UNIVERSITY OF TEXAS
SOUTHWESTERN MEDICAL CENTER

MEDICAL GRAND ROUNDS
JULY 7, 1994

AGING AND APOPTOSIS

W. C. YARBROUGH, M.D.

Apoptosis is a morphologically distinct form of cell death that is involved in many physiological and pathological processes. Because of its integral part in development it is frequently referred to as programmed cell death (PCD). This form of cell death has been shown to be involved in the development of the nervous system (1) and gut (2) and in the remodeling of the limb buds (3) and bones (4). PCD is also involved in homeostasis. It is particularly important in the immune system where lymphocytes are removed when they fail to correctly arrange their receptors, are self-reactive, or fail to recognize foreign antigen (5-7). One mechanism of protection against viral infection, since the host cell produces the viral particle, is to kill the infected cell (7,8). Cell death can also be used to minimize the risk of cell damage and mutation, such as gut epithelium (9,10) which is exposed to carcinogens in the diet, and skin which is exposed to UV radiation (11,12). If mutation does occur, TNF has been shown to induce apoptosis in transformed host cells (13,14).

Cells undergoing apoptosis become round from cell shrinkage, compact chromatin against the nuclear membrane, undergo enzymatic fragmentation of the nuclear DNA, and finally break up with disruption of the cellular membrane with budding to form membrane-bound fragments known as apoptotic bodies. Intracellular organelles are preserved until late in the process. This distinctive pattern was named apoptosis, after a Greek word describing a plant shedding its leaves (15). Some studies demonstrate that preventing the cell from engaging in protein synthesis blocks apoptosis (16-20), however others demonstrate that macro-molecular synthesis inhibitors do not protect the cell from death (21-23).

Investigation of embryonic development in the tiny, transparent roundworm Caenorhabditis elegans proved to be a valuable resource for identifying genes active in apoptosis. This microscopic worm has only 1090 cells, and during embryonic development a total of 131 cells undergo programmed cell death (24-26). The search for genes encoding proteins involved in apoptosis has led to the identification of 14 genes that are involved at various stages of PCD in C. elegans (27-29). Three of these genes are involved in the regulation and execution of all of the 131 cells. The activity of two of the three genes, ced-3 and ced-4 (called ced for cell death abnormal), are required for cells to die (30), while the third, ced-9, is involved in rescue of the cells from PCD (29). Further work showed that ced-9 is necessary for survival only when ced-3 and ced-4, the suicide genes, are functional suggesting that ced-9 works by preventing ced-3 and ced-4 activity (27,29).

In mammalian cells, a gene product of bcl-2 was shown to prolong cell survival in culture without affecting the growth of these cells (31). The bcl-2 gene was discovered only in the late 1980's and had been identified as a cancer causing antigen (32). BCL-2 is the acronym for the B cell lymphoma/leukemia-2 gene. This gene was first discovered to participate in the majority of follicular non-Hodgkins B cell lymphomas, where chromosomal translocations activate the gene. The bcl-2 gene is moved from its normal chromosomal location at 18 q 21 into the locus for the immunoglobulin heavy chain (IgH) at 14 q 32. This translocation results in overproduction of bcl-2 mRNAs and the encoded proteins (33). It was later shown to protect immune cells, especially lymphocytes, from apoptosis and later work showed that it protects neurons as well.

Hockenbery et al. formally demonstrated that bcl-2 was capable of blocking apoptosis (34). They also located the bcl-2 protein to the mitochondria, endoplasmic reticula, and paranuclear membrane. More recent studies of BCL-2 function have demonstrated that there is a role for this gene in the suppression of apoptosis in post-mitotic cells as well (35,36). Bcl-2 expression plasmids were micro-injected into neurons which normally undergo apoptosis when their normal growth factors are removed. bcl-2 functioned to markedly delay the rate of cell death that occurred upon removal of the neurotropic factors from the cultures (36).

Hengartner and Horvitz demonstrated that the ced-9 protein sequence was 23% identical to the mammalian protection gene, bcl-2 (37). Analysis of ced-9 genomic structure and transcripts suggested that ced-9 was an element of a complex genetic locus and shared a promoter with an upstream gene encoding for a mitochondrial cytochrome. They also demonstrated that bcl-2 could substitute for ced-9 to prevent programmed cell death. With this degree of conservation between the protection genes in worms and humans, the search for counterparts of the suicide genes, ced-3 and ced-4, was begun. A search of the GenBank, PIR, and SWISS-PROT databases revealed that the ced-3 protein was similar to both human and murine interleukin-1 converting enzyme (ICE) (38). Interleukin-1 beta (IL-1β) is a cytokine involved in mediating a wide range of biological responses including inflammation, septic shock, wound healing, hematopoiesis, and the growth of certain leukemias (39,40). ICE is a cysteine protease that cleaves the inactive 31 kd precursor of IL-1β, between Asp-116 and Ala-117, releasing a carboxy terminal 153 amino acid polypeptide, the mature IL-1β (41-44). The ced-3 protein was found to share 29% amino acid identity with the ICE from humans (38).

Active human ICE is composed of two sub-units (p20 and p10) that appear to be proteolytically cleaved from a single pro-enzyme (44). Both the carboxy terminal portion of the ced-3 protein and the p10 sub-unit of ICE were found to be similar to the protein product of the murine gene nedd-2, which is highly expressed during embryonic brain development, but is down-regulated in adult brain (45).

The similarities between ced-3 and ICE suggests that ced-3 might function as a cysteine protease, like ICE. However, it also suggests that ICE might function in programmed cell death in vertebrates (46). Hogoquist et al. demonstrated that murine peritoneal macrophages, when stimulated with lipopolysaccharide and induced to undergo programmed cell death by exposure to extracellular ATP, release mature active IL-1β into the culture supernatant, however when cells were injured by scraping, IL-1β was released exclusively as an inactive pro-enzyme (47). This is not firm evidence that ICE is involved in programmed cell death. However, IL-1β may not be the only substrate acted upon by ICE and ICE transcripts have been detected in cells that do not make IL-1β (43).

Stronger evidence was found when the ICE gene was transferred into rat cells in culture and showed that production of the ICE protein killed the cells (46). Mutant genes that coded for inactive enzymes did not kill the cells. This ICE-induced death could be blocked both by the known protective gene, bcl-2, and by crmA, a cow pox virus gene whose protein product inhibits ICE's protein splitting activity. Thus, overexpression of ICE induces programmed cell death in rat fibroblasts and this death can be suppressed by the cow pox virus crmA gene and by the bcl-2 proto-oncogene.

To test the possibility that ICE may be acting in vivo to cause cell death during development Gagliardini et al. performed experiments using cultured sensory neurons (36). The survival of sensory neurons during development depends on the presence of neurotrophic factors. When these neurotrophic factors are removed, the neurons are thought to undergo programmed cell death (48-50). A microinjected end-expression vector containing a crmA complimentary DNA (cDNA) under the control of the β-actin gene promoter was used. The cow pox virus crmA gene is a specific inhibitor of ICE (51). Control neurons in the presence of nerve growth factor (NGF) exhibited approximately 85% survival through day 6. However, upon removal of NGF more than 80% of the control neurons died within 3 days with fewer than 10% surviving to day 6. The cells injected with crmA, in contrast, exhibited a 60% survival through day 6 despite the absence of NGF.

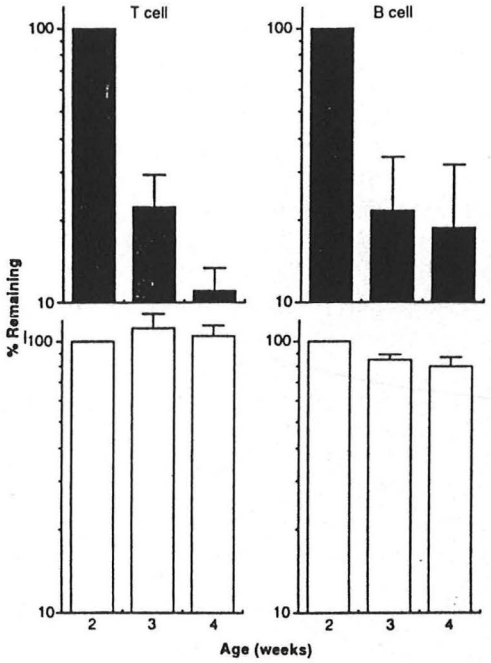
Since bcl-2 was found to prevent apoptosis in C. elegans, similar microinjection experiments were performed using bcl-2 instead of crmA in the neurons. About 60% of the bcl-2 injected neurons survived through day 6 in the absence of NGF, showing about equivalent ability, to crmA, to prevent the death of these neurons. To test whether bcl-2 and crmA acted through separate pathways they injected both into the neurons. The survival rate of these doubly injected neurons in the absence of NGF was no greater than that of the neurons injected singly with either crmA or bcl-2 (about 60%). Thus, crmA and bcl-2 may act through the same pathway.

Work done with C. elegans shows that ced-9 is necessary for survival only when ced-3 and ced-4, the suicide genes, are functional, suggesting that ced-9 works by preventing ced-3 and ced-4 activity (52). A similar relationship may exist between bcl-2 and ICE in mammalian cells. If BCL-2 levels are high, the conditions to activate ICE do not exist (53).

To further complicate matters, another mechanism of control was discovered when a search for proteins that might associate with BCL-2 was begun. BCL-2 had been found to associate with itself as a homodimer (54). However, another protein was found to associate with BCL-2. This protein was named BAX, for bcl-2 associated X. It was found that this protein exhibited homology with BCL-2 itself. It was found that Bax forms homodimers with itself but also forms heterodimers with BCL-2 in vivo. Overexpression of Bax was found to accelerate apoptosis as well as countering the death repressor activity of BCL-2 in both premitotic and postmitotic cells. This suggests that the BCL-2-Bax pair plays an important role in both positive and negative regulation of programmed cell death. The ratio of BCL-2 to Bax may determine whether the cell undergoes apoptosis. If the BCL-2 protein is in excess in the cell, it binds up all the Bax and the rest of the BCL-2 molecules form homodimers allowing the cell to survive. However, if Bax predominates it binds up all the BCL-2 and forms Bax/Bax homodimers resulting in cell death. In support of this concept, it was found that BCL-2 levels are high in immature T lymphocytes but decrease quickly at that point in development when self-reactive cells are signaled to undergo apoptosis (55-57).

Mice with a total lack of bcl-2 genes, often termed "knockouts", have been developed (58,59). The phenotype of these knockout mice is different from their normal litter mates being somewhat smaller with small external ears and immature facial features

which they keep throughout life. Hematopoietic differentiation including lymphocyte development is initially normal in these mice however after development a severe lymphopenia results while the absolute neutrophil count remains stable. This was shown to be secondary to a fulminant apoptosis of the thymus and spleen. Thymocytes from these animals demonstrated accelerated cell death following apoptotic stimuli. These mice also developed polycystic kidney disease with pathology resembling the human form of polycystic kidney disease.



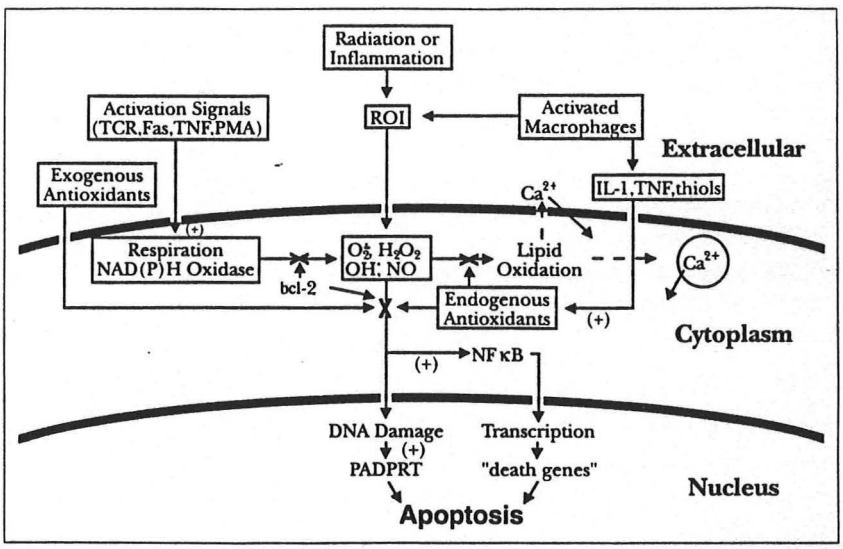
SCIENCE • VOL. 261 • 17 SEPTEMBER 1993

Another interesting characteristic of the bcl-2-/- (knockout) mice is that their hair coats turn gray during the second follicle cycle of hair growth. This graying occurred in mice with black coats and also Agouti coats. The graying was stable once it was completed (59). Key steps in melanin biosynthesis involve reactions converting tyrosine to L-DOPA and then DOPA-quinone, both catalyzed by tyrosinase. DOPA-quinone is an extremely reactive compound whose further cyclization and oxidative polymerization forms the insoluble heteropolymers of eumelanin. This has been shown to be regulated by the cellular redox potential (60). Over 150 mutations in more than 50 loci are known to affect hair pigmentation (61). Yohn et al. has shown that melanocytes have lower levels of superoxide dismutase and peroxide than fibroblasts or keratinocytes (62). Melanogenesis generates free radicals, and melanocytes therefore may be more vulnerable to free radical attack by melanin products. Changes in the metabolism of peroxides and free radicals can

impact both melanin synthesis and cellular survival (63,64).

BCL-2 protein has been shown to integrate into the outer membrane of the mitochondria with the carboxy terminal end functioning as a signal-anchor and the NH_2-terminus facing the cytosol (65,66). BCL-2 is also localized to the endoplasmic reticulum and the perinuclear membrane (65-67). Mitochondria are believed to be a major site of reactive oxygen species production in vivo as a by-product of mitochondrial metabolism. Electron transport through the mitochondrial respiratory chain is extraordinarily efficient and normally accounts for the vast majority (approximately 98-99%) of oxygen consumption (68). The principal loss of electrons that convert oxygen to $0_2^{\cdot-}$ involves coenzyme Q, ubiquinone, and its complexes (69). Loss of the superoxide radical, $0_2^{\cdot-}$ is potentially injurious to cellular constituents. Another site of electron transport is the endoplasmic reticulum in which the reduced form of NADPH cytochrome p450 reductase leaks electrons to 0_2, reducing it to $0_2^{\cdot-}$ (70). An electron transport chain has also been shown in nuclear membranes although its function is undetermined.

Mediators of apoptosis have been shown to increase oxidative stress in cells and also can be inhibited by the addition of certain anti-oxidants. Tumor necrosis factor alpha (TNFα) can serve as an example. Stimulation of the TNF receptor results in a rapid increase in intracellular levels of reactive oxygen species (or intermediates, ROS or ROI) (71,72). TNF induced apoptosis can be inhibited in various cells either by thioredoxin (73), an intracellular thiol reductant and free radical scavenger, or N-acetylcysteine (NAC), a thiol antioxidant and glutathione (GSH) precursor (74). A correlation has been shown between the sensitivity of cells to TNF and increased or decreased levels of superoxide dismutase (75). T-cells deficient in SOD have been shown to be more susceptible to killing by TNF, ionizing radiation, and hyperthermia (76). Therefore the balance between antioxidant defense and the extent of reactive oxygen species formation may determine whether the cell undergoes apoptosis or necrosis in response to TNF and other mediators of apoptosis.



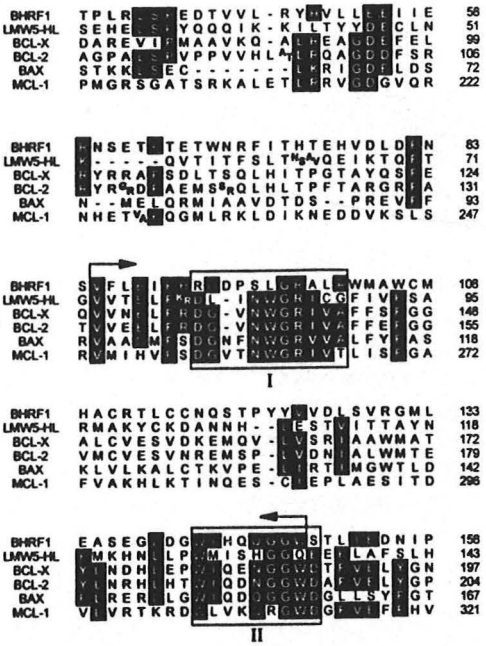
Oxidative signals for apoptosis can originate both extracellularly and intracellularly as shown in the figure above. Mitochondrial oxidation, the microsomal cytochrome P450 system, and plasma-membrane NADPH oxidases are all intracellular sources of ROI (77,78). Mitochondrial ROI are particularly important in apoptosis since cellular activation requires increased oxidative phosphorylation. Regulation of mitochondrial and/or cytosolic ROI levels in mammalian cells could be mediated in part by the bcl-2 gene product (34).

Recent studies with BCL-2 demonstrate that this molecule acts as a block between the generation of peroxide and lipid membrane peroxidation (79). Thus BCL-2 may be one of several proteins that regulate the redox state of the cell, thereby affecting the activity of several transcription factors including NF-kB, Fos/Jun, and helix-loop-helix members and others. These may mediate downstream effects resulting in cell death (80) (see figure above).

Support for BCL-2 acting as a free radical scavenger is that overexpression of glutathione peroxidase, a known inhibitor of lipid peroxidation, also represses programmed cell death (79). Also, two of the pathological features of the knockout mice described above are potentially a consequence of altered redox homeostasis, polycystic kidney disease, and hypopigmentation.

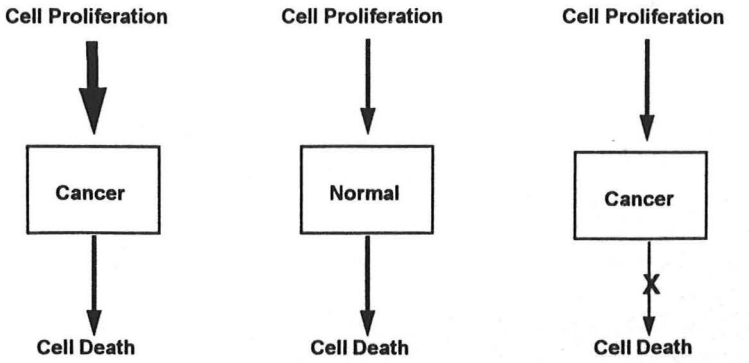
Most of the chemical and physical changes capable of inducing apoptosis are also known to increase oxidative stress. Examples include both ionizing and ultraviolet radiation which are capable of inducing apoptosis and also are known to generate reactive oxygen intermediates (ROI) such as H_20_2 and $OH^{\cdot}$. Doxorubicin, cisplatin, and ether-linked lipids are anti-neoplastic agents which increase ROI formation and induce both apoptosis and oxidative damage in sensitive cells (81-84). Nitric oxide (NO) has a single unpaired electron and reacts with molecular 0_2 to form $0_2^{\cdot-}$ and H_20_2, in addition to acting as a free radical itself (85). Recently NO has been implicated as an inducer of apoptosis in macrophages and monocytes (86). Decreasing anti-oxidant defenses in the cells also increases oxidative stress and induces apoptosis. For example, drugs such as buthionine sulfoxamine deplete intracellular stores of glutathione (GSH), thereby increasing susceptibility to oxidative stress and apoptosis (87). Extracellular catalase has been shown to decrease the sensitivity of a human T-cell line to H_20_2-induced apoptosis (88). HIV infection is associated with a decrease in anti-oxidant defenses in the infected cells. These cells display low levels of SOD, catalase, thioredoxin, and GSH peroxidase. These changes make HIV infected T-cells extremely susceptible to oxidative stress induced apoptosis (89).

Efficient activation of NF-kB dependent genes by TNF requires that a cell be in an oxidized redox state, suggesting that stimuli such as TNF and phorbol myristate acetate (PMA) may exert only a limited response if the cell is not in an appropriate redox equilibrium (90) (see figure above). Oxidant mediated DNA damage has been shown to lead to the activation of poly-ADP-ribose transferase and the accumulation of P53, both of which are associated with apoptosis (91,92). The polymerization of ADP-ribose to proteins leads to a rapid depletion of the cellular NAD/NADH pool. This results in collapse of ATP stores which leads to cell death. Reactive oxygen species readily interact with polyunsaturated fatty acids and cholesterol present in most cell membranes (77,93). These oxidized lipids alone have been shown to induce apoptosis.



The identification of several genes or protein products that show sequence homology with the bcl-2 gene makes it apparent that this is a growing family of genes that are important in the control of cell death (94). The overall amino acid homology between BCL-2 and others in this gene family is low. There is a concentration in two regions shown in the figure above. These have been termed BH1 and BH2 (27). It is important to note that there are other genes that do not show sequence homology to bcl-2 but can suppress apoptosis in at least some situations. These include adenovirus, E1-v, activated T24-ras, B-ABL, and the baculovirus genes P35 and IAP.

PATHS TO CANCER FORMATION



In the normal state of repair of tissues, cell proliferation is balanced against cell death as shown in the figure above. We are all aware of the increase in cell proliferation such as through the activation of certain oncogenes leading to cancer shown on the left of this figure (95). However, recent evidence shows that a loss of the ability to induce cell death will also lead to cancer even though there is no known signal for increased cell proliferation shown in the right on the figure. This is highlighted by the recent demonstration that the protein encoded by the P53 tumor suppressor gene apparently inhibits cell growth by turning on the production of a protein that blocks the cell cycle (96). It may be that decreased cell death allows the buildup of damage to the nuclear genome, or expression of other oncogenes, until cancer develops. This balance between cell proliferation and cell death may be dependent on the balance between oxidant and antioxidant forces within the cells. Lennon et al. (97) showed that exposure to low doses (10-100 mmol) of H_20_2 induces apoptosis in a variety of cell types, establishing oxidative stress as a direct mediator of apoptosis. High doses of H_20_2 were found to induce necrosis implying that the severity of the oxidative insult determines the form of cell death which occurs, as suggested by Duvall and Wyllie (98,99).

McDonnell, et al., looked at a transgenic mouse model that overexpressed bcl-2 primarily in B cells, the bcl-2 gene was coupled with the immunoglobulin gene causing overexpression, resulting in a four-fold increase in the number of B cells found in the spleen of these animals (95). This accumulation was due to a lack of cell death rather than an increase in proliferation. This was shown by transferring these B cells from the transgenic mice to the normal litter mates or by placing them in cell culture, both of which demonstrated prolonged cell survival compared to normal B cells. As might be expected, the B cell hyperplasia of these transgenic mice was initially polyclonal however the cost of prolonged cell survival in these animals was the development of a large cell lymphoma which was monoclonal and aggressive and life threatening. Other mechanisms of homeostasis may also be disrupted if this balance is lost.

This balance carries implications for the normal aging process of the cell as well as the aging of the animal. There are now more than 300 theories of aging. Many of these are either out of date or very selective while others co-exist without contradiction since they explain different and independent forms of senescence. A discussion of all, or even most of these is beyond the scope of this Grand Rounds. A listing and classification of theories of aging along with its proponents is contained in the reference by Z.A. Medvedev (100). Many of these theories of aging, however, have been of two types: 1) those related to by-products of differentiation and development, which have been called "developmentally-linked biosenescent processes," (101-103) examples of which are growth hormone, which was discussed by Dr. Craig Rubin, and testosterone covered in Dr. Jean Wilson's Grand Rounds on male menopause, or 2) those related to by-products of energy metabolism which have been called "continuously-acting biosenescent processes" (101). I will limit the present discussion to the second of these and mostly deal with the "free-radical theory of aging," which has the most supportive evidence and correlates very nicely with the balance implied by the discussion of apoptosis above.

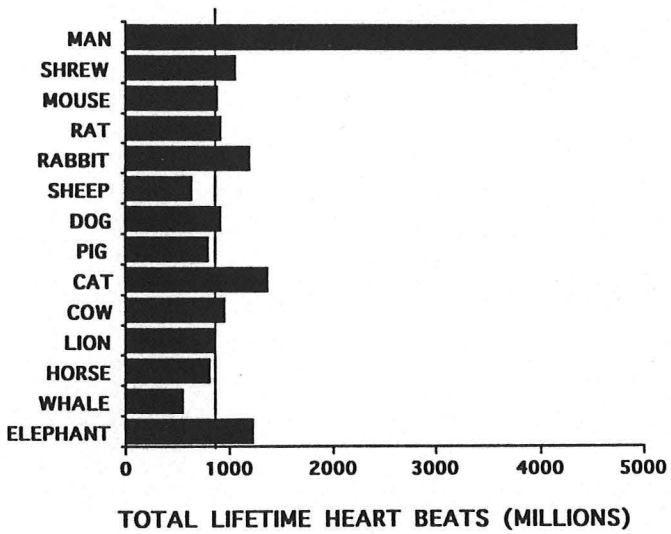
Over the span of mammals, from smallest to largest, there is a correlation between body size and breath rate defined by the following equation:

$$\text{Breath time} = .0000470(\text{weight})^{0.28}$$

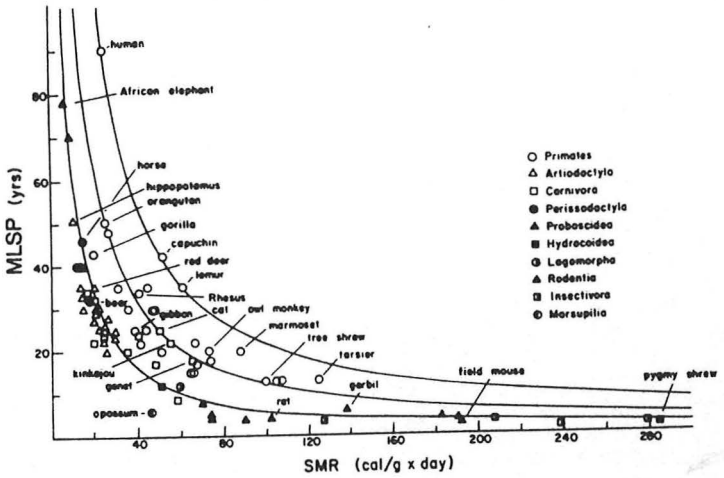
There is also a correlation between size and heart rate shown below:

$$\text{Heartbeat time} = .0000119(\text{weight})^{0.28}$$

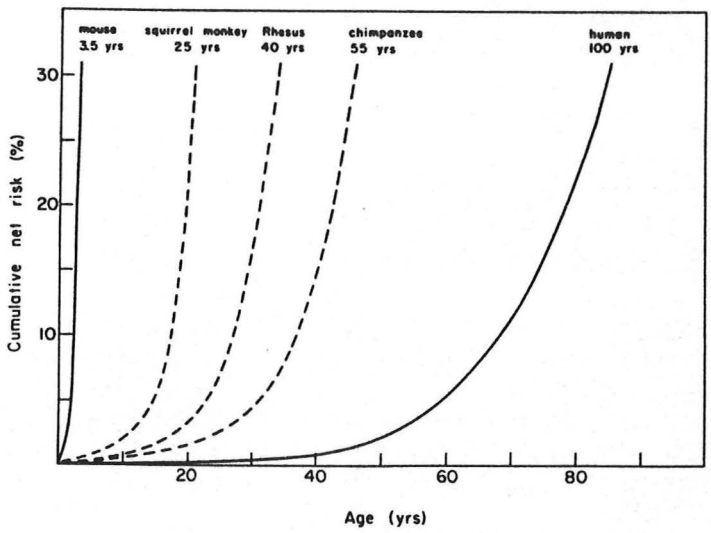
If these equations are divided, then body size cancels out, and it can be seen that all mammals, regardless of their size, breath once for every four heartbeats. Lifespan also increases at the rate of 0.28 times as fast as body size going from small to large mammals. This means that the ratio of heartbeat time to lifespan is constant. Thus, there is a constant for heartbeats over the life span of all mammals. With a little variation, all mammals are allotted approximately 800 million heartbeats over their lifetime (104).



The exception to this rule is the human who is allotted over 4 times this number of heartbeats in his lifetime. This relationship is better correlated to the specific metabolic rate of the animal. As shown below, there is a correlation between the specific metabolic rate and the maximum lifespan potential for various groups of animals (89).

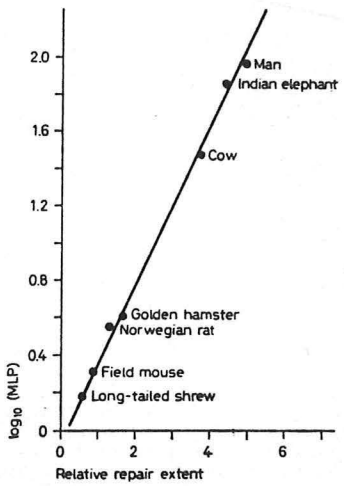


Thus, animals with a high metabolism do not live as long, chronologically as animals with a lower metabolism. This may be related to considerations of maintaining constant body temperature, which would be much easier for the large animal than the small animal. However, again, man is allowed almost twice as much energy expenditure in his lifetime as the closest mammalian relative. These findings led to the rate of living hypothesis of aging, that mammals may live different chronologic lifespans, but all live the same biologic lifespan (equal energy expenditure per unit of weight). Small animals live faster than large animals, and man gets to live more than any of them.



Another correlation among mammals is that cancer increases with about the fifth power of age (as a function of total lifespan) (105,106). The difference in metabolic rate could markedly affect the level of endogenous oxidants and other mutagens produced as a by-product of metabolism. The metabolic rate of the rat is about seven times higher than that of the human (89), and cancer rates are high in a two year old rat, but low in a two year old human. The level of DNA damage appears to be roughly related to metabolic rate in a number of animal species (101). Oxidative damage to DNA, proteins, and other macromolecules has been shown to accumulate with age. These oxidants are produced as a normal by-product of metabolism and are thought to be the major (and by some, the only) source of endogenous damage leading to aging (107). The repair of oxidatively damaged DNA is accomplished by enzymes that excise the oxidized lesions. The excised areas are then excreted in the urine and assays have been developed to measure several of these damaged products in the urine of rodents and humans (108-110).

Hart and Setlow measured the extent of unscheduled repair of damaged DNA in fibroblasts following exposure to ultraviolet light (104). The fibroblasts of long lived mammals were found to repair the UV damage to a greater extent than that of short lived mammals. The relationship between the life span of the species and the ^{3}H thiamine corporation into DNA of their fibroblasts is shown in the following graph. Thus, it appears that the longer lived species may be more efficient in their ability to repair DNA.



These repair enzymes are efficient and remove most, but not all of the oxidative lesions that form (111), therefore resulting in an accumulation of DNA oxidative "hits" with age. It has been estimated that the number of these DNA oxidative hits that occur per cell per day is about 100,000 in the rat and about 10,000 in the human, so that by the time a rat is 2 years old it has about 2,000,000 DNA lesions per cell, about twice that found in a young rat (112). The contribution of these oxidative lesions in DNA to somatic mutation frequency is unknown. However, somatic mutations are known to increase with age as well, the frequency in human lymphocytes being about 9 times greater

in elderly people than in neonates (113). The fact that specific repair glycosolases for DNA exist implies that these enzymes are important in the pathogenesis of cancer and aging. Oxidative damage to guanine residues in DNA leads to 8-oxo-2'-deoxyguanosine. If the glycosolase activity for this enzyme is lost an appreciable increase in spontaneous mutation rate occurs (114), suggesting this oxidative lesion has a mutagenic potential. The balance between oxidative damage to DNA and specific glycolate repair is likely to be important for other lesions as well.

Sources for oxidation products include both endogenous and exogenous sources. By far the largest endogenous source of reactive oxygen intermediates is as a consequence of normal aerobic respiration. Oxygen serves as the terminal electron receptor for oxidative phosphorylation being reduced in the mitochondria by sequential steps to produce water.

$$O_2 \xrightarrow{e^-} O_2^{\cdot -} \xrightarrow{e^-} H_2O_2 \xrightarrow{e^-} {\cdot}OH \xrightarrow{e^-} H_2O$$

This, however, leads to inevitable byproducts of a variety of reactive oxygen intermediates which have either unpaired electrons (i.e. $0_2^{\cdot -}$, ·OH) or the ability to steal electrons from other molecules (i.e. H_20_2, HOCl). These ROI rapidly react with cellular macromolecules resulting in either direct damage or the initiation of a chain reaction wherein the free radical is passed from one macromolecule to another. This latter chain reaction results in extensive damage to cellular structures particularly membrane structures including the cell membrane itself. Chance et al. have investigated this endogenous source of oxidation products in rat cells. They found about 10^{12} 0_2 molecules are processed by each rat cell per day. Leakage of partially reduced oxygen molecules was found to be approximately 2% which corresponded to about 2 x 10^{10} $0_2^{\cdot -}$ and H_20_2 molecules per cell per day (115).

The hydroxyl radical (·OH) is an extremely reactive oxygen species, and behaves with near diffusion limited rates of reactivity towards multiple types of biomolecules including DNA, a variety of proteins, as well as lipids (116). Due to its high reactivity under normal conditions that would occur in vivo and under most in vitro circumstances in intact cells, this molecule is probably able to diffuse at most 15-20 angstroms before it comes in contact with a biomolecule that it is able to oxidize (116). This has led to the theory of site-specific hydroxyl radical mediated injury, so that for a biomolecule to be damaged by this particular species, the origin of hydroxyl radical formation has to be in very close proximity to that molecule.

$$0_2^{\cdot -} + Fe^3 \rightarrow Fe^{+2} + 0_2$$

$$Fe^{+2} + H_20_2 \rightarrow Fe^{+3} + OH\text{-} + OH$$

Hydroxide serves as a reducing source of Fe^{3+} to generate Fe^{2+} which then reacts with hydrogen peroxide to generate hydroxyl radical, called the Fenton cycle (see above).

The trouble with the hydroxyl ion is that the general reaction by itself although thermodynamically possible would occur so slowly under physiologic circumstances that it is unlikely to play any particular role in biology. Thus, the need for a catalyst and given the availability of iron in biological systems, emphasis has been placed on this reaction. From the standpoint of this reaction there are essentially two ways to interrupt it enzymatically. One is to use superoxide dismutase which would remove superoxide from the system essentially removing the reducing equivalents that keep the reaction going and the other is to use a scavenger of hydrogen peroxide, most commonly catalase (117).

Another source of endogenous oxidants is through another by-product of a necessary function. Phagocytic cells are extremely efficient at destroying bacteria or virally infected cells with an oxidative burst of nitric oxide (NO), $O_2^{\cdot}$, H_2O_2, and $OCl^{\cdot}$ (85,118). These powerful oxidants help to protect us from an immediate death from infection but cause an increased oxidative load and damage to DNA contributing to mutation and carcinogenesis (119,120). Chronic infection, whether by viruses, bacteria or parasites leads to a chronic inflammatory process and a long-term increase in oxidant load (121-127).

Hydrogen peroxide is also a by-product of the degradation of fatty acids, most of which occurs in organelles called peroxisomes. There is evidence that some of this H_20_2 escapes degradation by catalase and makes its way into other compartments of the cell resulting in increased oxidative damage particularly to DNA (128).

Another endogenous source of oxidants could come from the cytochrome P450 enzyme system. In animals, these enzymes are one of the primary defense systems against toxic chemicals from plants eaten as part of the diet. Generation of oxidants through redox cycling may occur from natural phenolic compounds such as chlorogenic and caffeic acid which are a part of the normal diet. Again, there is a tradeoff between prevention of acute toxic effects from foreign chemicals and the production of oxidant by-products that damage DNA (129).

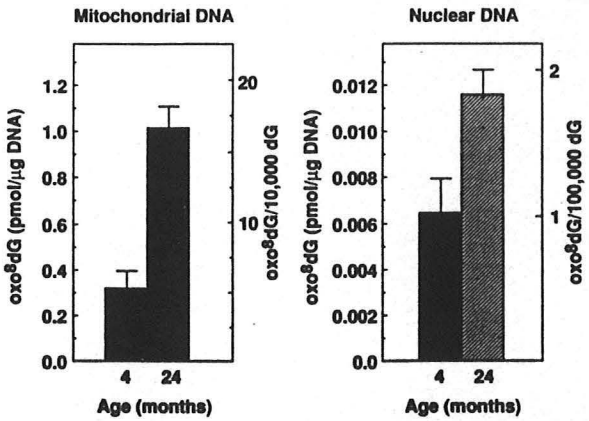
Exogenous sources of free radicals may significantly add to the endogenous oxidant load described above. Smoking is a major oxidant stress particularly from various oxides of nitrogen present at about 1000 ppm. These cause oxidation of proteins and lipids (130-133) and deplete antioxidant levels (134-136) in addition to being a major source of non-oxidative mutagens. Smoking is thought to contribute to approximately 1/3 of all cancers in the United States. It is also is a major risk factor for heart disease and is thought to contribute to approximately 1/4 of all heart disease in this country resulting in approximately 400,000 premature deaths per year in the United States. It is estimated that tobacco will cause approximately 3,000,000 deaths per year worldwide during this decade and at the present rates of smoking to cause 10,000,000 deaths per year a few decades from now (137). The low antioxidant levels found in smokers and the increased oxidative stress caused by smoking is likely involved in the pathology of smoking related illnesses including both cancer and heart disease.

There is recent concern about the long time practice of adding iron to various foods particularly flour and bread since iron (and copper) salts promote the generation of oxidizing radicals from peroxides through the fenton reaction. Patients with hemochromatosis absorb greater than normal amounts of dietary iron due to a genetic

defect and have been shown to be at increased risk for both cancer and heart disease (138). Too much dietary copper or iron may be a risk factor for cardiovascular disease and cancer in normal individuals as well (138-141).

Chronic infections may contribute to up to 1/3 of the world's cancer. Schistosomiasis and liver flukes are associated with increased risk of cancer in the organs in which they invade (142-145). More applicable to this country is hepatitis B and C viruses (146-148) and Helicobacter pylori bacteria (149-151) which are significant risk factors for cancer of the liver and stomach respectively. Chronic inflammation from non-infectious sources such as asbestos fibers may contribute to the reason this fiber represents such a significant factor for cancer of the lung (123,124).

Since mitochondria lack specific repair enzymes, protective histones, and are in close proximity to oxidants generated during oxidative phosphorylation, the accumulation of oxidative damage to mitochondrial DNA occurs at about 10 times the rate of nuclear DNA from the same tissue (152). It has been suggested that mtDNA mutations are involved in aging and carcinogenesis (153,154). The only defense against this high rate of damage is a constant turnover of mitochondria to remove those which are damaged or perhaps have increased leakage of oxidation products, although recent evidence suggests that mitochondria share DNA and act as an integrated cellular system (155). However, despite this mitochondrial replacement, oxidative lesions in the mitochondrial genome accumulate with age at a higher rate than nuclear DNA as shown in the figure below.



8-oxo-2'-deoxyguanosine (oxo^8dG) was analyzed in both nuclear and mitochondrial DNA from the livers of young and old rats. The graph on the left indicates that oxidative DNA damage increases with age in mitochondrial DNA almost 2 orders of magnitude greater than the nuclear DNA accumulation shown on the right graph (112).

Since the mitochondrial genome (mtDNA) is relatively small in comparison to the nuclear genome, several workers have studied the structural changes. One important finding was that large segments of the human mitochondrial genome are deleted as the body ages. Sugiyama et al. (156), found a 7,436-bp DNA segment is deleted from the mitochondrial DNA during the aging process. At age 80, 3% of mtDNA had this

deletion, whereas at age 90, 9% had this deletion. This segment of mtDNA encodes for certain sub-units of complexes of the respiratory chain implying that a loss of this segment would impair energy production by mitochondria.

Yen et al. (157) discovered another deletion of 4,977-bp segment of DNA from the mitochondrial genome in the liver of older persons. They found that the percentage of mitochondrial with this deletion increases with increasing age.

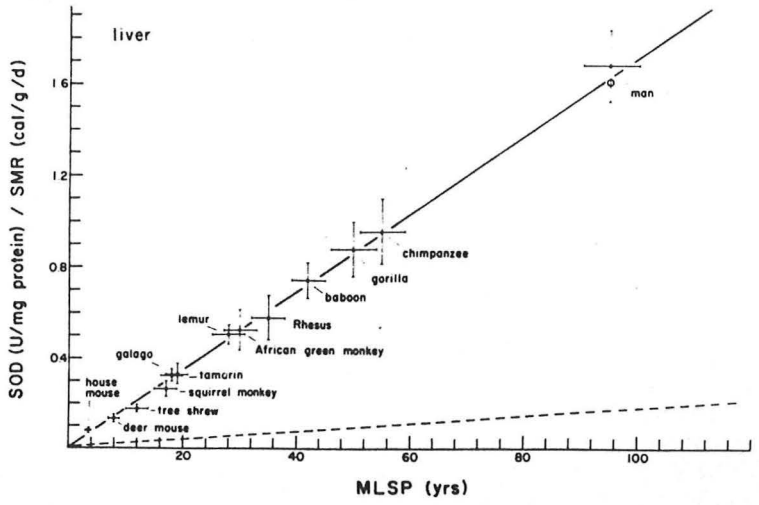
Nuclear and mitochondrial DNA are not the only targets for oxidation by free radicals. Stadtman and associates (158-160) have found at least 10 enzymes with important roles in cellular metabolism to be susceptible to oxidation which directly results in proteolytic degradation. The activity of the proteolytic enzymes that hydrolyze these oxidized proteins was found to be insufficient to prevent an age associated increase in oxidized proteins. Many of these proteins retain their antigenic reactivity but lose their enzymatic activity. Studies done in patients with Warner syndrome and progeria, diseases associated with premature aging, reveal that oxidized proteins increase at a much higher rate than is normal (158). Lipofuscin and fluorescent pigments representing cross links between protein and lipid peroxidation products were also found to increase with age (161,162).

To balance out the oxidant load from both endogenous respiration and exogenous sources there are several types of anti-oxidant defenses. Some of these defenses are produced within the cell while others are taken in as part of the diet. Some are confined to compartments within the cell or particular structures within the cell.

Superoxide dismutase (SOD) uses a free radical as a substrate and catalyzes the reduction of $0_2^{\cdot -}$ to H_20_2.

$$2\ O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$$

There are at least three known forms of SOD. An iron containing form is found only in lower life forms. Copper-zinc-SOD is found in the cytosol of most cells, and a manganese-containing variety is found in mitochondria (163). Mitochondrial SOD can be upregulated by stimulants which increase free radical generation such as paraquat (164), uncouplers of mitochondrial respiration (165), and hyperbaric oxygen (166). Superoxide dismutase activity per specific metabolic rate can be correlated to the maximum lifespan potential of various animals as shown below. This activity as a protectant against free radical production is conserved over a large range of animals.



Even though SOD reduces superoxide to H_2O_2, safety has not been achieved. H_2O_2 is more stable, however it can react with $O_2^{\cdot-}$ and transition metals to form the hydroxyl ($^{\cdot}OH$) radical, the most reactive of the oxygen species (167). H_2O_2 is also the product of a variety of cellular reactions such as amino acid oxidation and normal respiration. Most cells contain several types of enzymes to remove H_2O_2, the most important of which is catalase which dismutates H_2O_2 to H_2O and O_2. H_2O_2 is able to diffuse within the cell to damage cellular components that are a considerable distance from the site of the generation of H_2O_2. Peroxisomes appear to contain most of the catalase activity found in cells (168,169). Perhaps because of the diffusibility of H_2O_2 there is apparently no catalase activity in mitochondria (170,171) despite the fact that a significant portion of cellular H_2O_2 is produced within the mitochondria as a by-product of normal respiration. Sohal and associates at SMU here in Dallas have shown that catalase inhibition exerts no effect on the longevity of Drosophila except at near absent levels (172). A recent experiment involving overexpression of both SOD and catalase however caused a significant increase in longevity of these fruit flies (173). In humans, a genetic disorder that results in extremely low catalase activity produces few if any harmful effects (174). Likewise, inhibition of catalase in cell culture does not appear to exert harmful effects (175). Under conditions of increased oxidative stress, such as hyperbaric oxygen or exogenously added H_2O_2, cells in culture and insects exhibit more deleterious effects suggesting that catalase is important under these conditions (175,176).

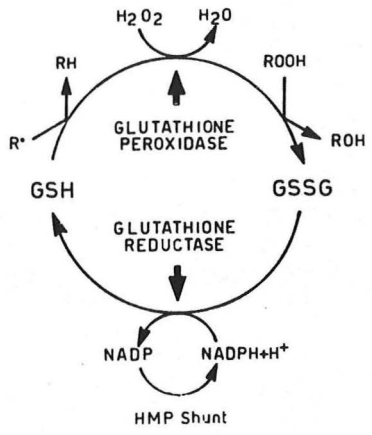
Glutathione is a tripeptide consisting of glycine, cysteine, and glutamic acid. It is a ubiquitous anti-oxidant found in all cells (177). It also acts as a co-factor for many of the enzymatic reactions that eliminate free radical by-products (178-180). Glutathione may represent the most important intracellular anti-oxidant since its concentration often exceeds 1 mmol/liter in several tissues (176). The intracellular ratio of oxidizing to reducing equivalents, (the redox state) is strongly influenced by the concentration of glutathione

(167). Glutathione is believed to absorb reactive oxygen radicals by reacting directly with them using the sulfhydryl group of the cysteine (181,182).

$$2\ GSH + 0_2^{\cdot -} \rightarrow GSSG + H_20_2$$

where GSSG is the oxidized form of glutathione. The fate of GSSG is to be excreted from the cell or reduced back to GSH (183), however, it may also interact with other cellular constituents. For example, the activity of the pentose phosphate shunt is modulated by GSSG which increases the activity of glucose - 6 - phosphate dehydrogenase (184). GSSG can also react with sulfhydryl containing enzymes to inactivate them. High concentrations of GSSG are toxic and have been shown to induce mitochondrial swelling, denature hemoglobin, and disrupt erythrocytes (178). Proteins which require the presence of disulfide bonds, such as insulin, can be inactivated by high concentrations of glutathione. A balance between glutathione and its oxidized form appears to be necessary for the health of the cell (185).

Several factors are known to disrupt this balance between GSSG and GSH. Exposure of cells to 100% oxygen for several hours completely depletes GSH (177). A smaller oxidative stress can stimulate GSH synthesis, which seems to be the primary response of human fibroblast to oxidative stress (186). Metabolic rate and mild oxidative stress have both been shown to modulate GSH concentration (187,188). Glutathione may play an important role in ion balance in the cell. Glutathione depletion either by oxidants or free radical generators causes mitochondrial release of calcium ion (189). This alteration of redox state and ion balance may result in an alteration in gene expression contributing to an increase in entropy and decrease in function and aging, possibly through changes in the cell's state of differentiation.

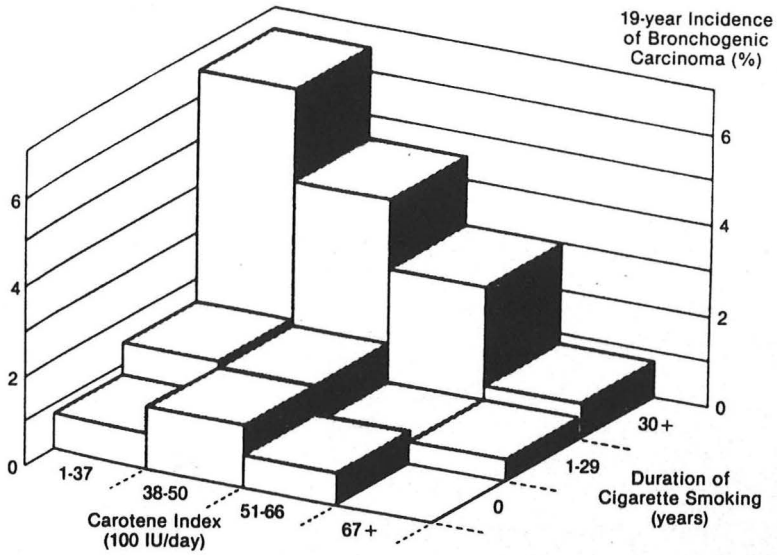


There are a couple of enzymes important in glutathione balance. One is glutathione reductase which reduces GSSG to GSH using NADPH as an electron donor. While GSH reductase does not have a direct anti-oxidant function, as with SOD and catalase, it does play a major role in the proper maintenance of the redox state of the cell (167). It may also indirectly control the pentose phosphate shunt through manipulation of GSSG levels (184).

Glutathione peroxidase works much like catalase except while catalase uses H_20_2 as an electron donor GSH peroxidase uses only glutathione as an electron donor. GSH peroxide can reduce H_20_2 and a variety of organic peroxides (190). GSH peroxidase requires selenium and may be upregulated by the addition of selenium to the diet (89). Selenium deficiency may accelerate many of the toxic effects associated with free radical damage. Cell culture studies have demonstrated that the addition of selenium permits the maximal expression of GSH peroxidase activity as well as an increased resistance to oxidation by organic peroxide addition (191).

Other defenses against oxidant damage include glutathione-S-transferases which inactivate reactive electrophilic mutagens, including the aldehyde products of lipid peroxidation. Peroxisomes can sequester peroxide producing enzymes to inhibit damage to other structures. Free iron is chelated by transferrin and ferritin and copper is taken up by ceruloplasmin both of which keep these metal ions from participating in fenton reactions (192). Ceruloplasm is a copper containing protein capable of oxidizing iron and has been found to effectively inhibit the iron catalyzed peroxidation of lipids (193). Urate may be a very important anti-oxidant in humans. It acts as a powerful anti-oxidant and scavenges singlet oxygen and other free-radicals (194). Bilirubin may also act as a non-enzymatic anti-oxidant (189). Ubiquinone, co-enzyme$_{10}$ (CoQ_{10}), is a small molecule necessary for transporting electrons in the rate limiting step of oxidative phosphorylation in mitochondria. Its reduced form, ubiquinol, can serve as an effective anti-oxidant in membranes (188,195-197). Several histidine derivatives such as carnosine, homocarnosine, and anserine exhibit potent anti-oxidant activity and are present in high concentrations in brain and muscle (197).

Exogenous anti-oxidants through dietary intake adds to the protection provided by these endogenous enzymatic anti-oxidant defenses. Fruits and vegetables are the main source of anti-oxidants in the diet and have been correlated with a lower risk of degenerative diseases and cancer (198). There is also mounting evidence in the literature that exogenous anti-oxidants are important in the prevention of cardiovascular disease and stroke (176,183). Low dietary intake of fruits and vegetables has been associated with an increased cancer rate for most types of cancer when compared with groups with high intake. This is demonstrated in the following graph in which the 19 year incidence of bronchogenic carcinoma in 2,100 men aged 40-55 years is shown as a function of the duration of cigarette smoking in years and the dietary intake of beta carotene through fruits and vegetables (not supplements) (199).



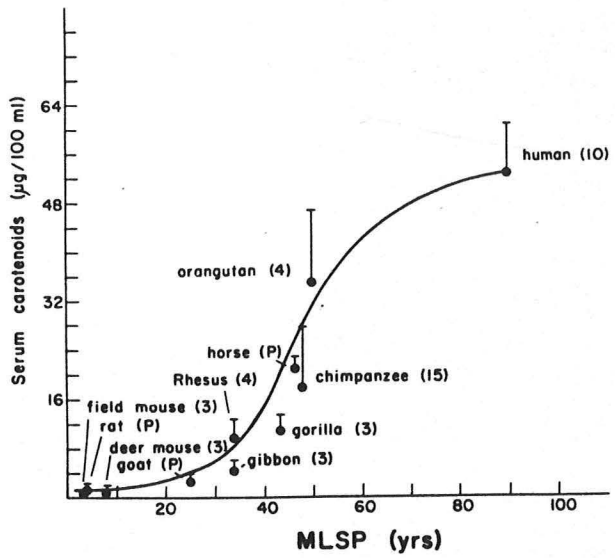
The non-smokers in the lowest quartile of carotene intake exhibited the same incidence of bronchogenic carcinoma as heavy smokers in the highest quartile of carotene intake. Block and colleagues have recently reviewed 172 studies in the epidemiologic literature that correlate inadequate consumption of fruits and vegetables to cancer incidence as shown in the table below. These studies show great consistency and good relative risk (198). Please refer to Dr. Gordon Luk's grand rounds on aspirin and colon cancer for a discussion of epidemiologic studies.

EPIDEMIOLOGIC STUDIES WITH FRUIT AND VEGETABLES

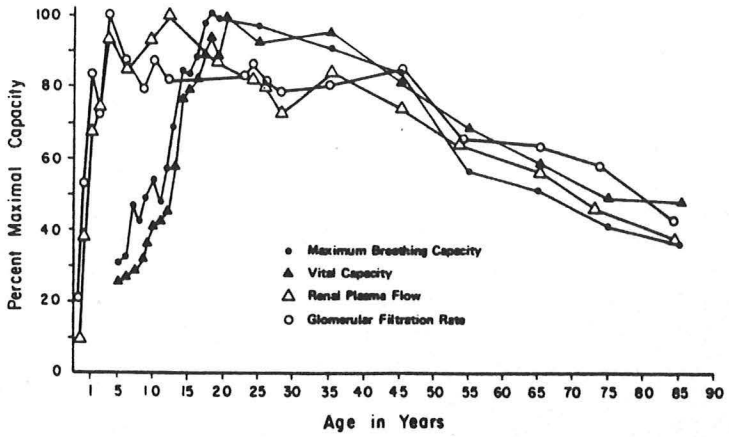
Cancer site	Studies with protection	Relative Risk
Epithelial		
– Lung	24/25	2.2
– Oral	9/9	2.0
– Larynx	4/4	2.3
– Esophagus	15/16	2.0
– Stomach	17/19	2.5
– Pancreas	9/11	2.8
– Cervix	7/8	2.0
– Bladder	3/5	2.1
– Colorectal	20/35	1.9
– Miscellaneous	6/8	-
Hormone-dependent		
– Breast	8/14	1.3
– Ovary/endometrium	3/4	1.8
– Prostate	4/14	1.3
Total	129/172	

Nutr. Cancer 18, 1992

Only 9% of Americans eat five servings of fruits and vegetables per day as recommended by the National Cancer Institute and the National Research Council (198,200). It is difficult to disentangle the effect of dietary intake of particular anti-oxidants such as ascorbate, alpha-tocopherol, and carotenoids from other vitamins or anti-oxidants found in fruits and vegetables. Dietary anti-oxidant vitamins, such as vitamin C, vitamin E, and carotenoids have shown particular promise as anticarcinogens and as defenses against degenerative diseases (201-204). Tocopherol appears to play a particularly important role in free radical protection because its hydrophobic structure permits incorporation into biologic membranes and is particularly prevalent in the membranes of endoplasmic reticula and mitochondria (119). Vitamin C plays an important role in the elimination of singlet oxygen and can regenerate oxidized vitamin E (115,201). Beta carotene, like most carotenoids, can be metabolized to vitamin A but has intrinsic anti-oxidant activity, particularly against singlet oxygen (205-208).



Several theoretical models of aging predict that normal human aging would result in a steady decline in functional capacity of essentially every physiological and mental process, beginning shortly after the age of sexual maturation. The change in organ function with age is shown in the figure below for the lung and kidney (209-212).



The slope of the decrease in organ capacity with age could be interpreted to reflect an average or overall aging rate for the body. It is well known that with increasing age there is also an increased incidence of disease which ultimately results in the death of the individual. The following table lists the ten leading causes of death in the United States for 1990 (213).

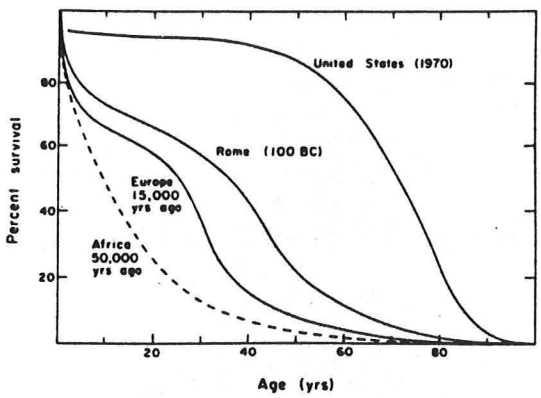
TEN LEADING CAUSES OF DEATH IN THE U.S.(1990)

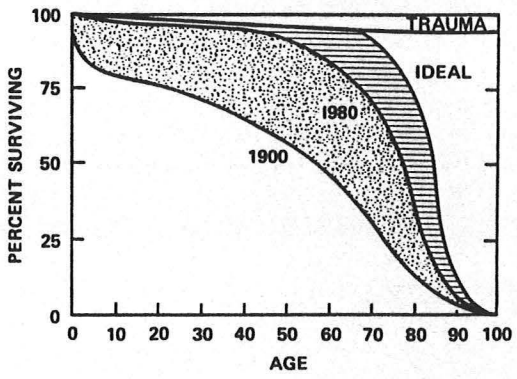
- HEART DISEASE
- MALIGNANT NEOPLASMS
- CEREBROVASCULAR DISEASES
- ACCIDENTS
- CHRONIC OBSTRUCTIVE PULMONARY DISEASE
- PNEUMONIA AND INFLUENZA
- DIABETES MELLITUS
- SUICIDE
- HUMAN IMMUNODEFICIENCY VIRUS
- HOMICIDE

Vital Statistics of the United States

Chronic illness accounts for more than 70% of all deaths and for an even higher percentage of cases of total disability. Arteriosclerosis which would include both coronary artery disease and stroke, cancer, chronic obstructive pulmonary disease, adult onset diabetes, and cirrhosis represent an overwhelming majority of our health problems. Most of these conditions exhibit an increased incidence with increasing age and can broadly be considered as problems of accelerated loss of organ reserve. These diseases develop slowly and asymptomatically until some clinical threshold is reached causing morbidity with progression and often culminating in death or disability.

This state is in complete contrast to mortality patterns that dominated at the turn of the century. Most deaths were from infectious disease processes such as tuberculosis, acute rheumatic fever, small pox, diphtheria, tetanus, polio myelitis, and pneumococcal pneumonia particularly in the young. Many factors contributed to the decrease in infectious diseases including less crowded living arrangements, improved nutrition, water sterilization, immunization and specific antibiotics. It is important to recognize that chronic diseases have replaced acute illness as a major cause of death and morbidity. Our extensive progress in medical care can be viewed as an exchange of acute medical problems for chronic medical problems. A patient surviving acute illness early in life will have more lingering problems later. Early death from the acute illness would cost relatively little in direct medical care dollars as compared with the expenses of a later, chronic problem. The exchange of acute illness for chronic illness has resulted in a massive need for additional medical services and has undoubtedly contributed to the increase in health care costs which now represent around 12% of the gross national product of this country. With increasing age every person is found to decline uniformly in essentially all body functions with increasing chronological age. Individuals living beyond 100 years do so because they age more uniformly rather than aging more slowly. Individuals dying before this time might be thought of as having a weak link in their body functions such as from heart disease or diabetes.





The shape of the human survival curve shown in the figure above provides several insights into human aging. For most of our time on this planet, human life expectancy is generally believed to have been around 20-30 years. In primitive cultures where life expectancy was this low, few people lived long enough to appreciably suffer from the processes of senescence or aging. Death was almost entirely due to environmental hazards and infectious diseases are usually considered to be a random factor in the mortality curves. Removal of these random factors over the past 400 years or so in developed nations has produced a dramatic increase in life expectancy of around 40-50 years. Sequential survival curves throughout this century show "progressive rectangularization" as elimination of these premature or random deaths results in an uncovering of a non-random factor which is thought to consist essentially of the aging process. Although people have life expectancies in the 70-80 year range they are not living younger for longer periods of time but are instead actually living older for longer periods of time (108). This condition largely reflects the fact that increased life expectancy occurred within the framework of an unchanging aging rate. The fact that the tail of the survival curve demonstrates the decline expected from a normal distribution rather than an additional peak of a few persons with notably long life spans implies that there is a fixed length to human life. In addition, despite this large increase in average life expectancy there has been no concomitant change in the number of persons living beyond 100 years of age (214). If relative immortality were possible, one would expect to find some persons genetically favored and fortunate enough to avoid disease and therefore live much longer than actually predicted. Data kept over the last 150 years fails to confirm the existence of such events (214).

Like so many other things that roll downhill, the signing of death certificates is usually done by the interns so they will know the truth of this statement: No one dies of old age anymore. It is simply not permitted as a diagnosis on the death certificate no matter how old the individual. Instead, the failing organ system is what keeps the death certificate from returning to the signer for correction. The continual decline of organ system function is one of the hallmarks of aging. A decline of one organ system out of proportion to the decline of other organ systems is often times the result of lifestyle choices such as tobacco or alcohol abuse or perhaps a distaste for broccoli. The longevity of individuals is the result of the uniformity of the loss of various organ functions some of which may be genetic. The following table demonstrates the dictum to choose your parents wisely.

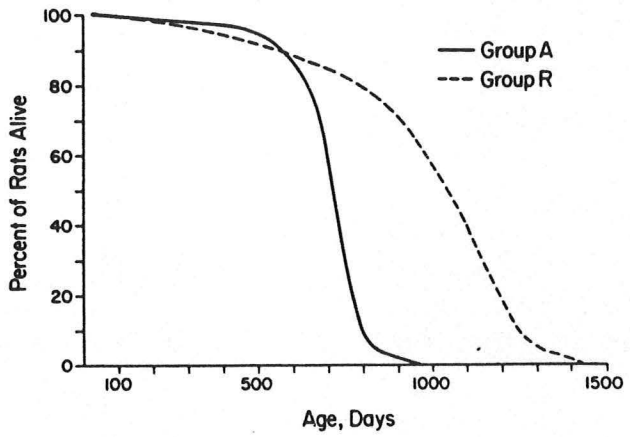
(1) Survivorship of offspring when father lived >90 years

Sex of offspring	Mother's age at death		
	<60	61–80	>81
Male	67.6	71.4	73.2
Female	73.8	74.1	77.2
Combined	70.9	72.8	75.1

(2) Survivorship of offspring when mother lived >90 years

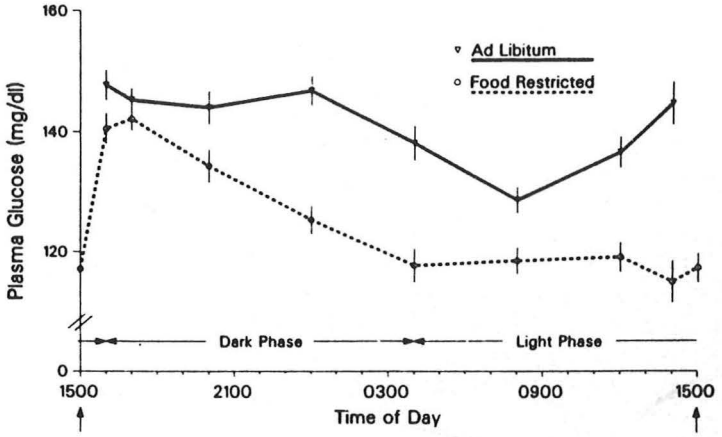
Sex of offspring	Father's age at death		
	<60	61–80	>81
Male	67.0	69.3	70.9
Female	73.0	73.5	73.3
Combined	69.8	71.4	72.1

Longevity of the individuals is correlated with the longevity of the parents. This correlation is stronger for the mother than for the father. This finding supports the notion that aging may be a disease inherited from your mother. Dr. Dennis Stone recently reviewed diseases inherited through abnormalities in the mitochondria. The same thing may be occurring in normal aging either through abnormalities in the mitochondria or antioxidant defenses for the mitochondria (see earlier discussion).



Taking antioxidants has been shown to extend the average lifespan of animals, but only food (or protein) restriction has been shown to extend the maximum lifespan potential of a species (215) (see figure above A = ad libitum; R = restricted).

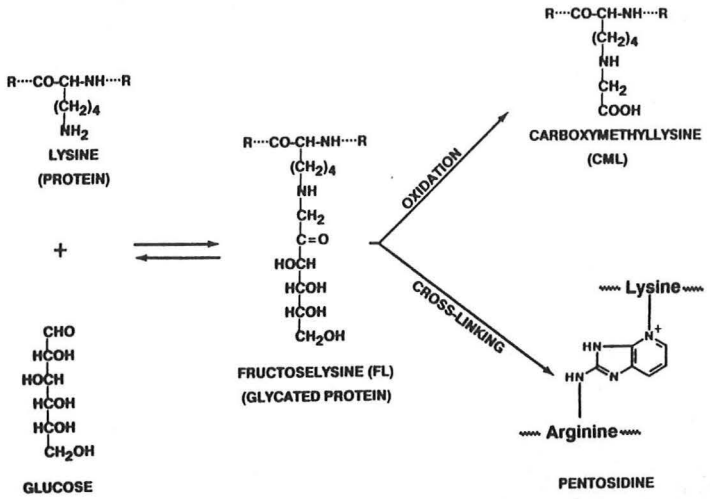
The extension is probably a protective mechanism for the species, to postpone reproduction during periods of famine (112). The explanation of how the added life is gained is not known. The metabolic rate of the animal does not decrease, except for a short time after initiation of the restriction, so it really is added time.



One observation may provide a possible mechanism for this life extension. These mice develop a sensitivity to insulin and have a lower plasma glucose than their well-fed counterparts (216) (see figure above). This lower glucose may decrease the glycation of proteins, lowering glycoxidation products, thereby maintaining their entropy at a lower state, and living longer. If this were the case, then we might expect diabetes to age faster. Certainly, this is the case for some of the "aging" diseases such as arteriosclerosis, and certain organ dysfunction (217).

Increased glucose concentrations, such as seen in diabetes results in the glycation of proteins. We use this clinically as a measure of control with the hemoglobin A1c. Glucose is an aldehyde and like other aldehydes, such as formaldehyde and glutaraldehyde, it reacts with proteins, especially lysine residues and alpha-amino groups. The reaction forms a Schiff base and undergoes what is called Amadori rearrangement to form a stable ketoamine adduct to the protein. But the reason that we can manage to circulate this compound in our blood at 5 mmol concentration is because 99.998% of it exists in a cyclic hemiacetone confirmation. This reaction is linear with time and linear with glucose concentration. It is stable with a half-life of about 4 months (218), which is why we can use it as a measure of long-term control.

If you take sugar and apply heat, it will brown. This is the mechanism to make caramel as seen on flan in most Mexican restaurants (personal experience). This reaction is called the Maillard reaction and involves oxidation (217-220). This same reaction occurs in the body although considerably slower than on your stove, but then the incubation period is much longer (a lifetime).



The oxidation of glycation products has been called glycoxidation (217). Oxidation, like resting or burning, is irreversible, while glycation is reversible. The products from glycoxidation have been termed AGE (for advanced glycosylation end products) (217-220). While glycation products do not increase with age, AGE products such as carboxymethyllysine and pentosidine do increase with age and increase at a greater rate in diabetes (217). This increased AGE accumulation can be blocked experimentally by antioxidants and the complications of diabetes prevented in experimental animals (217-220). Again defense against oxidant load may prevent increased aging from hyperglycemia.

SUMMARY

There is conservation of the aging phenomena and the underlying causes of aging across a wide range of species. This suggests that the difference in lifespan across species is the result of a quantitative difference in metabolism and the protection against oxidative by-products of metabolism. The balance between oxidant load and antioxidant defense may serve in the development of the organism, with apoptosis, and in the aging of the organism. Imbalance in either direction, as with bcl-2, may be detrimental to the health of the cell or the organism. Here's to a balanced life.

REFERENCES

1. Kimura KI, Truman JW: *J Neurosci* 10:403-411, 1990.

2. Williams L, Bell L: *Anat Embryol* 183:573-578, 1991.

3. Antalikova L, Kren V, Kasparek R, Bila V: *Folio Biol* 35:339-346, 1989.

4. Furtwangler JA, Hall SH, Koskinen ML: *Acta Anat* 124:74-80, 1985.

5. Golstein P, Ojcius DM, Young JD: *Immunol Rev* 121:29-65, 1991.

6. Rothenberg EV: *Adv Immunol* 51:85-214, 1992.

7. Cohen JJ, Duke RC, Fadok VA, Sellins KS: *Annu Rev Immunol* 10:267-293, 1992.

8. Clouston WM, Kerr JF: *Med Hypotheses* 18:399-404, 1985.

9. Duncan AM, Heddle JA: *Cancer Lett* 23:307-311, 1984.

10. Potten CS, Al BS, Hume WJ, Searle J: *Cell Tissue Kinet* 10:557-568, 1977.

11. Young AR: *Photodermatology* 4:127-134, 1987.

12. Danno K, Horio T: *Br J Dermatol* 107:423-428, 1982.

13. Laster SM, Wood JG, Gooding LR: *J Immunol* 141:2629-2634, 1988.

14. Heller RA, Song K, Fan N, Chang DJ: *Cell* 70:47-56, 1992.

15. Kerr JF, Wyllie AH, Currie AR: *Br J Cancer* 26:239-527, 1972.

16. Schwartz LM, Kosz L, Kay BK: *Proc Natl Acad Sci USA* 87:6594-6598, 1990.

17. Ju ST: *J Immunol* 146:812-828, 1991.

18. Odaka C, Kizaki H, Tadakuma T: *J Immunol* 144:2096-2101, 1990.

19. Kizaki H, Tadakuma T, Odaka C, Muramatsu J, Ishimura Y: *J Immunol* 143:1790-1794, 1989.

20. Martin DP, Schmidt RE, DiStefano PS, Lowry OH, Carter JG, Johnson EJ: *J Cell Biol* 106:829-844, 1988.

21. Sellins KS, Cohen JJ: *Radiat Res* 126:88-95, 1991.

22. Dutz JP, Walden PR, Eisen HN: *Int Immunol* 4:571-580, 1992.

23. Lin JK, Chou CK: *Cancer Res* 52:385-388, 1992.

24. Sulston JE, Horvitz HR: *Dev Biol* 56:110-156, 1977.

25. Sulston JE, Schierenberg E, White JG, Thomson JN: *Dev Biol* 100:64-119, 1983.

26. Hedgecock EM, Sulston JE, Thomson JN: *Science* 20:1277-1279, 1983.

27. Ellis HM, Horvitz HR: *Cell* 44:817-829, 1986.

28. Ellis RE, Jacobson DM, Horvitz HR: *Genetics* 129:79-94, 1991.

29. Hengartner MO, Ellis RE, Horvitz HR: *Nature* 356:494-499, 1992.

30. Yuan JY, Horvitz HR: *Dev Biol* 138:33-41, 1990.

31. Vaux DL, Cory S, Adams JM: *Nature* 335:440-442, 1988.

32. Tsujimoto Y, Cossman J, Jaffe E, Croce C: Involvement of the bcl-2 gene in human follicular lymphoma. *Science* 228:1440-1443, 1985.

33. Nowell, P.C. and Croce, C.M: Cytogenetics of neoplasia. In: *Development and recognition of the Transformed Cell*, edited by Greene, M.I. and Hamaoka, T. New York: Plenum Publishing Corporation, 1987,

34. Hockenbery D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ: Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 348:334-336, 1990.

35. Martin DP, Ito A, Horigome K, Lampe PA, Johnson EM,Jr.: Biochemical characterization of programmed cell death in NGF-deprived sympathetic neurons. *J Neurobiol* 23:1205-1220, 1992.

36. Gagliardini V, Fernandex PA, Lee RKK, Drexler HCA, Rotello RJ, Fishman MC, Yuan J: Prevention of vertebrate neuronal death by the crmA gene. *Science* 263:826-828, 1994.

37. Hengartner MO, Horvitz HR: C. elegans cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. *Cell* 76:665-676, 1994.

38. Yuan J, Shaham S, Ledoux S, Ellis HM, Horvitz HR: The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1B-converting enzyme. *Cell* 75:641-652, 1993.

39. Dinarello CA: Interleukin-1 and interleukin-1 antagonism. *Blood* 77:1627-1652, 1991.

40. diGiovine FS, Duff GW: Interleukin-1: the first interleukin. *Immunol Today* 11:13, 1990.

41. Kostura MJ, Tocci MJ, Limjuco G, Chin J, Cameron P, Hillman AG, Chartrain NA, Schmidt JA: Identification of a monocyte specific pre-interleukin-1B convertase activity. *Proc Natl Acad Sci USA* 86:5227-5231, 1989.

42. Black RA, Kronheim SR, Sleath PR: Activation of interleukin-1B by a co-induced protease. *FEBS Lett* 247:386-390, 1989.

43. Cerretti DP, Kozlosky CJ, Mosley B, Nelson N, Ness KV, Greenstreet TA, March CJ, Kronheim SR, Druck T, Cannizzaro LA, Huebner K, Black RA: Molecular cloning of the interleukin-1B converting enzyme. *Science* 256:97-100, 1992.

44. Thornberry NA, Bull HG, Calaycay JR, Chapman KT, Howard AD, Kostura MJ, Miller DK, Molineaux SM, Weidener JR, Aunins J, Elliston KO, Ayala JM, Cassano FJ, Chin J, Ding GJF, Egger LA, Gaffney EP, Limjuco G, Palyha OC, Raju SM, Rolando AM, Salley JP, Yamin TT, Lee TD, Shively JE, MacCross M, Momford RA, Schmidt JA, Tocci MJ: A novel heterodimeric cysteine protease is required for interleukin-1B processing in monocytes. *Nature* 356:768-774, 1992.

45. Kumar S, Tomooka Y, Noda M: Identification of a set of genes with developmentally down-regulated expression in the mouse brain. *Biochem Biophys Res Commun* 185:1155-1161, 1992.

46. Miura M, Zhu H, Rotello R, Hartwieg EA, Yuan J: Induction of apoptosis in fibroblasts by IL-1B-converting enzyme, a mammalian homolog of the C. elegans cell death gene ced-3. *Cell* 75:653-660, 1993.

47. Hogoquist KA, Nett MA, Unanue ER, Caplin DD: Interleukin 1 is processed and released during apoptosis. *Proc Natl Acad Sci USA* 88:8485-8489, 1991.

48. Davies AM: *Development* 100:185, 1987.

49. Martin DP, et.al. *J Cell Biol* 106:829, 1988.

50. Oppenheim RW, Prevette D, Tytell M, Homma S: *Devel Biol* 138:104, 1990.

51. Ray CA, Black RA, Kronheim SR, Greenstreet TA, Sleath PR, Salvesen GS, Pickup DJ: Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1B converting enzyme. *Cell* 69:597-604, 1992.

52. Hengartner M, Ellis RE, Horvitz HR: Caenorhabditis elegans gene ced-9 protects cells from programmed cell death. *Nature* 356:494-499, 1992.

53. Buttke TM, Sandstrom PA: Oxidative stress as a mediator of apoptosis. *Immunol Today* 15:7-10, 1994.

54. Oltvai ZN, Milliman CL, Korsmeyer SJ: Bcl-2 heterodimerizes in vivo with a conserved homolog, bax, that accelerates programmed cell death. *Cell* 74:606-619, 1993.

55. Sentman CL, Shutter JR, Hockenbery D, Kanagawa O, Korsmeyer SJ: Bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. *Cell* 67:879-888, 1991.

56. Strasser A, Harris AW, Cory S: Bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship. *Cell* 67:889-899, 1991.

57. Veis DJ, Sentman CL, Bach EA, Korsmeyer SJ: Expression of the bcl-2 protein in murine and hyman thymocytes and in peripheral T lymphocytes. *J Immunol* in press:1994.

58. Nakayama K-I, Nakayama K, Negishi I, Kuida K, Shinkai Y, Louie MC, Fields LE, Lucas PJ, Stewart V, Alt FW, Loh DY: Disappearance of the lymphoid system in bcl-2 homozygous mutant chimeric mice. *Science* 261:1584-1588, 1993.

59. Veis DJ, Sorenson CM, Shutter JR, Korsmeyer SJ: Bcl-2 deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. *Cell* 75:229-240, 1993.

60. Prota G: Recent advances in the chemistry of melanogenesis in mammals. *J Invest Dermatol* 75:122-127, 1980.

61. Silvers, W.K. *The Coat Colors of Mice*, New York:Springer-Verlag, 1979.

62. Yohn JJ, Norris DA, Yrastorza DG, Bruno IJ, Jeff JA, Hake SS, Repine JE: Disparate antioxidant enzyme activites in cultured human cutaneous fibroblasts, keratinocytes, and melanocytes. *J Invest Dermatol* 97:405-409, 1991.

63. Spanakis E, Lamina P, Bennett DC: Effects of the developmental colour mutations silver and recessive spotting on proliferation of diploid and immortal mouse

melanocytes in culture. *Development* 114:675-680, 1992.

64. Piatelli M, Fattorusso I, Magno S, Nicholaus RA: Structure of melanins and melanogenesis. 2. Sepoimelanin and synthetic pigments. *Tetrahedron* 18:941-949, 1962.

65. Hockenbery DM, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ: Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 348:334-336, 1990.

66. Nguyen M, Millar DG, Yong VW, Korsmeyer SJ, Shore GC: Targeting of Bcl-2 to the mitochondrial outer membrane by a COOH-terminal-signal-anchor sequence. *J Biol Chem* in press:1993.

67. Jacobson MD, Burne JF, King MP, et.al. : . *Nature* 361:365-369, 1993.

68. Boveris A, Chance B: The mitochondrial generation of hydrogen peroxide. *Biochem J* 134:707-716, 1973.

69. Forman, H.J. and Boveris, A. Superoxide radical and hydrogen peroxide in mitochondria. In: *Free Radicals in Biology*, edited by Pryor, W.A. New York: Academic Press, 1982, p. 65-90.

70. Cross AR, James OTG: Enzymic mechanisms of superoxide production. *Biochim Biophys Acta* 1057:281-298, 1991.

71. Larrick JW, Wright SC: Cytotoxic mechanism of tumor necrosis factor-alpha. [Review]. *FASEB Journal* 4:3215-3223, 1990.

72. Matthews N, Neale ML, Jackson SK, Stark JM: Tumour cell killing by tumour necrosis factor: inhibition by anaerobic conditions, free-radical scavengers and inhibitors of arachidonate metabolism. *Immunology* 62:153-155, 1987.

73. Matsuda M, Masutani H, Nakamura H, Miyajima S, Yamauchi A, Yonehara S, Uchida A, Irimajiri K, Horiuchi A, Yodoi J: Protective activity of adult T cell leukemia-derived factor (ADF) against tumor necrosis factor-dependent cytotoxicity on U937 cells. *J Immunol* 147:3837-3841, 1991.

74. Chang DJ, Ringold GM, Heller RA: Cell killing and induction of manganous superoxide dismutase by tumor necrosis factor-alpha is mediated by lipoxygenase metabolites of arachidonic acid. *Biochemical & Biophysical Research Communications* 188:538-546, 1992.

75. Hirose K, Longo DL, Oppenheim JJ, Matsushima K: Overexpression of

mitochondrial manganese superoxide dismutase promotes the survival of tumor cells exposed to interleukin-1, tumor necrosis factor, selected anticancer drugs, and ionizing radiation. *FASEB Journal* 7:361-368, 1993.

76. Wong GH, McHugh T, Weber R, Goeddel DV: Tumor necrosis factor alpha selectively sensitizes human immunodeficiency virus-infected cells to heat and radiation. *Proc Natl Acad Sci USA* 88:4372-4376, 1991.

77. Halliwell B, Gutteridge JM: Role of free radicals and catalytic metal ions in human disease: an overview. [Review]. *Methods Enzymol* 186:1-85, 1990.

78. Ramasarma T: Generation of H2O in biomembranes. [Review]. *Biochimica et Biophysica Acta* 694:69-93, 1982.

79. Hockenbery DM, Oltvai ZN, Yin X-M, Milliman CL, Korsmeyer SJ: Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* 75:241-251, 1993.

80. Schreck R, Albermann KAJ, Baeuerle PA: *Free Rad Res Comm* 17:221-237, 1992.

81. Benchekroun MN, Pourquier P, Schott B, Robert J: Doxorubicin-induced lipid peroxidation and glutathione peroxidase activity in tumor cell lines selected for resistance to doxorubicin. *Eur J Biochem* 211:141-146, 1993.

82. Diomede L, Colotta F, Piovani B, Re F, Modest EJ, Salmona M: Induction of apoptosis in human leukemic cells by the ether lipid 1-octadecyl-2-methyl-rac-glycero-3-phosphocholine. A possible basis for its selective action. *Int J Cancer* 53:124-130, 1993.

83. Wagner BA, Buettner GR, Burns CP: Increased generation of lipid-derived and ascorbate free radicals by L1210 cells exposed to the ether lipid edelfosine. *Cancer Res* 53:711-713, 1993.

84. Takagi Y, Shikita M, Terasima T, Akaboshi S: *Rad Res* 60:292-301, 1974.

85. Stamler JS, Singel DJ, Loscalzo J: Biochemistry of nitric oxide and its redox-activated forms. [Review]. *Science* 258:1898-1902, 1992.

86. Albina JE, Cui S, Mateo RB, Reichner JS: Nitric oxide-mediated apoptosis in murine peritoneal macrophages. *J Immunol* 150:5080-5085, 1993.

87. Zhong LT, Sarafian T, Kane DJ, Charles AC, Mah SP, Edwards RH, Bredesen DE: bcl-2 inhibits death of central neural cells induced by multiple agents. *Proc Natl Acad Sci USA* 90:4533-4537, 1993.

88. Sandstrom PA, Buttke TM: Autocrine production of extracellular catalase prevents apoptosis of the human CEM T-cell line in serum-free medium. *Proc Natl Acad Sci USA* 90:4708-4712, 1993.

89. Cutler, R.G. Antioxidants, aging, and longevity. In: *Free Radicals in Biology*, edited by Pryor, W.A. New York: Academic Press, 1984, p. 371-428.

90. Israel N, Gougerot-Pocidalo MA, Aillet F, Virelizier JL: . *J Immunol* 149:3386-3393, 1992.

91. Schwartzman RA, Cidlowski JA: . *Endocrine Rev* 14:133-151, 1993.

92. Maltzman W, Czyzyk L: . *Mol Cell Biol* 4:1689-1694, 1984.

93. Halliwell B: . *Drugs* 42:569-605, 1991.

94. Williams GT, Smith CA: Molecular regulation of apoptosis: Genetic controls on cell death. *Cell* 74:777-779, 1993.

95. Reed JC: Bcl-2 and the regulation of programmed cell death. *J Cell Biol* 124:1-6, 1994.

96. Marx J: How p53 suppresses cell growth. *Science* 262:1644-1645, 1993.

97. Lennon SV, Martin SJ, Cotter TG: Dose-dependent induction of apoptosis in human tumour cell lines by widely diverging stimuli. *Cell Proliferation* 24:203-214, 1991.

98. Duvall E, Wyllie AH: . *Immunol Today* 7:115-119, 1986.

99. Wyllie AH, Kerr JFR, Currie AR: . *Int Rev Cytol* 68:251, 1980.

100. Medvedev ZA: An attempt at a rational classification of theories of ageing. *Biol Rev* 65:375-398, 1990.

101. Cutler, R.G. *Interdisciplin Topics Gerontol*, Basel:Karger, 1976. Ed. 9 pp. 83-133.

102. Cutler, R.G. *Testing the Theories of Aging*, Boca Raton:CRC Press, 1982. pp. 25-114.

103. Cutler, R.G. *Aging and Cell Structure*, New York:Plenum Press, 1984. Ed. 2

104. Hart RW, Setlow RB: Correlation between deoxyribonucleic acid excision repair and life span in a number of mammalian species. *Proc Natl Acad Sci USA* 71:2169-2173, 1974.

105. Doll R: . *J Royal Stat Soc* A134:133-166, 1971.

106. Portier CJ, Hedges JC, Hoel DG: . *Cancer Res* 46:4372-4378, 1986.

107. Harman D: The aging process. *Proc Natl Acad Sci USA* 78:7124-7128, 1981.

108. Cutler RG: Human Longevity and Aging: Possible role of reactive oxygen species. *NY Acad Sci* 638:1-28, 1991.

109. Adelman R, Saul RL, Ames BN: . *Proc Natl Acad Sci USA* 85:2706-2708, 1988.

110. Shigenaga MK, Gimeno CJ, Ames BN: . *Proc Natl Acad Sci USA* 86:9697-9701, 1989.

111. Ames BN, Shigenaga MK: Oxidants are a major contributor to aging. [Review]. *Annals of the New York Academy of Sciences* 663:85-96, 1992.

112. Ames BN, Shigenaga MK, Hagen TM: Oxidants, antioxidants, and the degenerative diseases of aging. *Proc Natl Acad Sci USA* 90:7915-7922, 1993.

113. Grist SA, McCarron M, Kutlaca A, Turner DR, Morley AA: In vivo human somatic mutation: frequency and spectrum with age. *Mutation Research* 266:189-196, 1992.

114. Michaels ML, Cruz C, Grollman AP, Miller JH: Evidence that MutY and MutM combine to prevent mutations by an oxidatively damaged form of guanine in DNA. *Proceedings of the National Academy of Sciences of the United States of America* 89:7022-7025, 1992.

115. DiLuzio NR: Antioxidants, lipid peroxidation and chemical induced liver injury. *Fed Proc* 32:1875, 1973.

116. Pryor, W.A. Free Radicals in Autoxodiation and in Aging. In: *Free Radicals in Molecular Biology, Aging, and Disease*, edited by Armstrong, D., Sohal, R.S., Cutler, R.G. and Slater, T.F. New York: Raven Press, 1984, p. 13-41.

117. Mahaffey JW, Griswold CM, Matthews AL: Molecular genetic mechanisms in oxidative damage and aging. *Toxicol Ind Health* 9:215-222, 1993.

118. Ischiropoulos H, Zhu L, Beckman JS: Peroxynitrite formation from

macrophage-derived nitric oxide. *Archives of Biochemistry & Biophysics* 298:446-451, 1992.

119. Shacter E, Beecham EJ, Covey JM, Kohn KW, Potter M: . *Carcinogenesis* 9:2297-2304, 1988.

120. Yamashina K, Miller BE, Heppner GH: . *Cancer Res* 46:2396-2401, 1986.

121. Morrison DG, Daniel J, Lynd FT, Moyer MP, Esparza RJ, Moyer RC, Rogers W: Retinyl palmitate and ascorbic acid inhibit pulmonary neoplasms in mice exposed to fiberglass dust. *Nutr Cancer* 3:81-85, 1981.

122. Sanders CL, Mahaffey JA: Action of vitamin C on pulmonary carcinogenesis from inhaled 239PuO2. *Health Physics* 45:794-798, 1983.

123. Marsh JP, Mossman BT: Role of asbestos and active oxygen species in activation and expression of ornithine decarboxylase in hamster tracheal epithelial cells. *Cancer Res* 51:167-173, 1991.

124. Korkina LG, Durnev AD, Suslova TB, Cheremisina ZP, Daugel-Dauge NO, Afanas'ev IB: . *Mutat Res* 265:245-253, 1992.

125. Sobala GM, Pignatelli B, Schorah CJ, Bartsch H, Sanderson M, Dixon MF, Shires S, King RF, Axon AT: Levels of nitrite, nitrate, N-nitroso compounds, ascorbic acid and total bile acids in gastric juice of patients with and without precancerous conditions of the stomach. *Carcinogenesis* 12:193-198, 1991.

126. Kneller RW, Guo W-D, Hsing AW, Chen J-S, Blot WJ, Li J-Y, Forman D, Fraumeni FJ,Jr.: . *Cancer Epidemiol Biomarkers Prev* 1:113-118, 1992.

127. Arroyo PL, Hatch-Pigott V, Mower HF, Cooney RV: Mutagenicity of nitric oxide and its inhibition by antioxidants. *Mutat Res* 281:193-202, 1992.

128. Kasai H, Okada Y, Nishimura S, Rao MS, Reddy JK: Formation of 8-hydroxydeoxyguanosine in liver DNA of rats following long-term exposure to a peroxisome proliferator. *Cancer Res* 49:2603-2605, 1989.

129. Boobis, A., Caldwell, J., Dematteis, F. and David, D. *Microsomes and Drug Oxidation*, London:Taylor & Francis, 1985.

130. Kiyosawa H, Suko M, Okudaira H, Murata K, Miyamoto T, Chung MH, Kasai H, Nishimura S: Cigarette smoking induces formation of 8-hydroxydeoxyguanosine, one of the oxidative DNA damages in human peripheral leukocytes. *Free Radical Research Communications* 11:23-27, 1990.

131. Pryor WA, Dooley MM, Church DF: . *Adv Free Radical Biol Med* 2:161-188, 1986.

132. Frei B, Forte TM, Ames BN, Cross CE: Gas phase oxidants of cigarette smoke induce lipid peroxidation and changes in lipoprotein properties in human blood plasma. Protective effects of ascorbic acid. *Biochem J* 277:133-138, 1991.

133. Reznick AZ, Cross CE, Hu ML, Suzuki YJ, Khwaja S, Safadi A, Motchnik PA, Packer L, Halliwell B: Modification of plasma proteins by cigarette smoke as measured by protein carbonyl formation. *Biochem J* 286:607-611, 1992.

134. Schectman G, Byrd JC, Hoffmann R: Ascorbic acid requirements for smokers: analysis of a population survey. *Am J Clin Nutr* 53:1466-1470, 1991.

135. Duthie GG, Arthur JR, James WP: Effects of smoking and vitamin E on blood antioxidant status. *Am J Clin Nutr* 53:1061S-1063S, 1991.

136. Bui MH, Sauty A, Collet F, Leuenberger P: . *J Nutr* 122:312-316, 1991.

137. Peto R, Lopez AD, Boreham J, Thun M, Heath C,Jr.: Mortality from tobacco in developed countries: indirect estimation from national vital statistics [see comments]. *Lancet* 339:1268-1278, 1992.

138. Lauffer, R.B. *Iron and Human Disease*, Boca Raton:CRC, 1992.

139. Salonen JT, Nyyssonen K, Korpela H, Tuomilehto J, Seppanen R, Salonen R: High stored iron levels are associated with excess risk of myocardial infarction in eastern Finnish men [see comments]. *Circulation* 86:803-811, 1992.

140. Salonen JT, Salonen R, Korpela H, Suntioinen S, Tuomilehto J: Serum copper and the risk of acute myocardial infarction: a prospective population study in men in eastern Finland [see comments]. *Am J Epidemiol* 134:268-276, 1991.

141. Sullivan JL: The iron paradigm of ischemic heart disease [see comments]. [Review]. *Am Heart J* 117:1177-1188, 1989.

142. Chen M: . *Trop Dis Bull* 85:2-56, 1988.

143. Chen M, Mott K: . *Trop Dis Bull* 86:2-36, 1989.

144. Srivantanakul P, Sontipong S, Chotiwan P, Parkin DM: . *J Gastroenterol Hepatol* 3:413-420, 1988.

145. Shanmugaratnam K: . *Br J Cancer* 10:232-245, 1956.

146. Beasley RP: Hepatitis B virus. The major etiology of hepatocellular carcinoma. [Review]. *Cancer* 61:1942-1956, 1988.

147. Tabor E, Kobayashi K: Hepatitis C virus, a causative infectious agent of non-A, non-B hepatitis: prevalence and structure--summary of a conference on hepatitis C virus as a cause of hepatocellular carcinoma. *J Natl Cancer Inst* 84:86-90, 1992.

148. Yu MW, You SL, Chang AS, Lu SN, Liaw YF, Chen CJ: Association between hepatitis C virus antibodies and hepatocellular carcinoma in Taiwan. *Cancer Res* 51:5621-5625, 1991.

149. Parsonnet J, Friedman GD, Vandersteen DP, Chang Y, Vogelman JH, Orentreich N, Sibley RK: Helicobacter pylori infection and the risk of gastric carcinoma [see comments]. *N Engl J Med* 325:1127-1131, 1991.

150. Dooley CP, Cohen H, Fitzgibbons PL, Bauer M, Appleman MD, Perez-Perez GI, Blaser MJ: Prevalence of Helicobacter pylori infection and histologic gastritis in asymptomatic persons. *N Engl J Med* 321:1562-1566, 1989.

151. Cover TL, Blaser MJ: . *Annu Rev Med* 43:135-145, 1992.

152. Richter C, Park JW, Ames BN: Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proc Natl Acad Sci USA* 85:6465-6467, 1988.

153. Shay JW, Werbin H: Are mitochondrial DNA mutations involved in the carcinogenic process? *Mutat Res* 186:149-160, 1987.

154. Shay JW, Werbin H: New evidence for the insertion of mitochondrial DNA into the human genome: significance for cancer and staging. *Mutat Res* 275:227-235, 1992.

155. Hayashi JI, Takemitsu M, Goto Y-I, Novaka I: Human mitochondria and mitochondrial genome function as a single dynamic cellular unit. *J Cell Biol* 125:43-50, 1994.

156. Sugiyama S, Hattori K, Hayakawa M, Ozawa T: . *Biochem Biophys Res Commun* 180:894-899, 1991.

157. Yen TC, Su JH, King KL, Wei YH: . *Biochem Biophys Res Commun* 178:124-131, 1991.

158. Stadtman ER: Protein oxidation and aging. [Review]. *Science* 257:1220-1224,

1992.

159. Oliver CN, Ahn BW, Moerman EJ, Goldstein S, Stadtman ER: Age-related changes in oxidized proteins. *J Biol Chem* 262:5488-5491, 1987.

160. Oliver CN, Starke-Reed PE, Stadtman ER, Liu GJ, Carney JM, Floyd RA: Oxidative damage to brain proteins, loss of glutamine synthetase activity, and production of free radicals during ischemia/reperfusion-induced injury to gerbil brain. *Proc Natl Acad Sci USA* 87:5144-5147, 1990.

161. Halliwell, B. and Gutteridge, J.M.C. *Free Radicals in Biology and Medicine/*, Oxford:Clarnedon, 1989.

162. Brunk UT, Jones CB, Sohal RS: A novel hypothesis of lipofuscinogenesis and cellular aging based on interactions between oxidative stress and autophagocytosis. [Review]. *Mutat Res* 275:395-403, 1992.

163. *Free Radicals in Biology*, edited by Pryor, W.A. New York: Academic Press, 1976, p. 1-50.

164. Autor AP: Reduction of paraquat toxicity by superoxide dismutase. *Life Sci* 14:1309-1319, 1974.

165. Dryer SE, Dryer RL, Autor AP: Enhancement of mitochondrial, cyanide-resistant superoxide dismutase in the livers of rats treated with 2,4-dinitrophenol. *J Biol Chem* 255:1954-1057, 1980.

166. Crapo JD, McCord JM: Oxygen-induced changes in pulmonary superoxide dismutase assayed by antibody titrations. *Am J Physiol* 231:1196-1203, 1976.

167. Chance B, Sies H, Boveris A: Hydroperoxide metabolism in mammalian organs. *Physiol Rev* 59:527-605, 1979.

168. Halliwell, B. Free radicals, oxygen toxicity, and aging. In: *Age Pigments*, edited by Sohal, R.S. New York: Elsevier/North Holland, 1981, p. 1-62.

169. Tolbert NE: Microbodies-peroxisomes and glycoxysomes. *Ann Rev Plant Physiol* 22:45-74, 1971.

170. Allen JF: Effect of washing and osmotic shock on the catalase activity of intact chloroplast preparations. *FEBS Lett* 84:221-224, 1977.

171. Day DA, Arron GP, Lattes GG: Enzyme distribution in potato mitochondria. *J Exp Biol* 30:539-549, 1979.

172. Orr WC, Arnold LA, Sohal RS: Relationship between catalase activity, life span and some parameters associated with antioxidant defenses in Drosophila melanogaster. *Mech Ageing Dev* 63:287-296, 1992.

173. Orr WC, Sohal RS: Extension of life-span by overexpression of superoxide dismutase and catalase in Drosophila melanogaster. *Science* 263:1128-1130, 1994.

174. Masters C, Holmes R: Peroxisomes. New aspects of physiology and biochemistry. *Physiol Rev* 57:816-882, 1977.

175. Lesko, S., Trpis, L. and Yang, S. Cytotoxicity and somatic mutation induced in mammalian cells in culture by hyperoxia and ionizing radiation: Effect of superoxide dismutase and catalase inhibitors. In: *Free Radicals in Molecular Biology, Aging and Disease*, edited by Armstrong, D., Sohal, R.S., Cutler, R.G. and et.al., New York: Raven Press, 1984, p. 391-396.

176. Nicolosi RJ, Baird MB, Massie HR, et.al. : Senescence in Drosophila. II. Renewal of catalase activity in flies of different ages. *Exp Gerontol* 8:101-108, 1973.

177. Kosower, N.S. and Kosower, E.M. The glutathione-glutathione disulfide system. In: *Free Radicals in Biology*, edited by Pryor, W.A. New York: Academic Press, 1976, p. 55-84.

178. Meister A: Metabolism and function of glutathione. *TIBS* 6:231-234, 1981.

179. Meister A: Selective modification of glutathione metabolism. *Science* 220:472-477, 1983.

180. Meister A, Anderson ME: Glutathione. *Ann Rev Biochem* 49:711-760, 1983.

181. Foreman, H.J. and Fischer, A.B. Antioxidant defenses. In: *Oxygen and Living Processes*, edited by Gilbert, D.L. New York: Springer-Verlag, 1981,

182. Wefers H, Seis H: Oxidation of glutathione by the superoxide radical to the disulfide and the sulfonate yielding singlet oxygen. *Eur J Biochem* 137:29-36, 1983.

183. Nishiki K, Jamieson D, Oshino N, et.al. : Oxygen toxicity in the perfused rat liver and lung under hyperbaric conditions. *Biochem J* 160:343-355, 1976.

184. Eggleston EV, Krebs HA: Regulation of the pentose phosphate cycle. *Biochem J* 138:425-435, 1974.

185. Balin AK, Goodman DG, Rasmussen H: The effect of oxygen tension on the growth and metabolism of WI-38 cells. *J Cell Physiol* 89:235-250, 1976.

186. Balin, A.K., Allen, R.G. and Reimer, R. Human fibroblast antioxidant defense response to alteration in oxygen tension. In: *Oxygen Radicals in Biology and Medicine*, edited by Simic, M.G. New York: Plenum Press, 1989,

187. Allen, R.G. and Sohal, R.S. Role of glutathione in aging and development of insects. In: *Comparative Biology of Insect Aging: Strategies and Mechanisms*, edited by Collatz, K.G. and Sohal, R.S. Berlin: Springer-Verlag, 1986, p. 168-181.

188. Sohal, R.S. and Allen, R.G. Relationship between metabolic rate, free radicals, differentiation and aging: A unified theory. In: *The Molecular Biology of Aging*, edited by Woodhead, A.V., Blackett, A.D. and Hollaender, A. New York: Plenum Press, 1985, p. 75-104.

189. Jewell SA, Bellomo G, Thor H, et.al. : Bleb formation in hepatocytes during drug metabolism is caused by disturbances in thiol and calcium ion homeostasis. *Science* 217:1257-1258, 1982.

190. Flohe, L. Glutathione peroxidase brought into focus. In: *Free Radicals in Biology*, edited by Pryor, W.A. New York: Academic Press, 1982, p. 223-255.

191. Hornsby, P.J., Aldern, K.A. and Harris, S.E. Adrenocortical cultures as model systems for investigating cellular aging. In: *Free Radicals in Molecular Biology, Aging and Disease*, edited by Armstrong, D., Sohal, R.S., Cutler, R.G. and et.al., New York: Raven Press, 1984, p. 203-222.

192. Biemond P, van Eijk HG, Swaak AJG, Koster JF: . *J Clin Invest* 73:1576-1579, 1984.

193. Gutteridge JMC, Richmond R, Halliwell B: Oxygen-free radicals and lipid peroxidation: Inhibition by protein caeruloplasm. *FEBS Lett* 112:269-272, 1980.

194. Ames BN, Cathcart R, Schwiers E, et.al. : Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer. *Proc Natl Acad Sci* 78:6858-6862, 1981.

195. Scheibel MR, Lindsay RD, Tomiyasu O, et.al. : Progressive dendritic changes in aging human cortex. *Exp Neurol* 47:392-403, 1975.

196. Schroder HC, Messer R, Bachmann M, et.al. : Superoxide radical induced loss of

nuclear restriction of immature mRNA: A possible cause of ageing. *Mech Age Devel* 41:251-266, 1987.

197. Kohen R, Yamamoto Y, Cundy KC, et.al. : Antioxidant activity of carnosine, homocarnosine, and anserine present in muscle and brain. *Proc Natl Acad Sci* 85:3175-3179, 1988.

198. Monnier VM, Cerami A: Nonenzymatic glycosylation and browning in diabetes and aging. Studies on lens proteins. *Diabetes* 31:57-63, 1982.

199. Shekelle RB, Liu S, Raynor WJ, et.al. : Dietary vitamin A and risk of cancer in the Western Electric study. *Lancet* 2:1185-1190, 1981.

200. Northrop JH: Carbon dioxide production and duration of life of Drosophila cultures. *J Gen Physiol* 9:319-324, 1926.

201. Packer JE, Slater TF, Wilson RL: Direct observation of a free radical interaction between vitamin E and vitamin C. *Nature* 278:737-738, 1979.

202. Pearl, R. *The Rate of Living*, New York:Knopf Press, 1928.

203. Poplin L, DeLong R: Accelerated aging due to enzymatic racemization. *Gerontology* 24:365-368, 1978.

204. Pongor S, Ulrich PC, Bencasath FA, et.al. : Aging of proteins: Isolation and identification of a fluorescent chromophore from the reaction of polypeptides with glucose. *Proc Natl Acad Sci* 81:2684-2688, 1984.

205. *Free Radicals in Biology*, New York:Academic Press, 1976. pp. 1-50.

206. Ragland SS, Sohal RS: Mating behavior, physical activity and aging in the housefly, Musca domestica. *Exp Gerontol* 8:135-145, 1973.

207. Richie JP, Lang CA: Acetaminophen toxicity increases with aging in the mosquito. *Fed Proc* 38:2303, 1979.

208. Roth GS, Hess GD: Changes in the mechanisms of hormone and neurotransmitter action during aging, current status of the role of receptor and postreceptor alterations. *Mech Aging Dev* 20:175-194, 1982.

209. Comfort, A. *The Biology of Senescence*, New York:Elsevier, 1978.

210. Kohn, R.R. *Principles of Mammalian Aging*, Englewood Cliffs:Prentice-Hall,Inc., 1978.

211. *Handbook of the Biology of Aging*, New York:Academic Press, 1990. Ed. 3

212. Bafitis H, Sargent F: Human physiological adaptability through the life sequence. *J Gerontol* 32:402-410, 1977.

213. Boring CC, Squires TS, Tong T, Montgomer S: . *CA* 44:7-26, 1994.

214. Comfort, A. *The Biology of Senescence*, New York:Elsevier Press, 1979. Ed. 3

215. Yu BP, Lee DW, Marler CG, Choi JH: Mechanism of food restriction: Protection of cellular homeostasis. *Proc Soc Exp Biol Med* 193:13-15, 1990.

216. Masoro EJ: Retardation of aging processes by nutritional means. *Ann NY Acad Sci* 628:29-35, 1991.

217. Dyer DG, Dunn JA, Thorpe SR, Bailie KE, Lyons TJ, McCance DR, Baynes JW: Accumulation of maillard reaction products in skin collagen in diabetes and aging. *J Clin Invest* 91:2463-2469, 1993.

218. Lyons TJ, Bailie KE, Dyer DG, Dunn JA, Baynes JW: Decrease in skin collagen glycation with improved glycemic control in patients with insulin-dependent diabetes mellitus. *J Clin Invest* 87:1910-1915, 1991.

219. Monnier VM, Sell DR, Nagaraj RH, Miyata S, Grandhee S, Odetti P, Ibrahim SA: Maillard reaction-mediated molecular damage to extracellular matrix and other tissue proteins in diabetes, aging, and uremia. *Diabetes* 41:36-41, 1992.

220. Boynes JW: Glycation and oxidation of collagens in diabetes and aging. *Clin Res* 42:313, 1994.