Healthy ageing: a question of stress, damage and repair

Meeting on mechanisms of biological ageing

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Received July 18, 2002; revised September 26, 2002; accepted October 3, 2002

A group of unstressed individuals from different age groups discussed the links between stress and ageing on the Greek island of Spetses, May 18–22, 2002. The conference was sponsored by EURESCO and entitled 'Biological Ageing: 2nd EuroConference on Normal Ageing, Longevity and Age-Related Diseases'.

Introduction

The recent EURESCO meeting on biological ageing provided an excellent overview of several trends emerging in this field of research. After many years during which the field has been dominated by controversy over the role of predetermined genetic programmes versus stochastic damage, it is now becoming clear that ageing is the result of a combination of mechanisms that are genetically modulated via pathways of maintenance and repair. This realization raises many important challenges. T. Kirkwood (Newcastle-upon-Tyne, UK) illustrated this with data from a range of model systems (e.g. *Caenorhabditis elegans*, *Drosophila melanogaster*, mice, humans) that showed

how intrinsic stochastic factors, such as the accumulation of random mutations, combine with genetic and environmental factors, such as the age-related expression of specific 'ageing genes', to shape the individual phenotype. In fact, the challenge of dealing with the heterogeneity of ageing at the molecular, cellular and organismal levels proved to be a recurring theme at this meeting. Integrating these multiple mechanisms also calls for new multidisciplinary approaches, as illustrated by the Biology of Ageing e-Science Integration and Simulation (BASIS) system being developed in Newcastle. This is planned to be a user-friendly interactive Internet interface that will involve collaborators around the world. Its main goal is to link theoretical ageing models with experimental data. For example, the 'virtual ageing cell' will be a module within which different elements can be activated or suppressed by the user in order to explore predicted outcomes. This module will focus on exploring the mechanisms of deterioration and damage resulting in age-related impairment and death.

Cellular senescence: the role of telomeres and telomerase

The catalytic subunit of telomerase (known as TERT, a cellular reverse transcriptase expressed in germline cells, proliferative stem cells and many cancer cells) catalyses the addition of telomeric sequences to the ends of chromosomes. Due to the absence of telomerase activity in most somatic cells, incomplete synthesis of the lagging strand leads to telomere shortening every time these cells divide. Once a critical shortened telomere length is attained, cell senescence is triggered. This phenomenon is called telomere-driven senescence, as opposed to stress-induced premature senescence (see below). The expression of TERT in cultured normal cells ('telomerization') leads to the reconstitution of telomerase activity and circumvents the induction of senescence.

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Although telomerase has been the focus of intensive research, some aspects of basic telomere biology are still unknown, hindering the efficient translation of this basic understanding into practical applications.

The laboratory of J. Shay (Dallas, TX) used the human TERT (hTERT) gene to 'telomerize' a large variety of human cell types. These studies revealed that immortalization via the activity of telomerase depends on optimal tissue culture conditions that must be experimentally adapted for each cell type. Under such conditions, telomerized cells can be used to produce organotypic cultures, which behave similarly if not identically to those derived from normal non-telomerized cells except that they can be grown for an infinite length of time. The ability to create hTERT-engineered tissues could potentially be used to treat a variety of diseases and age-related medical conditions that are the result of telomere-based replicative senescence.

It was initially hypothesized that telomere length is directly correlated with the number of elapsed cell divisions, but this picture has now changed: telomeres are no longer viewed as simple cell division counters. The rate of telomere shortening, and thus the cell's replicative lifespan, is not determined solely by incomplete lagging strand synthesis, but is also largely dependent on telomeric DNA damage. In support of this, data reported by T. von Zglinicki (Newcastle-upon-Tyne, UK) showed that telomere shortening is strongly modulated by the ratio of oxidative stress and antioxidant defence. It appears that telomeres contain single-stranded DNA regions and, according to the current theory, single-stranded DNA is particularly vulnerable to attack by reactive oxygen species (ROS). This may explain the increased rate of telomere shortening in cells under oxidative stress. Telomeres may therefore be the cell's sentinels for oxidative stress-induced DNA damage, and mutational risk and telomere-driven replicative senescence might serve primarily as a stress response rather than being the result of an inborn counting mechanism (von Zglinicki, 2002). In evolutionary terms, such a response might have developed as a means of blocking the growth of cells that have a high risk of mutation or those that have been exposed to DNA-damaging agents.

Telomere attrition due to multiple rounds of cell division is now recognized as a factor contributing to age-dependent loss of organ function, as was discussed by G. Butler-Browne (Paris, France) in relation to human skeletal muscle. She addressed a potential role for telomeres in the deterioration of muscle satellite cells during ageing and through exercise, particularly strenuous exercise. These cells are progenitor cells and are responsible for muscle growth and repair. During normal ageing, a progressive decline in muscle mass and strength is accompanied by a decrease in the absolute number of these satellite cells. In contrast, the mean number of myonuclei within the muscle fibres remains constant. Apparently, the proliferative capacity of the satellite cells is limited by a mitotic clock determined by telomere length. *In vitro* studies have shown that the proliferative capacity of isolated satellite cells decreases with donor age, accompanied by progressive telomere shortening (Decary *et al.*, 1997).

Biochemical changes that have been only recently associated with replicative senescence were also discussed at the meeting. As cells age in culture, they lose methyl-cytosine from their genomic DNA and such changes can have profound effects on the pattern of gene expression. For example, as reported by

J. Smith (San Antonio, TX), decreasing DNA methyltransferase (MeTase) activity induces the expression of the cyclin-dependent kinase inhibitor, p21. While this apparently involves no changes in the methylation status of the p21 promoter, genes encoding upstream regulators of the p21 promoter are probably affected. Therefore, the observed changes in MeTase activity/DNA methylation could account for some of the changes in gene expression seen during cellular ageing. Cells expressing hTERT maintain DNA MeTase activity. These findings suggest that hTERT may prevent changes in gene expression that normally occur during cellular ageing. It will now be interesting to elucidate the mechanisms by which telomerase or telomere length regulate DNA MeTase activity and DNA methylation and to determine whether there is a causal link between decreasing DNA MeTase activity, DNA methylation and cellular ageing. P. Jansen-Dürr (Innsbruck, Austria) provided data from a metabolome analysis of senescent human fibroblasts. This technique involves a systematic characterization of the level of intracellular metabolites and metabolic enzymes by biochemical analysis, based on the assumption that metabolic regulation contributes significantly to the cellular phenotype. The analysis of glycolytic pathways in young and old cells revealed age-associated changes in the activity of these enzymes, which leads to a severe metabolic imbalance that induces growth arrest through an as yet uncharacterized AMP-dependent pathway.

Forever young—no stress please!

Increasing evidence suggests that the process of replicative senescence can be prematurely induced through a variety of stress-inducing agents, e.g. hyperoxia or UV irradiation, that probably all involve the generation of intracellular ROS. These aggressive compounds induce damage to cellular macromolecules such as DNA, lipids and proteins. According to the current hypothesis, only a certain proportion of these damaged macromolecules can be removed, and if their concentration exceeds a certain threshold, the cell is then unable to remove any more. Apparently, the accumulation of damaged macromolecules also limits the activity of the cellular repair systems (see below), thereby creating a positive feedback loop, which is one primary cause of cellular ageing. These data provide a possible molecular explanation for the long-standing assumption that the high oxygen concentration in our environment is a major inducer of age-associated changes (Figures 1 and 2). Another important aspect discussed at the meeting was that ROS, considered primarily as damaging agents, appear to play important house-keeping roles as signalling molecules, for example in the NF- κ B pathway.

O. Toussaint (Namur, Belgium) described his latest work on the mechanisms of signal transduction responsible for the appearance of stress-induced premature senescence (SIPS) in human diploid fibroblasts exposed to subcytotoxic levels of oxidative stress. He presented evidence that SIPS induced by oxidative stress involves signalling along the transforming growth factor β -1 (TGF β -1) pathway, which is thought to impinge on the regulation of senescence-associated genes. Based on recent evidence of SIPS *in vivo*, he proposed that this process may contribute to the changes observed in ageing tissues, and thereby participate in the ageing process.

Fig. 1. Correlation between stress, damage, ageing and longevity. As discussed at the meeting, oxygen exposure and telomere shortening drive normal ageing processes. When an organism encounters stressful conditions, intracellular damage to various macromolecules may arise, which accelerates the ageing process (upper arrow). However, most organisms have evolved multiple systems to repair and/or eliminate damaged material and hence protect from stress-induced premature senescence (lower arrow). In addition to these environmental factors, several genetic traits predispose individuals to either premature ageing or to a naturally increased lifespan (longevity). While the genetic basis of premature ageing syndromes (such as the Werner syndrome) is well understood, the identity of the genes that specify longevity is largely unknown. Currently, populations of centenarians are being intensely studied to identify longevity-associated human genes.

K. Scharffetter-Kochanek (Ulm, Germany) presented a new model of SIPS using dermal fibroblasts, and showed that their proliferative capacity unexpectedly recurred after a growth arrest of more than 3 months. In a different set of experiments, stable *in vitro* overexpression of the gene coding for the manganese-containing mitochondrial superoxide dismutase (SOD2) resulted in an imbalance in the antioxidative cellular defence system. This in turn increased hydrogen peroxide levels and enhanced the synthesis of matrix-degrading metalloproteases, a process that is characteristic of senescent fibroblasts. These data are important for future attempts to substitute and balance antioxidant deficiencies in ageing processes.

P. Pelicci (Milano, Italy) discussed the mechanistic connections between an increase in lifespan and the observed increase in resistance to oxidative stress in p66^{Shc} knockout mice. He reported that p66^{Shc} acts as a downstream target of the tumour suppressor p53 and is indispensable for the ability of stress-activated p53 to induce intracellular oxidants, cytochrome c release and apoptosis (Figure 2). Results obtained from the characterization of p66^{Shc} and p53^{-/-} cells suggest that steady-state levels of intracellular oxidants and oxidative damage are genetically determined and regulated by a stress-induced signal transduction pathway involving p53 and p66^{Shc} (Migliaccio *et al.*, 1999).

H. Osiewacz (Frankfurt, Germany) described investigations into the complex network of mitochondrial–nuclear interactions governing the ageing of the fungal model system *Podospora anserina*. He explained that changes in different combinations of factors and pathways relevant for the ageing process result in different increases in lifespan. The type of respiration is important, whether it is the standard cytochrome oxidase (COX)-dependent respiration or the alternative COX-independent pathway, which

is used in COX-deficient strains (Figure 2). Combined with different levels of ROS-scavenging enzymes, this can have a tremendous effect on lifespan. However, long-lived mutants are often characterized by a significant decrease in growth rate and fertility. These negative characteristics seem to be due to impairments in energy transduction pathways. Interestingly, some strains of fungi are able to overcome the negative effects of the different longevity mutations (Osiewacz, 2002). The understanding of the mechanistic basis of this ability may hold an important key to unravelling the connection between old age and health impairment in humans, which may also be related to defects in energy transduction.

Damage induced by stress and ageing

Several presentations demonstrated that proteins, DNA and telomeres are all actively damaged by ROS during ageing, leading to cell death by apoptosis, necrosis or other mechanisms unless repair systems can limit the damage to a tolerable degree. T. Cowen (London, UK) studies the role of ROS and diet in age-related neurodegeneration, with particular emphasis on the cell signalling mechanisms involved. He showed that age and diet conspire to induce neurodegeneration, that neurodegeneration is preceded by elevated intracellular ROS, that ROS induce apoptotic cell death in ageing neurons and, finally, that neurotrophic factors and the associated phosphatidylinositol 3-kinase signalling pathways are involved in neuroprotective mechanisms that may counteract age-related neurodegeneration. Neuronal apoptosis is thought to contribute to brain ageing and the etiology of several age-associated forms of dementia.

C. Soti (Budapest, Hungary) reviewed the relevance of oxidative stress-induced protein damage during ageing. He outlined the stress-related and housekeeping roles of molecular chaperones and their potential to interfere with the ageing process. He also provided *in vitro* evidence that chaperone overload during ageing, due to the accumulation of too many damaged protein molecules, could have potentially deleterious effects.

The value of repair and maintenance systems for healthy ageing

Survival rate is reduced as a result of damage to various cellular constituents by oxidative stress and during ageing. Several presentations at the meeting addressed the molecular pathways and repair strategies that the cell uses to counteract these detrimental effects. Strategies to repair damaged DNA are complemented by strategies for eliminating damaged protein; of particular importance is the repair of damaged telomeres, which are most sensitive to oxidative stress.

A. Bürkle (Newcastle-upon-Tyne, UK) focused on the relationship between DNA damage, repair and longevity, with special emphasis on poly(ADP-ribosylation), a post-translational modification that is triggered by DNA strand breaks and is linked with base excision repair (BER). Recent data indicate that (i) L-selegiline, a drug with life-extending effects in animals, can potentiate cellular poly(ADP-ribosylation), and (ii) there is a positive correlation between single-strand break repair in γ -irradiated lymphocytes and mammalian lifespan. J. Hoeijmakers (Rotterdam, The Netherlands) reported new studies on mice with a mutation

Fig. 2. Molecular events related to normal and stress-induced senescence. Reactive oxygen species (ROS) are capable of damaging both proteins and DNA and are generated in mitochondria, both as by-products and by p66^{Shc}. Scavenging systems are, however, able to cope to some extent with these agents of damage. For example, superoxide dismutases (SOD1, SOD2) convert superoxide (one type of ROS) to hydroxyl peroxide, which can then be converted to water and oxygen (not indicated in detail) giving rise to a lower ROS load. Moreover, once cellular components such as proteins are damaged (red asterisks) they may be removed and replaced by newly synthesized proteins. This molecular remodelling of the respiratory chain is possible if mitochondrial DNA (mtDNA) is functional. However, during ageing subtle mtDNA mutations and gross mtDNA rearrangements accumulate. Point mutations can be repaired by a repair system (e.g. OGG1) in mitochondria. If functional damage to mitochondria is too severe (e.g. complex IV deficiency), a retrograde response signals to the nucleus and induces the expression of additional genes that can rescue lost functions. This rescue may, at least in some systems, occur via the replacement of the damaged oxidase with an alternative oxidase (AOX) in the respiratory chain, or by a more general change in metabolism. Damage may, however, also lead to the induction of apoptosis via the opening of a permeability transition pore (PTP) in the outer mitochondrial membrane and to leakage of cytochrome *c* and other proteins (not shown). Age-related changes in the nuclear genome due to damage, methylation or other processes, influence not only mitochondrial pathways but also numerous pathways in the cytoplasm and other cellular compartments (not shown in detail). Repair of nuclear DNA via different enzyme activities [e.g. PARP [poly(ADP)ribosyl polymerase], or WRN, a helicase], ROS scavenging in the cytoplasm and degradation of damaged proteins via proteasomes consequently have an important impact on biological ageing.

in XPD (derived from xeroderma pigmentosum group D, a sun-sensitive and cancer-prone genetic disorder in humans), a gene encoding a DNA helicase that functions in both repair and transcription. This gene is also mutated in the human disorder trichothiodystrophy (TTD) and TTD mice were found to exhibit various symptoms of premature ageing. The hypothesis was put forward that ageing in the TTD mice is caused by unrepaired DNA damage, which compromises transcription and thus may lead to functional inactivation of certain critical genes and apoptosis. In a model derived from these findings, DNA damage can lead to heritable mutations that contribute to neoplastic transformation. Furthermore, the effects of DNA damage on cell death and cell cycle arrest may cause (accelerated) ageing (de Boer and Hoeijmakers, 2000). E. Seeberg (Oslo, Norway) reported new findings on DNA repair mechanisms of oxidative DNA damage in mammalian cells. Of particular interest is the hOGG1 enzyme, which is responsible for removing the most abundant and potentially mutagenic oxidation product, 8-oxoguanine (8-oxoG). hOGG1 and other DNA glycosylases initiate the BER pathway by removing modified base residues.

This is the major mechanism for repair of oxidative DNA damage and appears to be extremely important in human cells. T. Stevnsner (Aarhus, Denmark) reported new findings on the mitochondrial repair of 8-oxoG and the ageing process. She explained that 8-oxoG is removed more efficiently from the mitochondrial DNA, as compared with 8-oxoG removal from nuclear DNA. By studying nuclear and mitochondrial liver extracts of *Ogg1*^{-/-} mice, it was found that OGG1 is responsible for mitochondrial removal of 8-oxoG. Furthermore, the mitochondrial capacity to remove 8-oxoG in both liver and heart in mice was found to increase with ageing, whereas the repair of the nuclear DNA remains unchanged.

Protein turnover is essential to preserve cell function. The proteasome, a multicatalytic complex, recognizes and selectively degrades damaged (and ubiquitinated) proteins. In recent studies with rat myocardial cells, the group of B. Friguet (Paris, France) observed that the accumulation of chemically modified (e.g. carbonylated) proteins is associated with decreased proteasome activity. The available data suggest that the age-related accumulation of damaged proteins leads to a saturation of the

capacity of the proteasome, rendering it unable to remove all damaged proteins. Proteasomes therefore become blocked by damaged proteins, leading to their inactivation and, consequently, to the accumulation of even more damaged proteins. Purified peptidase-specific proteasome components were found to exhibit an age-related decrease in activity and indicated that the observed decline in proteasome activity with age may also be related to loss and/or inactivation of specific subunits.

Werner syndrome is the hallmark premature ageing disease in which patients appear much older than they should on the basis of their chronological age (Figure 1). The disease results from inherited mutations in the gene coding for the Werner protein (WRN), which is a helicase and an exonuclease. V. Bohr (Baltimore, MD) reported new studies on the biochemistry of both these activities, which can function either separately or jointly on the same DNA substrate. The helicase can unwind various substrates including many that are DNA recombination intermediates. These characteristics suggest that WRN serves as an anti-recombinase. Protein interaction studies indicate that WRN interacts with proteins involved in different DNA repair pathways and in telomeric maintenance. Recent studies on DNA helicases, including the WRN protein, suggest that specific mechanisms repair the damage to telomeres and that these pathways are therefore important in preventing ageing.

Having the right genes may delay ageing

Although it is general knowledge that a healthy lifestyle can delay the onset of age-associated dysfunctions, it has been known for some time that certain cohorts of people have an extended lifespan that is independent of their behaviour (for a review, see Perls *et al.*, 2002). Several contributions at the meeting addressed this phenomenon and the data presented suggest some trends concerning the genetic determinants of longevity. In particular, several presentations suggested a potential correlation between increased stress resistance and lifespan extension (Figure 1).

T. Johnson (Boulder, CO) presented an overview of 20 years of ageing research on *C. elegans*, which has resulted in the identification of more than 100 so-called gerontogenes. All long-lived mutants tested were relatively stress resistant. Microarray studies and experiments using a fluorescence-activated worm sorter—similar to a cell sorter—are now being used to identify genes specifying the increased longevity and increased stress resistance and to identify long-lived worms from isogenic populations of nematodes to determine why they live so long. E. Gonos (Athens, Greece) reported the isolation of several senescence-associated genes, which are overexpressed during both serial passaging of human fibroblasts and stress-induced premature senescence. One of the cloned genes, encoding apolipoprotein J, is overexpressed under a variety of stress conditions and confers chemotherapeutic drug resistance, and may therefore represent a novel 'survival factor'. His group has also established cell banks from healthy centenarians. Analysis of tissue culture characteristics, proteasome status and function, telomere shortening and gene expression levels shows that most of the centenarian samples have characteristics similar to those from samples originating from younger 'control' donors. D. Kletsas (Athens, Greece) provided evidence that, when cultured *in vitro* in the

absence of exogenous stress, fibroblasts from patients with hyperadrenocorticism (Cushing's syndrome, a condition that mimics permanent stress exposure) exhibit a significant increase in their proliferative lifespan, i.e. the number of population doublings that are achieved in *in vitro* cultures. These cells exhibit an intense heat shock protein (Hsp) 70 activation in response to stress and, furthermore, secrete lower levels of TGF- β , the latter being related to accelerated cellular senescence. These data agree with similar observations in calorie-restricted animals, and indicate the beneficial role of stress response activation in longevity and tissue homeostasis, a phenomenon known as hormesis. This view was supported by S. Rattan (Aarhus, Denmark), who reported on experiments where human skin fibroblasts were exposed to repeated mild heat shock. This treatment slows down the accumulation of abnormal proteins, stimulates proteasome activities, increases the levels of Hsp27, Hsp70 and heat shock cognate (Hsc) 70, and improves tolerance to other stresses. While mild heat shock treatment is unable to increase the *in vitro* lifespan of cells, it can delay the appearance of markers of the senescent phenotype.

Evolutionary theories of ageing predict trade-offs between reproductive success and longevity, but the underlying mechanisms have not yet been identified. R. Westendorp (Leiden, The Netherlands) reported a trade-off between human fertility and protection against infectious disease that might explain an observed inverse association between reproduction and longevity under conditions where infections are a major cause of early death. It appears that women with maximal fecundity exhibit a heritable cytokine profile that drives naïve T-cells towards a Th2 tolerance (anti-inflammatory) phenotype. Women with this innate characteristic, however, are susceptible to infectious disease and are less likely to survive. In contrast, survivors are more likely to be characterized by a Th1-type (pro-inflammatory) immune response and are at increased risk of developing age-associated diseases, e.g. neurodegenerative disorders, type-2 diabetes and stroke. Taken together, these observations provide an immunogenetic explanation for how selection for human reproductive success has increased the likelihood of early death and limited our maximal lifespan. E. Slagboom (Leiden, The Netherlands) discussed genetic studies of human mortality and age-related disease such as osteoarthritis. Functional gene variants affecting gene expression are being investigated for their contribution to mortality in the population. Single nucleotide polymorphisms (SNPs) have been found in several genes that increase mortality risk. An example is a common mutation in the methylene tetrahydrofolate reductase gene involved in DNA methylation, which is associated with increased risk of death from cancer, especially in male homozygotes with low folate and high alcohol intake. Other genes associated with mortality and comorbidity (i.e. negative influence on several aspects of healthy ageing) were the insulin, interleukin-1 β and peroxisome proliferator-activated receptor (PPAR) γ genes (Heijmans *et al.*, 2000). G. Wick (Innsbruck, Austria) presented new data on the link between infectious load and atherosclerosis. He also presented evidence to support the Darwinian evolutionary hypothesis of ageing. In terms of atherosclerosis, this model predicts that the benefit of increased immunity to infections by various microorganisms or immune reactivity to biochemically altered autologous material is paid for by an age-related increased risk of vascular disease. The common

antigenic denominator for cross-reactivity of pre-existent humoral and cellular immunity with surface antigens on stressed arterial endothelial cells is Hsp60, which is expressed by both the pathogen (e.g. *Chlamydia* species) and stressed endothelial cells.

Conclusions

In conclusion, the EURESCO meeting provided valuable new insight into the interdisciplinary research activities aimed at elucidating the mechanistic basis of biological ageing. Although it is clear that the problem of ageing is very complex, it is also obvious that various forms of stress and the ability of each biological system to deal with these are major contributing factors. It appears that this theme holds true for all organisms and is therefore of major importance, although a number of other species-specific ageing mechanisms also play a role in this process. A detailed picture of how these act is now emerging, in particular in terms of the role of scavenging systems and the pathways capable of repairing damage. It seems that interdisciplinary research in this field is on track towards the ultimate goal of being able to intervene with the various age-related disabilities and severe diseases of the elderly.

Acknowledgements

Due to space limitations, we could only focus on those talks that are directly related to the role of stress, damage and repair for

healthy ageing. We apologize to all colleagues whose work was omitted from this report. The conference was organized by the European Science Foundation and supported by the European Commission, Research DG, Human Potential Programme, High-Level Scientific Conferences, Contract: HPCF-CT-1999-00115.

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DOI: 10.1093/embo-reports/kvf247