and neuromuscular junctions. It is also well recognized that the immune system undergoes major age-associated restructuring which includes both enhanced and diminished function of immune cells.

Introduction of cell culture and molecular biology techniques has provided new experimental tools for the analysis of ageing at the molecular level. As early as in 1907 Harrison reported the ability to maintain cells in culture. In 1920s, on the basis of experiments performed with explanted chick heart fibroblast, Carrel suggested that cells in culture could divide indefinitely (4). He claimed that normal cells, when relieved from *in vivo* regulatory mechanisms and placed in appropriate *in vitro* environment, were essentially immortal. However, Carrel's experiments proved to be seriously flawed since an extract from chick embryos used for feeding the cells at that time was contaminated with fresh living cells which could resume proliferation in culture. In the 1960s Hayflick challenged the Carrel's legacy and in a series of elegant experiments convincingly demonstrated that normal human fibroblasts displayed a limited capacity to proliferate *in vitro* (5). He showed that normal cells could double only a finite number of times and after a phase of exponential growth they reached a point, now known as the Hayflick limit, when they stopped dividing. This and other studies with different cell types demonstrated that replicative senescence *in vitro* could be used as a valuable and convenient model for investigating molecular changes in aged cells.

One of the fundamental Hayflick's findings was the observation that the proliferative potential of cells in culture was related to the number of cell doublings rather than chronological age of the culture (6). It has been demonstrated by preserving cells cryogenically for extended periods of time. When reconstituted, these populations could further divide and achieve the same limit of doublings as cells that had not been frozen (7). Later studies have provided some evidence that the loss of cellular proliferative potential *in vitro* may reflect or correlate with *in vivo* ageing processes. It has been observed that cell cultures established from young individuals achieve more population doublings *in vitro* compared to cells from older donors (8). Human fibroblasts isolated from fetuses are able to double 70 – 80 times, while fibroblasts obtained from the elderly divide only few times. In addition, cells derived from patients with premature ageing disorders such as Werner or Hutchinson-Gilford syndromes display a significantly diminished growth capacity *in vitro* (9;10). After a predetermined number of cell divisions (the Hayflick limit), normal somatic cells enter a period of prolonged quiescence (mortality stage 1 or M1), in which they stop replicating but maintain viability and basal metabolic activity for a long period of time (11). Senescent cells require mitogens to remain in a viable state but fail to proliferate in response to growth promoting stimuli (12). At this stage cells display enlarged and flattened morphology and accumulate lipofuscin pigment. But why do they stop proliferating and degenerate?

Ageing is associated with two overlapping processes which ultimately lead to death. These are (i) progressive degeneration of cells and (ii) loss of regenerative capacity. Degeneration and regeneration of cells are processes that occur at every

stage of life and remain well balanced under normal conditions. The ‘mitotic homeostasis’ enables swift replacement of damaged cells and effective preservation of functional integrity of tissues and organs. However, in the aged this balance shifts towards degeneration. Mechanisms of degeneration appear to be primarily related to generation of reactive oxygen species and non-enzymatic glycosylation of proteins; both processes being closely linked to environmental factors. In turn, loss of proliferative and regenerative capacity could be accounted for by genetically determined telomere shortening and apoptosis. This classification highlights the role of both exogenous and endogenous factors in ageing which unites two renowned but apparently contradictory theories of ageing. The ‘catastrophe theory‘ focuses on environmental factors that produce injuries the consequences of which accumulate with time and cause dysfunction of vital systems. In contrast, the ‘biological clock theory’ postulates that cells age according to predetermined genetic program which gets activated at a certain point in life.

Free radicals in ageing

It is now widely believed that degeneration occurs primarily in response to oxidative stress. Free radicals or reactive oxygen species (ROS) are molecules characterized by the presence of an unpaired electron in their outer orbit. Small quantities of ROS are formed spontaneously under normal conditions as the byproducts of redox processes such as oxidative phosphorylation in the mitochondria and β-oxidation of fatty acids. However, generation of ROS is significantly enhanced by either irradiation, chemicals or infection. Free radicals are extremely reactive species since by reacting with other molecules they try to stabilize their configuration and obtain lacking electrons. These processes often occur as uncontrolled chain reactions which may lead to structural damage and functional elimination of the molecules encountered (13-16). The macromolecules that are extremely susceptible to ROS-mediated damage are nucleic acids, phospholipids and proteins. Oxidation of both nuclear and mitochondrial DNA may result in mutations and aberrant protein synthesis. Oxidative damage to cell membrane phospholipids may impair transmembrane transport mechanisms and signal transduction while oxidized enzyme proteins may display abnormal metabolic activity.

Ultrastructural alterations caused by ROS are recognized and eliminated by cellular repair mechanisms. Detailed characterization of these systems is beyond the scope of the current review. It is, however, important to emphasize that the cumulative damage exerted by ROS may - with time - exceed repairing potential of the cellular machinery which ultimately leads to a decline in physiological capacity of the whole organism. Morphological evidence of increased oxidative stress may be accumulation of lipofuscin which consist to a large extent of oxidized unsaturated fatty acids. There is compelling evidence that ROS play a significant role in ageing *in vivo*. It has been known for decades that the mean life

span of rats fed with a diet that provided essential nutrients but restricted caloric intake by 30–50% could be significantly increased compared to animals fed ad libitum (17). Such restricted caloric intake has also been reported to delay several age-associated biochemical and functional changes (18;19). According to a 'free radical theory' these effects can be attributed to a decrease in metabolic rate and lowering of ROS generation. Studies in comparative physiology have revealed that species with short life span that age quickly have higher rates of metabolism and ROS generation compared to long-living animals that display low metabolic rate and ROS accumulation. Under normal conditions a network of cellular defense systems including catalase, superoxide dismutase, and glutathione peroxidase effectively scavenges small amounts of ROS generated. However, the capacity of the antioxidant system is limited and the rate at which antioxidant enzyme are resynthesized declines with age. In fact, it has been suggested that longevity depends largely on the efficient antioxidant status ameliorating the effects of oxidative stress (20). Strong support for this hypothesis has come from studies in insects (*Drosophila melanogaster*) in which overexpression of antioxidant enzymes led to increased longevity and preservation of motor capacity (21;22). More recently, it has been demonstrated that also mammalian life-span can be increased by mutation of a gene encoding the signaling molecule *p66shc* that influences the extent to which cells resist oxidative damage (23).

Glycation in ageing

Another pathway that has been implicated in age-related cell degeneration is the accumulation of advanced glycosylation end-products (AGE). AGE are formed (26) by non-enzymatic reaction between aldehyde groups of reducing sugars and amino groups of proteins (*Fig. 2,* upper part) (24). Under *in vivo* conditions the process depends predominantly on the blood glucose level which accounts for increased AGE formation in diabetes. It has been demonstrated that the physiological control of blood glucose becomes less tight with age which leads to impaired glucose tolerance and prolonged periods of elevated glucose that favor AGE formation. Generation of AGE induces pathology via a number of mechanisms (25). Glycosylation has been documented for many important proteins such as hemoglobin, fibrinogen, lipoproteins, and ferritin. Of particular importance is glycosylation of extracellular matrix proteins with long half-life as exemplified by collagen (*Fig. 2,* lower part). AGE formation on collagen leads to cross-linking that results in expansion of molecular packing, abnormalities in extracellular matrix and impaired cell-matrix interactions. Consequences of AGE-induced abnormalities include narrowing of blood vessels, capturing of other plasma proteins such as LDL, thickening of basement membranes, decreased elasticity of blood vessels, skin, and ligaments. In addition AGE bind to specific receptors on immune cells which triggers the release of inflammatory mediators and generation of ROS which further increase AGE-induced damage.

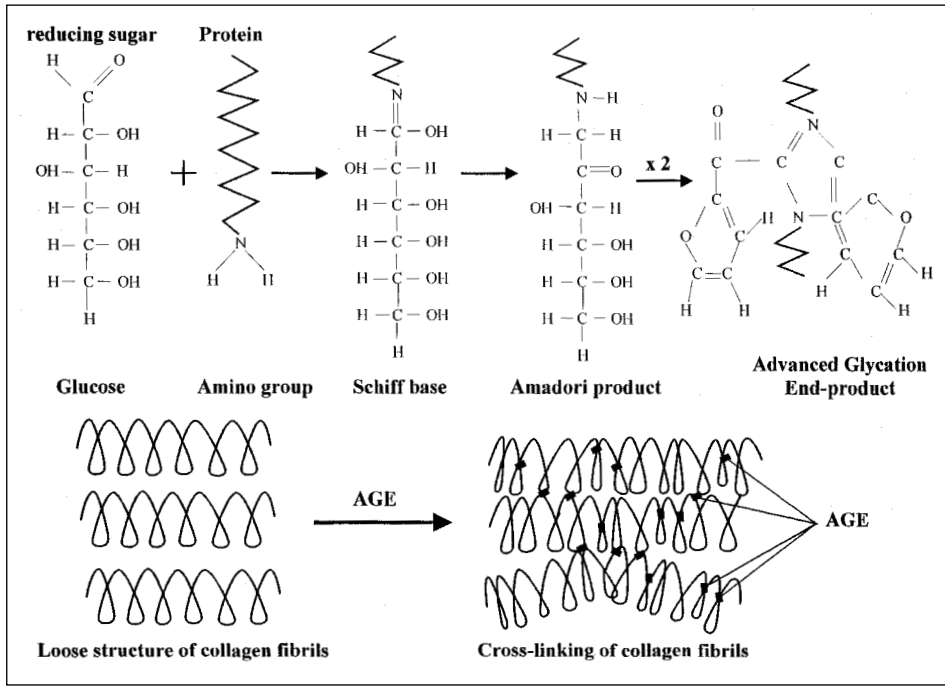

Fig. 2. Advanced glycosylation end-product formation (upper part) and cross-linking on collagen fibrils (lower part).

Cell cycle and ageing

Atrophy of organs and tissues or protracted wound healing are well recognized features of ageing. However, the process of organ atrophy during normal uncomplicated ageing is very slow. Therefore, these changes observed in the aged can be attributed to decreased physical activity and less active lifestyle rather than to ageing itself. In recent years our understanding of the mechanisms that underlie reduced regenerative capacity has increased significantly. They involve two processes that are closely linked to cell cycle control: (i) the regulation of cell proliferation and (ii) the induction of programmed cell death or apoptosis (*Fig. 3*). Matured tissues maintain proliferative homeostasis that optimizes the number of cells within these tissues. Thus, death of a cell initiates the mitotic cycle in other tissue-forming cells. Cell division produces two daughter cells whose fate may be different. Roughly speaking, the cell may be destined to commit to a tissue-specific function and to replace the loss. Alternatively, it may get committed to the preservation of proliferative potential and next divisions if necessary. These cells are sometimes referred to as ‘stem cells’.

Cell cycle is tightly controlled at different stages by specific regulatory proteins. Recent studies have revealed that the expression of many genes involved in cell cycle progression is significantly altered in senescent populations compared

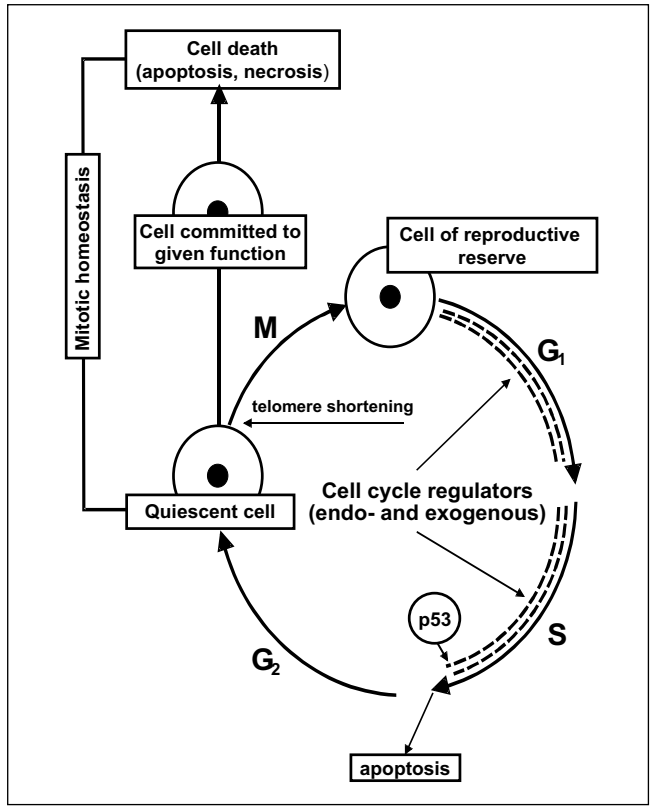

Fig. 3. Cell cycle and tissue regeneration.

to young cells (26). These changes may be exemplified by the retinoblastoma gene product - a protein with tumor suppressing activity. The *Rb* protein appears to be a negative regulator of the cell cycle acting in the G_1 phase through interactions with transcription factors, such as E2F. These transcription factors control genes required for DNA synthesis. The *Rb* protein executes its inhibitory function in the unphosphorylated form while phosphorylation inactivates *Rb* allowing the cell to complete cell cycle. It has been demonstrated that in senescent cells the *Rb* protein remains permanently unphosphorylated and may therefore contribute to the induction of growth arrest (12). Other alterations in the *Rb* pathway involve the system of cyclins, cyclin-dependent kinases (CDK), and CDK inhibitors (CDI). The process of *Rb* phosphorylation depends on the activity of kinases CDK4 and CDK6. These kinases are under control of CDK inhibitors, exemplified by $p21^{WAF1}$ protein. The $p21^{WAF1}$ molecule has been shown to inactivate cyclin-CDK complexes and induce senescence-like proliferation arrest (27). Interestingly, $p21^{WAF1}$ appears to be regulated by *p53* which is a chief protein that guards integrity of the genome and is capable of promoting cell cycle arrest in response to DNA damage (28). Embryonic fibroblasts from *p53*-deficient animals proliferate vigorously and do not senesce (29).

The M1 barrier to replication can be overcome by several viral oncoproteins such as SV40 T-antigen or adenovirus E1A (30). By neutralizing the function of *pRb* and *p53*, these stimuli drive cells to continue dividing until they reach a state of crisis (mortality stage 2 or M2). At this phase cells become affected by massive genomic instability (chromosomal fusions, breaks, degradation) leading to cell death (30;31). Increasing evidence points to telomere erosion as an event triggering signals for the onset of cellular senescence and death.

Telomeres are DNA fragments that form the ends of chromosomes extending up to 12,000 base pairs (32). These GT-rich motifs are highly conserved in eukaryotes; in humans the telomere sequences are simple repeats of the hexanucleotides TTAGGG. Telomeres guard chromosome stability protecting them against degradation and rearrangement. However, in normal somatic cells telomeres are progressively shortened during DNA replication at a rate of approximately 100 base pairs per cell division. This is due to the properties of DNA polymerase which cannot fully replicate the 3' end of linear DNA; a phenomenon commonly referred to as the 'end-replication problem'. The idea that repeated shortening of the telomeres may be responsible for the Hayflick limit and may represent hypothetical molecular clock was proposed by Olovnikov in the early 1970s (33). However, the direct link between telomere attrition and cellular senescence has only recently been proved. First, it has been demonstrated that telomeres decrease as human fibroblasts divide *in vitro* and that telomere lengths in human tissues correlate with donor's age. Further confirmation came from studies of Greider and Blackburn who discovered telomerase - the enzyme that is capable of synthesizing telomeric sequences and, thus, of restoring telomere length (34). Telomerase activity has been documented in immortal cancer-derived cell lines and great majority of human tumors (35). Also 'immortal' germ cells of testes and ovaries, and some stem and progenitor cells have been found to express telomerase. In sharp contrast, telomerase activity has not been detected in normal somatic cells.

All these observation form the basis of the telomere hypothesis of cell ageing (5;35). This theory suggests that as the somatic cells divide and approach the Hayflick limit (M1 stage), their shortened telomeres are recognized as damaged DNA which initiates *p53* and *pRb* pathways. Activation of these checkpoints in the absence of telomerase results in growth arrest in a viable senescent state. Viral oncogene proteins and mutations may allow cells to bypass those checkpoints and proliferate further until erosion of telomeres becomes critical. Cells enter crisis (M2 stage) leading to cell death.

While the role of telomere shortening in ageing seems to be established, the contribution of apoptosis remains poorly defined (36-38). However, it is easy to imagine that in individuals with diminished regenerative capacity, induction of apoptosis for whichever reason may lead to cell deficit and tissue atrophy. Apoptosis in the aged is probably closely related to cell cycle regulation with a link provided by the *p53* protein. As mentioned above, *p53* controls the integrity of

DNA during cell replication. When DNA damage is beyond repair, *p53* is capable of inducing apoptosis by means of specific signaling pathways. Since the damage accumulates during ageing the incidence of apoptosis in the elderly is grater than in young individuals. The processes aims at eliminating damaged cells but may also lead to tissue atrophy. Interestingly, if a cell becomes destined to undergo apoptosis it receives an initiating signal at least twice: in the middle (end of phase S) and at the end (phase M) of the cycle. This phenomenon appears to facilitate elimination of damaged cells in the aged.

In summary, it is laborious and challenging task to examine mechanisms of ageing. However, even the data obtained from well-defined cell culture systems may be difficult to interpret. For example, although the existence of a replicative limit to cell growth in culture appears to be established, the critics argue that the progressive loss of proliferative potential may be related not only to the function of an internal mitotic clock but may also result from cumulative damage produced by tissue culture environment. They suggest that some culture conditions may be imperfect enough to induce DNA damage, cell cycle checkpoint activation and growth arrest (31;39). The situation is even more difficult when analyzing multicell systems or whole organisms. If human body may be compared to a complex system, ageing could be described as progressive loss of its functional modules in the order of increasing importance. There are still significant gaps in our knowledge of how the process of ageing is initiated and controlled. One of the unresolved issues is the question of why ageing may occur selectively with great intensity in certain tissues or even cell clones. For example, it is believed that ageing itself diminishes the number of dopamine-producing cells in the substantia nigra. However, increased degeneration of these cells may lead to Parkinson's disease (40). Likewise, an important feature of Alzheimer's disease (AD) is the degenerative loss of cholinergic neurons in the basal nucleus of Meynert. Similar changes, although of distinctly lower intensity, can be detected in healthy aged individuals (41). Therefore, the mechanisms controlling the selectivity and intensity of ageing are likely to be the primary goal of gerontology research in the nearest future.

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Author's address: Prof. Jan Knapowski, Department of Pathophysiology, University of Medical Sciences, 60-781 Poznań, Poland, Tel. +48-61-865 95 02, Fax. +48-61-865 88 79