Implications of oxidative stress on premature cellular senescence and development of Alzheimer's disease

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IMPLICATIONS OF OXIDATIVE STRESS ON PREMATURE CELLULAR SENESCENCE AND DEVELOPMENT OF ALZHEIMER’S DISEASE

By Danijela Domazet-Damjanov

A Thesis Submitted to the Faculty of Graduate Studies through the Department of Chemistry and Biochemistry in Partial Fulfilment of the Requirements for the Degree of Master of Science at the University of Windsor

Windsor, Ontario, Canada
2008

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Abstract

In response to external oxidative stress/DNA damaging agents, mammalian cells may choose one of the following pathways to avoid propagation of the damaged cells: repair the DNA and proceed with the normal cell cycle; trigger apoptosis; or undergo senescence to block cell division. Working with NHFs, we have observed that quiescent fibroblasts, unlike dividing fibroblasts, do not undergo apoptosis when subjected to a high dose of external oxidative stress but become senescent instead. Our results have indicated that p21 and MnSOD over-expression in quiescent cells is highly correlated to resistance to external oxidative stress and senescence induction. Furthermore, we observed that fibroblasts harvested from individuals diagnosed with Alzheimer's disease have a higher amount of endogenous ROS and double stranded DNA breaks than NHFs, leading to an earlier onset of replicative senescence. Consistent with higher ROS levels, AD fibroblasts have up-regulated expression of MnSOD and decreased levels of non-selenium glutathione peroxidase.
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<table>
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<th>Definition</th>
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<tbody>
<tr>
<td>4-HNE</td>
<td>4-Hydroxy-2-Nonenal</td>
</tr>
<tr>
<td>8-OHdG</td>
<td>8-Hydroxyl-2-Deoxyguanosine</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s Disease</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced Glycation End Products</td>
</tr>
<tr>
<td>APAF</td>
<td>Apoptosis Protease Activating Factor</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid Beta</td>
</tr>
<tr>
<td>DFO</td>
<td>Desferrioxamine</td>
</tr>
<tr>
<td>DISC</td>
<td>Death Inducing Signalion Complex</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas Associated Death Domain</td>
</tr>
<tr>
<td>MMP</td>
<td>Mitochondrial Membrane Potential</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Manganese Superoxide Dismutase</td>
</tr>
<tr>
<td>NHF</td>
<td>Normal Human Fibroblasts</td>
</tr>
<tr>
<td>NT</td>
<td>Nitro-tyrosine</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptors for Advanced Glycation End Products</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive Nitrogen Species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SAPK</td>
<td>Stress Activated Protein Kinase</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF Related Apoptosis Inducing Ligand</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal dUTP Nick End Labelling</td>
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Chapter I: Introduction

1.1 Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) is a collective term referring to oxygen radicals (superoxide and hydroxyl radicals) and non-radical derivatives (hydrogen peroxide) of molecular oxygen (Le Bras et al., 2005). Superoxide anion is formed by reduction of triplet state molecular oxygen. This reaction is mediated by NAD(P)H oxidases or xanthine oxidase, or non-enzymatically by redox reactive compounds (Dröge, 2002). Superoxide dismutase (SOD) converts superoxide radical into hydrogen peroxide (Deby and Goutier, 1990). In the presence of reduced transition metals (Fe$^{2+}$, Cu$^+$), hydrogen peroxide can be converted into the highly reactive hydroxyl radical (·OH) (Chance et al., 1979). On the other hand, hydrogen peroxide can be converted to water by the enzymes catalase or glutathione peroxidase. When the reaction is catalyzed by glutathione peroxidase, glutathione is oxidised to glutathione disulfide, while hydrogen peroxide is converted to water (Dröge, 2002). The summary of cellular ROS production and removal is shown in Schematic Diagram 1 (Sayre et al., 2008).
1.1.1. Sources of ROS

Major sources of ROS are mitochondrial electron transport chain, xanthine oxidase catalyzed reactions and oxidation of dopamine in the central nervous system (Dröge, 2002). The mitochondrial electron transport chain is a multi-component system that houses a series of oxidation-reduction reactions between redox pairs involving transfer of electrons from electron donor to electron acceptor. These redox reactions involve either only transfer of electrons, or transfer of electrons and protons together (as in the case of NADH and FAD). The only part of
the electron transport chain that actually uses oxygen is cytochrome oxidase, a terminal oxidase enzyme (Le Bras et al., 2005). Cytochrome oxidase does not release any detectable oxygen radicals, but rather transfer of electrons through earlier components of electron transport chain might cause leakage of electrons that flow directly to oxygen, forming superoxide radical. According to this view, damaged mitochondria with severely compromised electron flow have an increased production of superoxide radical (Le Bras et al., 2005). A major site for reduction of molecular oxygen to superoxide in mitochondrial electron transport chain is ubisemiquinone (Turrens et al., 1985; Boveris et al., 1976). NADH-ubiquinone and ubiquinol-cytochrome-c reductase, which contain ubisemiquinone, were shown to generate superoxide and hydrogen peroxide (Cadenas et al., 1977).

Furthermore, xanthine oxidase produces superoxide by converting hypoxanthine to xanthine, and xanthine to uric acid. Xanthine oxidase is derived from xanthine dehydrogenase by proteolytic cleavage. Under normal conditions, xanthine oxidase is only a minor source of ROS, but it becomes a major source of oxidative stress in certain diseases, like ischemia (Dröge, 2002). Moreover, dopamine oxidation leading to ROS production has been implicated in aging-related destruction of dopaminergic neurons, especially in Parkinson’s disease.

1.1.2. ROS Scavengers

In eukaryotic cells, intracellular concentration of superoxide radical is tightly regulated by SOD enzymes: Cu/Zn SOD and MnSOD. Cu/Zn SOD is primarily located in the cytosol, whereas MnSOD is located in the mitochondria. SOD catalyzes the reaction of two superoxide anions, as shown in Scheme 1 (Le Bras et al., 2005):

Scheme 1:
$O_2^* + O_2^* + 2H^+ \rightarrow H_2O_2 + O_2$ (ground state)

Hydrogen peroxide gets removed by either catalase or glutathione peroxidise (GPx). Catalase consists of four units each containing a ferric heme group bound to its active site that directly catalyzes the conversion of hydrogen peroxide to ground state $O_2$, with hydrogen peroxide acting as both electron donor and electron acceptor (Halliwell, 1999). This is shown bellow in Scheme 2.

Scheme 2:

$$\text{Catalase-Fe(III) + H}_2O_2 \rightarrow \text{compound I + H}_2O$$

$$\text{Compound I + H}_2O_2 \rightarrow \text{Catalase-Fe(III) + H}_2O + O_2$$

The exact structure of compound I is still unknown (Halliwell, 1999). GPx consists of four subunits with each unit containing one atom of selenium in the active site. GPx scavenges hydrogen peroxide by coupling the reaction with oxidation of GSH, as shown in Scheme 3 (Halliwell, 1999).

Scheme 3:

$$H_2O_2 + 2\text{GSH} \rightarrow \text{GSSG} + 2H_2O$$

1.1.3. Oxidant-Antioxidant Balance

Free radicals and reactive non-radical species derived from radicals exist in the cells at low but measurable concentrations. The amount of ROS is determined by rate of their production and rate of their clearance by various antioxidant enzymes, such as SOD, glutathione peroxidase
(GPx) and catalase, and antioxidant compounds, such as glutathione, α-Tocopherol (Vitamin E), β-carotene, and ascorbate (Vitamin C) (Halliwell and Gutteridge, 1989). The exact measurement of a cell's overall redox state is difficult to achieve. To that purpose representative redox couples are often used: PSSP/PSH (thiols), NADP+/NADPH, GSSG/GSH, TrxSS/Trx(SH)2 (thioredoxin) and ascorbate/dehydroascorbate (LeBras et al., 2005). Cells and tissues are considered to be in a stable state if their rates of ROS production and scavenging are constant and in balance. Redox signalling happens if this balance is disturbed by either increase in ROS levels or decrease in ROS scavenging capacity. This can happen by stimulating endogenous ROS production or by environmental oxidative stress. If an increase in ROS is relatively low, an antioxidant response may be sufficient to restore the original balance; however, if the ROS increase is high and chronic pathological conditions may develop (Dröge, 2002).

1.1.4. Consequences of ROS

ROS causes protein modifications that may lead to changes in protein function, chemical fragmentation, or increased susceptibility to proteolytic attack (Davies, 1987). Proteolysis is carried out by the proteasome and it has been reported that the rate of proteolysis increases about 11 times after exposure to superoxide or hydrogen peroxide (Davies and Goldberg, 1987). Oxidative modifications of proteins are indicated by high concentrations of carbonyl groups (Nunomura et al., 2001) and increased nitration of tyrosine residues (Castegna et al., 2003). Oxidative modifications can also lead to cross linking of proteins, which might slow down or stop their intracellular and extracellular removal, even when targeted with ubiquitin (Cras et al., 1995). Lipid peroxidation is indicated by high concentrations of isoprostane, 4-hydroxy-2-nonenal, malondialdehyde, and thiobarbituric acid reactive substances, as well as altered phospholipid composition (Markesbery et al., 1998; Lovell et al., 1995; Tamaoka et al., 2000;
Palmer et al., 1994; Guan et al., 1999). Furthermore, DNA and RNA oxidation results in increased concentrations of 8-hydroxyl-2-deoxyguanosine (8-OHdG) and 8-hydroxyguanosine (Nunomura et al., 1999). Modification to sugars is indicated by increased glycooxidation and glycation (Castellani et al., 2001).

1.2. Apoptosis

1.2.1. Mitochondria

Cell death is closely linked to mitochondrial structure and function. In healthy cells, the inner mitochondrial membrane (IMM) is almost completely impermeable to all ions including protons, which enables complexes I-IV of the electron transport chain to build up a proton gradient across IM that is required for oxidative phosphorylation (Kroemer et al., 2007). The electrochemical gradient caused by charge imbalance is the basis of inner mitochondrial transmembrane potential (∆ψm). This proton gradient is used by complex V of the electron transport chain to drive ATP synthesis. Maintenance of the proton gradient is therefore essential for cellular bioenergetics (Mitchell and Moyle, 1965), and long lasting and permanent dissipation of ∆ψm is associated with cell death (Kroemer et al., 2007).

Permeability of the outer mitochondrial membrane (OMM) is also well regulated. It is assumed that OMM is freely permeable to small solutes and metabolites (up to approximately 5kDa) due to presence of the voltage-dependent anion channel (VDAC). During cell death, OMM permeability increases, leading to release of soluble proteins that are usually retained within the inter-membrane space (IMS) of the mitochondria. Cell death related permeabilization of OMM is a tightly regulated process with major consequences for both health and disease (Kroemer et al., 2007).
1.2.2. Apoptosis

Cells undergoing apoptosis break down into small secular apoptotic bodies that are surrounded by membranes and contain intact cellular organelles and nuclear content. These apoptotic bodies are easily engulfed by neighbouring phagocytic cells. Apoptosis is characterized by nuclear condensation (pyknosis) and nuclear fragmentation (karyorhexis) (Kroemer et al., 2005). Apoptosis is a part of normal embryonic development; disabled apoptosis contributes to oncogenesis and cancer progression. On the other hand, unwarranted apoptosis in post-mitotic cells (such as neurons) also causes disease (Reed, 2002). Apoptosis may proceed through either intrinsic or extrinsic pathway.

The extrinsic pathway or death-receptor pathway is triggered by ligand-induced activation of death receptors on the cell surface. Those death receptors include tumour necrosis factor (TNF) receptor-1, CD95/Fas, and TNF related apoptosis inducing ligand (TRAIL) receptors 1 and 2. The intrinsic or mitochondrial pathway results from an intracellular cascade of events triggered by mitochondrial permeabilization or DNA damage (Scaffidi et al., 1998). Both extrinsic and intrinsic pathways ultimately lead to cell shrinkage, chromatin condensation, nuclear fragmentation, blebbing, and phosphatidylserine flipping from inner to outer leaflet of cell membrane (Kroemer et al., 2007). Activation of caspases (specific class of proteases) is a key in the development of these apoptotic features. Apoptosis ultimately results from activation of a subset of executioner caspases (caspases 3, 6, and 7) (Fuentes-Prior and Salvesen, 2004).

1.2.3. Extrinsic Apoptotic Pathway

In this pathway, ligation of death receptors (TNF receptor-1, CD95/Fas, and TRAIL receptors 1 and 2) causes recruitment and oligomerization of the adapter molecule Fas-
associating-death-domain containing protein (FADD) within the death-inducing-signaling-complex (DISC). Following activation, FADD binds to initiator caspase 8 and/or 10 causing their dimerization and subsequent activation (Kroemer et al., 2007).

1.2.4. Intrinsic Apoptotic Pathway

In the intrinsic pathway, executioner caspases are cleaved and activated by initiator caspase-9. Caspase-9 gets activated by multimerization on APAF-1 (adapter molecule: apoptosis protease activating factor 1) within a protein complex called the apoptosome. APAF-1 is present in the cytosol as a monomer and its activation depends on the presence of cytochrome c (Cyt c) and ATP/dATP (Cain et al., 2002). Cyt c is normally present in the mitochondrial intermembrane space, where it functions as an electron carrier in the electron transport chain, and it gets released into the cytoplasm upon mitochondrial membrane permeabilization. Mitochondrial membrane permeabilization is therefore a critical event in triggering the intrinsic apoptotic pathway (Kroemer et al., 2007). Interestingly, mitochondrial membrane permeabilization may commit a cell to apoptosis even without caspase activation. Caspase independent apoptosis can occur due to loss of mitochondrial function or by release of caspase-independent death effectors, such as AIF (apoptosis inducing factor), EndoG (endonuclease G) etc. from the mitochondria (Susin et al., 1999; Li et al., 2001; Kroemer and Martin, 2005).

Interaction between intrinsic and extrinsic pathways has been thoroughly investigated. There are two types of extrinsic apoptosis depending on the cell involved. In type I cells, ligation of death receptors causes the activation of effector caspases without mitochondrial membrane permeabilization; however, in type II cells the signalling cascade depends heavily on mitochondrial membrane permeabilization. This cascade involves caspase-8
activation \rightarrow \text{truncation and activation of Bid} \rightarrow \text{tBid mediated Bax activation} \rightarrow \text{mitochondrial membrane permeabilization} \rightarrow \text{Cyt c dependent caspase-3 activation (Scaffidi et al., 1998).}

Mitochondria receive a myriad of death inducing and life preserving signals, and when lethal signals predominate, mitochondria undergo membrane permeabilization. Signs of mitochondrial membrane permeabilization are release of Cyt c, release of AIF and EndoG, change in mitochondrial membrane potential, oxidative phosphorylation arrest, and ROS accumulation (Kroemer et al., 2007).

1.2.5. Bcl-2 Family of Proteins and Apoptosis

The Bcl-2 family of proteins can be divided into: anti-apoptotic multidomain proteins (Bcl-2, Bcl-XL) that contain four BH domains (BH1234); pro-apoptotic multidomain proteins (Bax, Bak) that contain three BH domains (BH123); and pro-apoptotic BH3-only proteins (Bid, Bad) (Letai et al., 2002). The main site of action of Bcl-2 family of proteins is the mitochondria (Kroemer and Reed, 2000). BH1234 proteins mainly reside in the outer mitochondrial membrane where they protect the mitochondria against membrane permeabilization, presumably by binding to and neutralizing pro-apoptotic proteins from the Bcl-2 family. Pro-apoptotic Bcl-2 family proteins work to induce mitochondrial membrane permeabilization (Kroemer et al., 2007).

In healthy cells, Bak is associated with the outer mitochondrial membrane, and Bax resides in the cytosol. Activation of at least one of these two proteins is needed to induce mitochondrial membrane permeabilization and subsequent apoptosis (Wei et al., 2001). During apoptosis, Bax inserts itself into the outer mitochondrial membrane, where it is assumed to form supramolecular openings, alone or in association with Bak or tBid (truncated Bid) (Kuwana et al., 2002). It is still largely unclear how these molecular openings mediate Cyt c release.
According to some studies, Bax engages in close molecular interaction with proteins from the PTPC (permeability transition pore complex), such as ANT (adenine nucleotide translocase) or VDAC (voltage dependent anion channel), to induce mitochondrial membrane permeabilization. Other studies on isolated mitochondria and liposomes suggest that Bax permeabilizes mitochondria and directly releases Cyt c without PTPC involvement (Kroemer et al., 2007).

It is unclear how anti-apoptotic proteins from the Bcl-2 family inhibit mitochondrial permeabilization. Some studies suggest that suppression of mitochondrial membrane permeabilization is achieved either directly by interaction with pore-forming Bcl-2 family members, or indirectly by neutralizing BH3-only proteins (Letai et al., 2002). However, other reports indicate that Bcl-2 and Bcl-XL interact with ANT and VDAC inhibiting pore formation by pro-apoptotic proteins (Marzo et al., 1998; Shimizu et al., 2000).

1.2.6. Necrosis

While apoptosis requires a minimum amount of cellular ATP, necrosis happens when cellular ATP levels are completely depleted (Nicotera et al., 1998). Necrosis can be viewed as accidental cell death: it is not genetically predetermined and it occurs within 2-4 hours after injury. Main features of a necrotic cell include increased cellular volume that eventually leads to cell rupture and disorganized elimination of swollen organelles (Kroemer et al., 2007). Necrosis is considered harmful since it is often associated with local inflammation and pathological cell loss (Vakkila and Lotze, 2004).

1.3. Senescence

Cellular senescence is described as the finite replicative span (Campisi, 2005). Cells will stop dividing and become senescent once they reach their “Hayflick limit” (a maximum number
of population doublings for a cultured cell line). This happens due to depletion of telomere length caused by the lack of telomerase enzyme (Shay and Wright, 2000). Senescence ensures that cells with dysfunctional, shortened telomeres permanently withdraw from the cell cycle. Cells that continue to proliferate despite dysfunctional telomeres develop chromosomal aberrations, which could lead to malignant transformation (Artandi and DePinho, 2000). In this way, senescence is an anti-tumorigenic response posted by damaged cells (Campisi, 2005).

Cells cultured from donors 50 years or older tend to senesce after fewer population doublings than cells from young donors; cells from short-lived species will senesce after fewer population doublings than cells from long-lived species (Hayflick, 1977). These observations suggest that cells deplete their potential for replication as they age. It has also been reported that cells from humans with hereditary premature aging syndromes senesce faster than their normal, age-matched counterparts (Goldstein, 1978). Cells entering senescence stop responding to mitogenic stimuli; they undergo changes in chromatin structure and gene expression, and morphologically they become enlarged and flattened (Ben-Porath and Weinberg, 2004). Senescent cells remain alive and metabolically active for a long time, but they differ from replication competent cells with respect to telomere length, morphology, gene expression patterns, and Senescent Associated (SA) β-galactosidase activity (Dimri et al., 1995). Senescent human fibroblasts also produce higher levels of ROS like hydrogen peroxide, and because of this they have higher levels of oxidative damage to their DNA (Chen et al., 1995).

Normal cells in culture undergo senescence in response to over-expression of some oncogenes (such as RAS) or of its downstream effectors, such as RAF, activated MAP kinase, and PML oncoprotein (Ben-Porath and Weinberg, 2004). It has therefore been suggested that senescence is a tumour-suppressive mechanism by which cells avoid uncontrolled proliferation.
due to abnormal activation of proliferation-driving oncogenes. It seems that extremely high levels of RAS expression and its downstream effector proteins are needed to induce senescence. It has also been suggested that p16 is activated in response to high levels of RAS activity, leading to senescence (Ben-Porath and Weinberg, 2004). In human cells, RAS causes parallel activation of p53 and p16, and it seems that p16 plays a more prominent role in committing the cell to senescence (Serrano et al., 1997). In some cell lines, inactivation of p16 is enough to prevent “RAS induced” senescence (Brookes et al., 2002). Cells that express low levels of p16, such as freshly isolated primary human fibroblasts, do not enter senescence as a response to over-expressed RAS; instead, they have enhanced proliferation abilities (Benanti and Galloway, 2004). It has also been reported that p38-MAPK proteins play a crucial role in RAS induced senescence by activating p16 and p53 (Wang et al., 2002). Lee et al. (1999) reported that RAS over expression induces generation of ROS, leads to p38-MAPK activation and ultimately senescence. According to this, RAS over-expression induces DNA damage, which has never been reported. ROS involvement in this type of senescence needs more investigation (Ben-Porath and Weinberg, 2004).

1.3.1. Senescence as a cellular stress response

Multicellular organisms contain two different cell types: post-mitotic cells, which are incapable of dividing, and mitotic cells that do divide. Renewable tissues are repaired and replenished by cell proliferation, and the process of DNA replication may be accompanied by mutations, which, when occurring in excess, is a major cause of tumourigenesis. Tumour suppressors prevent cancer by protecting the genome from mutations (Campisi, 2005). Tumour suppressors can be classified into either caretakers or gatekeepers (Kinzler and Vogelstein, 1997). Caretaker tumour suppressors prevent cancer by protecting the genome from mutations by
preventing DNA damage and promoting DNA repair (Campisi, 2005). Caretakers also prevent aging related phenotypes, and as such, are longevity assurance genes (Hasty et al., 2003). On the other hand, gatekeeper tumour suppressor genes prevent cancer by acting on intact mitotic cells that are at risk of transformation. These proteins eliminate potential cancer cells by initiating apoptosis, or by inducing permanent withdrawal from the cell cycle (cellular senescence) (Campisi, 2005). By either inducing senescence or apoptosis, gatekeepers could eventually cause depletion of both post-mitotic cells in non-renewable tissues, and proliferating cells in renewable tissues. Therefore, gatekeepers may be an example of evolutionary antagonistic pleiotropy: genes that are beneficial early in life (by suppressing cancer), but are detrimental later in life (Campisi, 2003).

Many kinds of oncogenic or stressful stimuli (such as certain types of DNA damage, like DNA breaks and oxidative lesions caused by either environmental stimuli or genetic defects) can induce a senescent response (Samper et al., 2003; Hasty et al., 2003). Many normal cells senesce when they over-express some oncogenes such as activated components of RAS-RAF-MEK signalling cascade (Campisi, 2005). Mutational activation of RAS without over-expression stimulates cell proliferation, not senescence (Campisi, 2005). It appears that a myriad of stimuli that are responsible for the onset of senescence belong to one of the two pathways: p53 pathway or Rb pathway (Bringold and Serrano, 2000). Both p53 and Rb are transcriptional regulators.

p53 is a main mediator of cellular responses to DNA damage, including the senescence response (Wahl and Carr, 2001). Loss of p53 function delays or prevents the onset of cellular senescence of human cells (Itahana et al., 2001). According to this, dysfunctional telomeres may trigger a p53 dependent DNA damage response. Dysfunctional telomeres activate p53 mediated damage response and senescence (Itahana et al., 2001). p53 pathway leading to senescence also
gets activated in response to over-expression of some oncogenes such as activated RAS, which is signalled by ROS generation (Pearson et al., 2000). Generation of ROS is required for this pathway's mitogenic effects, as well as its ability to induce cellular senescence. Therefore, over-expressed oncogenic RAS may trigger p53 dependent damage response by producing high levels of damaging ROS (Campisi, 2005). Oncogenic RAS can also induce p16, which is an activator of pRb pathway, an additional barrier to proliferation of potentially malignant cells (Campisi, 2005).

It was also reported that inactivation of p53 in some replicatively senescent human cells reverses the senescence growth arrest (Gire and Wynford-Thomas, 1998). In these cases, p53 inactivation causes post-mitotic senescent cells to resume cell proliferation in spite of short telomeres (Beausejour et al., 2003). Similarly, inactivation of p21 gene, an inhibitor of cell cycle and a target for p53 transactivation, blocks cells from entering telomere-dependent replicative senescence (Brown et al., 1997). Since p53 pathway is involved in induction of senescence by DNA damage, telomere dysfunction and oncogene overexpression, loss of p53 function might lead to reversal of cellular senescence (Campisi, 2005).

However, p53 inactivation fails to reverse senescence in some cell lines (Beausejour et al., 2003). It appears that this differential behaviour of some cell lines regarding p53 inactivation and senescence depends on the level of p16 expression. p16 is a tumour suppressor and a positive regulator of pRb and is induced by variety of stressful stimuli such as over-expression of RAS oncogene and abnormal culturing conditions (Lowe and Sherr, 2003). Some cells spontaneously reduce or eliminate expression of p16, often by promoter methylation. This can happen during long-term culture of human epithelial cells, and even in human fibroblast cultures (Holst et al., 2003). In those cells, senescence response depends primarily on the p53 pathway
In vivo studies indicate that p16 suppresses the development of spontaneous cancer (Sharpless et al., 2001), and cell culture studies indicate that p16 prevents the reversal of senescence by p53 inactivation (Beausejour et al., 2003). Therefore, p16 tumour suppressor, and pRb pathway it activates, provide the ultimate barrier to cell proliferation, one that cannot be overcome by p53 inactivation (Campisi, 2005).

The relationship between p53 and pRb pathways in regulating cellular senescence remains unclear. There are known molecular interactions linking these two pathways, such as induction of p21 over-expression (Campisi, 2005). p21 is a more general inhibitor of cyclin dependent kinases than p16, and it is also able to hyperphosphorylate and activate Rb. According to this, p53 pathway activation should engage the pRb pathway as well. However, consequences of pRB activation by p21 and p16 differ in some respects. For example, p53 inactivation can induce cell proliferation in senescent fibroblasts expressing p21 but not in fibroblasts expressing p16. Also, p16 is more efficient than p21 in inducing cell cycle arrest in human fibroblasts (McConnell et al., 1998). In addition, when in the senescent state, some human fibroblasts express p21 or p16, but rarely express both (Herbig et al., 2004). These findings suggest that there are differences between senescent states induced by p53 and pRb pathways, and there is an emerging consensus that senescence occurs through p53 pathway in response to telomere dysfunction and DNA damage, and that senescence occurs by p16/pRB pathway in response to oncogenes, chromatin disruption and other stresses (Ben-Porath and Weinberg, 2004). To date, not enough is known about senescence markers to be able to distinguish between senescent states caused by p53 and pRb pathways (Campisi, 2005).

1.3.2. The p53 and p16/pRB pathways: cellular senescence and aging
In addition to being a crucial tumour suppressor, p53 also contributes to aging by enhancing a cell’s response to senescence stimuli. To illustrate, genetically modified mice with enhanced p53 function were shown to be remarkably resistant to developing cancer, but they also showed signs of accelerated aging (Maier et al., 2004). Cells from these modified mice were more susceptible to both p53 mediated apoptosis (Tyner et al., 2002) and p53 mediated senescence (Maier et al., 2004). These findings suggest that senescent cells contribute to organismal aging, and enhanced protection from cancer might be coming at the cost of accelerated aging (Campisi, 2005).

Interestingly, there is still no evidence that enhanced pRb function accelerates aging as enhanced p53 function does (Campisi, 2005). Even so, there is still evidence that incidence of p16 positive cells and p16 expression increase with age in many rat and mouse tissues (Krishnamurthy et al., 2004; Zindy et al., 1997). p16 positive cells are most likely senescent cells, since p16 expression parallels that of senescence-associated β-galactosidase (SA-β-gal), which is highly associated with the senescent phenotype (Dimri et al., 1995). Combined results from both p16 and SA-β-gal indicate that the number of senescent cells accumulate with age in various human and rodent tissues. Also, senescent cells are found in excess at cites of certain age-related pathologies, such as atherosclerotic lesions, etc (Campisi, 2005). Since p16 activates the pRb pathway, it is possible that the pRb pathway, like the p53 pathway, may contribute to aging phenotypes by inducing cellular senescence (Campisi, 1995).

Accumulation of senescent cells may compromise tissue structure and function due to the inability of senescent cells to proliferate, and repair and renew tissues. Also, many genes that are upregulated in senescence code for secreted proteins that can change tissue microenvironment leading to a change in structure and function (Zhang et al., 2003). Senescent fibroblasts secrete
high levels of matrix metalloproteinases, epithelial growth factors, and inflammatory cytokines (Campisi, 2005). It seems that the secretory phenotype of senescent fibroblasts resembles that of fibroblasts undergoing wound response (Grinnell, 2003). Wound response includes local inflammation, which is a frequent occurrence in aging tissues either as a cause or a consequence of age related diseases such as atherosclerosis and cancer (Longo and Finch, 2003).

1.3.3. Senescence and Oxidative Stress

Oxidative stress and accumulation of intracellular ROS play a major role in the onset of senescence (Ben-Porath and Weinberg, 2004). It has been reported that human diploid fibroblasts undergo early senescence when grown in high oxygen conditions (40-50% oxygen), and experience an extended replicative life span when grown in low oxygen conditions (2-3% oxygen) (Chen et al., 1995). Premature senescence can also be caused by increasing internal ROS through treatment with hydrogen peroxide, or through inhibition of ROS scavenging enzymes such as superoxide dismutase SOD1 (Blander et al., 2003). This increase in internal ROS causes damage to cellular macromolecules and is also known to act as secondary messengers involved in regulation of specific cellular pathways (Saitoh et al., 1998).

Some studies suggest that the proportion of p21 (not p16) expressing cells is reduced when these cells are grown in low oxygen ambient, which indicates that oxidative stress acts through the p53 pathway to induce premature senescence (Itahana et al., 2003). On the other hand, some studies indicate that p16 does indeed get activated in response to oxidative stress, possibly through activation of p38-MAPK, a member of the stress activated protein kinase (SAPK) family (Iwasa et al., 2003). SAPKs are a group of kinases that respond to multiple physiological stresses (including oxidative stress) and induce apoptosis or senescence in
response to those stresses (Kyriakis and vruch, 2001). The SAPK family includes JNK1/2/3 and p38-MAPKα/β/γ/δ, with p38-MAPKα/β/γ/δ being mostly implicated in senescence. p38-MAPKα/β/γ/δ and its upstream activators mediate the activation of p16 in response to various stressful stimuli (Ben-Porath and Weinberg, 2004).

ROS may promote senescence through DNA damage. It has been reported that the rate of telomere shortening is accelerated in fibroblasts grown in high oxygen ambient (Forsyth et al., 2003). It has been proposed that ROS cause single stranded breaks in telomeric DNA, which may lead to deletions of telomeric repeats (von Zglinicki et al., 2000; Baird et al., 2003). However, oxidative stress does not cause loss of telomeric overhang and does not cause formation of telomeric DNA foci (Keys et al., 2004; Sedelnikova et al., 2004). Another study showed that ROS damages genomic DNA, not telomeres, and subsequently activates the p53 pathway. DNA damage foci were indeed detectable in Human Fibroblasts in response to hydrogen peroxide treatment (Sedelnikova et al., 2004).

Activation of the p53 pathway in response to DNA damage has been studied extensively (Wahl and Carr, 2001). It is still unclear how a cell decides to commit to apoptosis or senescence when DNA is damaged enough to induce the p53 pathway. It has been suggested that this might be determined by differences in post-translational modifications of p53 in response to various stimuli (Webley et al., 2000); by different binding partners of p53; and by activation of different sets of transcriptional targets by p53 (Wahl and Carr, 2001). According to this theory, as opposed to apoptosis, senescence occurs in response to lower levels of DNA damage. Activities of p53 and p21 are induced to high levels immediately after DNA damage and their levels decrease after a several days. Alternatively, p16 levels increase over a period of several days after DNA damage was induced (Robles and Adami, 1998). According to this finding, p53 and p21 are
needed to initiate a senescence response, while p16 (together with activated Rb) work at maintaining it (Ben-Porath and Weinberg, 2004).

1.4. **Alzheimer’s disease (AD)**

Alzheimer’s disease affects several million people worldwide, and as such, it is the most common form of dementia. Both incidence and prevalence of AD increase with age and the disease is gaining social and economic relevance, mainly in developed countries where the overall life span is continuously increasing (Hampel et al., 2003). Familial AD is an autosomal dominant disease and is associated with specific mutations in the genes encoding for amyloid precursor protein (APP) and presenilin-1 (PS-1) mapped on chromosome 14, and presenilin-2 (PS-2) mapped on chromosome 1 (Cecchi et al., 1999).

Alzheimer’s disease hallmarks are Amyloid β plaques and neurofibrillary tangles; it is marked by neuronal and synaptic loss in the cerebral cortex and hippocampus. Susceptible neurons are subject to both oxidative and mitotic injuries, which ultimately leads to neuronal cell death (Zhu et al., 2004). There is evidence that early neuronal and pathological changes associated with AD show oxidative damage, indicating that oxidative stress is a very important contributor to the disease. (Nunomura et al., 2000).

1.4.1. **AD and Oxidative Stress**

Markers of oxidative damage are present in susceptible neurons even without evidence of neurofibrillary pathology (Nunomura et al., 2000). Free radicals produced due to oxidative stress, as well as oxidative modifications of proteins, lipids and nucleic acids are pathologically essential for the onset of AD (Smith et al., 1995).
Oxidative modifications of proteins are indicated by high concentrations of carbonyl groups (Nunomura et al., 2001) and increased nitration of tyrosine residues (Castegna et al., 2003). Oxidative modifications can also lead to cross linking of proteins, which might slow down or stop their intracellular and extracellular removal even when targeted with ubiquitin (Cras et al., 1995). Lipid peroxidation is indicated by high concentrations of isoprostane, 4-hydroxy-2-nonenal, malondialdehyde, and thiobarbituric acid reactive substances, as well as altered phospholipid composition (Markesbery et al., 1998; Lovell et al., 1995; Tamaoka et al., 2000; Palmer et al., 1994; Guan et al., 1999). Furthermore, DNA and RNA oxidation results in increased concentrations of 8-hydroxyl-2-deoxyguanosine (8-OHdG) and 8-hydroxyguanosine (Nunomura et al., 1999). Also, high DNA fragmentation, nicking and DNA breaks are observable in AD patients indicating deficient DNA repair mechanisms (Colurso et al., 2003; Adamec et al., 1999). Modification to sugars is indicated by increased glycoxidation and glycation (Castellani et al., 2001); however, neuronal cells do have an antioxidant defence system in the form of up-regulated antioxidant enzymes such as heme oxygenase I, catalase, glutathione peroxidase, glutathione reductase, Cu-Zn superoxide dismutase, peroxiredoxins, as well as some heat shock proteins (Aksenov et al., 1998).

Oxidative stress induces changes in gene expression and enzyme activities that are mediated through the communication of various signalling pathways such as stress activated protein kinase (SAPK) pathways, JNK-SAPK and p38-SAPK2, which propagate stress signals from the membrane to the nucleus. SAPKs and their downstream effectors are involved in the ambiguous response to oxidative stress: they will provoke a stress response that either leads to apoptosis or to defensive protective adaptations depending on cellular and environmental conditions (Mielke et al., 2000). The entire JNK-SAPK pathway is affected in AD: JNK1 is
related to Hirano bodies in AD, while JNK2 and JNK3 are related to neurofibrillary pathology. JNK-SAPK is activated in AD and redistributed from nuclei to the cytoplasm as the disease progresses. Nuclear localization of active JNK-SAPK is detected in susceptible neurons in the early stages of AD, while phospho-JNK-SAPK is only localized in association with neurofibrillary tangle formation in advanced stages of AD (Zhu et al., 2001). The nuclear localization of active JNK-SAPK suggests that oxidative signalling possibly affects gene expression. This is supported by the fact that activation of JNK-SAPK pathway can modulate the induction of some anti-oxidant enzymes that are induced in AD, such as heme oxygenase 1 and superoxide dismutase 1 (Popolla et al., 1992).

1.4.2. Causes of Oxidative Stress in AD

Sources of damage-inducing ROS include interaction of abnormal mitochondria, redox transition metals, proteolysis dysfunction, and activated microglia (Zhu et al., 2007). Many studies implicate that metabolic defects in AD, such as reduced rate of brain metabolism, precede brain atrophy and functional impairment (Blass, 2000). Also, it has been documented that AD patients have increased oxidative utilization compared to glucose utilization supporting the role of oxidative stress in the onset of AD (Fukuyama et al., 1994).

1.4.3. Amyloid β (Aβ) lesions and oxidative stress

It was initially thought that lesions found in the brain of AD patients were the source of ROS in AD. However, it now appears more likely that oxidative damage is a cause of AD, and leads to activation of defence mechanisms (such as Aβ and hyperphosphorylated tau protein deposition) in an attempt to restore the redox balance and avoid neuronal death initiated by oxidative damage (Smith et al., 2002). However, as the disease progresses, both Aβ and
hyperphosphorylated tau through gain-of-function transformation, shift their activity from anti-
oxidant to pro-oxidant ultimately leading to an increase in ROS and decrease in defence 
mechanisms. Aβ is considered a dangerous by-product of APP processing by β- and γ-secretases 
even though it is present in healthy neurons (Zhu et al., 2007). It was recently reported that Aβ 
gets secreted from healthy neurons in response to synaptic activity and that it down-regulates 
excitatory synaptic transmission (Kamenetz et al., 2003). Furthermore, Aβ has strong chelating 
properties for zinc, iron and copper, which suggests that one function of Aβ is to sequester these 
metals (Rottkamp et al., 2001). Theoretically, this sequestering of redox active metals can serve 
to inhibit metal-catalyzed oxidation of biomolecules. However, the role of Aβ can be considered 
to be both anti-oxidant and pro-oxidant since methionine at residue 35 (Met35) can both 
scavenge free radicals (Unnikrishnan et al., 1990) and reduce metals to their highly active low-
valence form (Hiller et al., 1981). Therefore, the overall activity of Aβ with respect to oxidation 
is a combination of metal chelation, metal reduction, and free radical scavenging resulting in 
either pro-oxidative or anti-oxidative activity. In order for Aβ to induce oxidation, three 
conditions have to be satisfied: presence of Met35, presence of transition metals, and fibrillation 
(Zhu et al., 2007). Fibrillation will only occur if Aβ is aged and present in large (micromolar) 
quantities (Iversen et al., 1995). Presence of redox metals is also required for Aβ aggregation and 
its pro-oxidant activity (Rottkamp et al., 2001). Methionine 35 is necessary for pro-oxidant 
activity of Aβ, and it has been demonstrated that substitution of this residue greatly diminishes or 
even aborts the pro-oxidant activity of Aβ. In its role as a pro-oxidant, Aβ induces peroxidation 
of membrane lipids (Varadajan et al., 2000) and lipoproteins (Kontush et al., 2001), generates 
hydrogen peroxide and 4-HNE in neurons (Behl et al., 1994; Mark et al., 1997a), inactivates 
transport enzymes (Mark et al., 1997b) and damages DNA (Xu et al., 2001).
1.4.4. Mitochondria and oxidative stress

Since metabolic impairments (such as hypoxia or hypoglycaemia) are sufficient to induce mental and neurological impairments similar to the symptoms of AD, it is clear that mitochondrial dysfunction may play a role in the onset of AD (Blass et al., 1999). A characteristic of AD is damaged mitochondria that show increased oxidative stress, which is consistent with the fact that damaged mitochondria produce more ROS and less ATP comparing to their normal counterparts (Castellani et al., 2002). The functionality of mitochondria is dependent on maintaining their membrane integrity, and it has been found that mitochondria in AD are deficient in some key enzymes involved in oxidative metabolism such as α-ketoglutarate dehydrogenase complex, pyruvate dehydrogenase complex (both enzymes of Krebs cycle) and cytochrome oxidase (COX, an enzyme responsible for reducing molecular oxygen in the electron transport chain) (Gibson et al., 1998). Different stages of mitochondrial abnormality were detected in almost all AD neurons. Quantitative morphomeric measurements of the percentage normal, partially damaged and completely damaged mitochondria show that AD neurons have significantly higher proportion of damaged mitochondria and significantly lower percentage of normal mitochondria compared to normal cells (Zhu et al., 2007). Factors that contribute to mitochondrial dysfunction in AD are the following: low vascular blood flow in the brain (de la Torre, 1997); increased sporadic mutations in mitochondrial DNA, which negatively affect mitochondrial stability (Coskun et al., 2004); amyloid-β and amyloid-β precursor protein (APP) processing machinery are located in mitochondria with APP present in mitochondrial import channel, thus potentially affecting mitochondrial transport (Seshadri et al., 2002); and increased homocysteine (hyperhomocysteinemia) observed in AD inhibits several genes that encode mitochondrial proteins ultimately promoting ROS production (Streck et al., 2003).
1.4.5. Iron and copper and their role in oxidative stress

Most types of oxidative damage in AD are linked to metal-catalyzed hydroxyl radical formation. Due to this, loss of iron and copper homeostasis in the brain is accompanied by serious neurological consequences. Iron is involved in hydroxyl radical formation through the Fenton reaction and has been noted to accumulate in the brain where it participates in the formation of increased oxidative stress (Smith et al., 1997). Over-accumulation of iron in AD is found in the cerebral cortex, hippocampus, and basal nucleus of Meynert, which coincides with the location of neurofibrillary tangles, senile plaques and lesions in an AD brain (Smith et al., 1997). RNA bound iron plays a role in RNA oxidation in the neurons of AD (Honda et al., 2005). rRNA is present in large amounts in neurons and is considered to have the largest number of iron binding sites among all cytoplasmic RNA species. Oxidation of rRNA by iron-dependent Fenton reaction leads to the formation of 8-hydroxyguanosine (8-OHG). Also, ribosomes obtained from the hippocampus of an AD brain contain significantly higher levels of RNase sensitive iron compared to the controls, as well as 8-OHG detected by immunoprecipitation, which is present exclusively in an AD brain. rRNA serves as binding site for redox active iron and serves as redox center in the cytoplasm of AD neurons. Oxidized ribosomes in turn show significant reduction in protein synthesis. These ribosomal changes due to iron mediated oxidation are detectable well in advance versus other morphological changes in AD neurons that indicate neurodegeneration (Honda et al., 2005).

Copper is required by many oxidation-reduction enzymes. It is found in Cu-Zn SOD, and in the catalytic site of COX within the electron transport chain etc. Copper entry into the brain is mainly mediated by ceruloplasmin, a copper binding protein responsible for protecting the neurons against oxidative stress, and abnormalities in ceruloplasmin may lead to oxidative
damage in the brain (Zhu et al., 2007). Ceruloplasmin is also involved in the regulation of the redox state of iron by converting ROS catalytic Fe(II) to the less reactive Fe (III). Levels of ceruloplasmin are increased in cerebrospinal fluid and brain tissue of AD patients, while concentration of ceruloplasmin in neurons remains unchanged (Loeffler et al., 1996). Increased ceruloplasmin concentration in the cerebrospinal fluid may be an attempt to respond to increased oxidative stress in AD, while failure to get up-regulated in the neurons may explain the metal catalyzed oxidative damage in AD neurons (Castellani et al., 1999). Furthermore, copper can play a role in ROS generation through its binding to APP. APP is able to reduce Cu(II) to Cu(I) through formation of the AβPP-Cu(II)-hydroxyl radical intermediate which ultimately enhances production of hydroxyl radical. Copper, like iron, is highly concentrated in Aβ plaques (Zhu et al., 2007).

1.4.6. Glycation, glycoxidation and oxidative stress

Advanced glycation end products (AGEs) are generated by non-enzymatic reaction of ketone or aldehyde group of a sugar with the free amino group of a protein or free amino acids, especially arginine and lysine (Zhu et al., 2007). AGEs are formed as a mixture of protein bound nitrogen and oxygen containing heterocyclic compounds through a complex cascade of dehydration, oxidation, fragmentation and cyclization reactions (Harrington and Colaco, 1994). Monosaccharides in the equilibrium with their enediol can undergo auto-oxidation in the presence of transition metals to form enediol radical. Enediol radical can reduce molecular oxygen to form the superoxide radical. Advanced glycation end-products result in formation of oxygen-derived free radicals, and as such are a main source of oxidative stress in AD. Furthermore, AGEs accumulate in the aging brain (Munch et al., 1997), and in vitro studies showed that AGE-modified Aβ promotes rapid aggregation, which is a feature associated with
AD (Vitek et al., 1994). AGEs were detected in neurofibrillary tangles as well. It is hypothesized that glycation of tau protein leads to stabilization of helical filaments and tau aggregation, ultimately leading to neurofibrillary tangles (Ko et al., 1999). The role of AGEs in AD is also demonstrated by their neurotoxicity and their ability to increase levels of Aβ (Mruthinki et al., 2006). Moreover, AGEs and Aβ activate specific receptors such as class A scavenger receptors (El Khouri et al., 1996) and receptors for advanced glycation end products (RAGE) (Yan et al., 1996) leading to an increase in intracellular ROS levels and modulation of gene transcription (Zhu et al., 2007).

1.4.7. Proteasomes, lysosomes and their role in oxidative stress

Degradation of oxidatively modified non-functional proteins is an important part of the cellular anti-oxidant defence system. The proteasome is a large intracellular protease responsible for degradation of misfolded, oxidized or truncated proteins through the process of ubiquitination of the proteins targeted for degradation (Zhu et al., 2007). Components of the proteasomal complex are affected by oxidative stress with 26S proteasome being the most vulnerable (Reinhekel et al., 2000). It has been found that proteasomal activity declines with age (Keller et al., 2002). The fact that PHF-τ is heavily ubiquitinated indicates involvement of the proteasome in the pathogenesis of AD (Zhu et al., 2007). Further studies found decreased activity of the proteasome in AD (Keller et al., 2000), and indeed, inhibition of proteasomal activity has been found to lead to neuronal death and neuropathology similar to that of AD. Moreover, chronic proteasome inhibition together with increased protein insolubility induces increased levels of protein oxidation and potentially leads to the increased oxidative stress that is observed in AD (McNaught et al., 2002).
Cellular oxidative levels can also be increased by lysosomal dysfunction. Lysosomal degradation of iron-containing proteins found in various organelles results in intra-lysosomal formation of redox active iron, which can lead to increased lysosomal oxidative stress and consequent rupture of lysosomal membrane and release of intra-lysosomal contents. This leads to an increase in cellular oxidative stress and mitochondrial damage, which in turn leads an increase in ROS production by mitochondria (Zhu et al., 2007).

1.4.8. Activated microglia, astrocytes and oxidative stress

Inflammation in the AD brain can be caused by injured neurons, amyloid plaques and neurofibrillary tangles, which is supported by the fact that both microglia and astrocytes are attracted to the sites of Aβ deposition. Attracted microglia are activated as seen through the altered morphology and increased expression of MHCII. Astrocytes express a wide range of inflammatory mediators such as cyclooxygenase, complement system and cytokines (Akiyama et al., 2000). RNS (reactive nitrogen species) and ROS are used to attack the targets in inflammation, and activated microglia and astrocytes are capable of producing large amounts of both RNS and ROS by various mechanisms. In addition, Aβ can directly activate NADPH oxidase of microglia leading to an increased production of hydrogen peroxide and superoxide radicals (Klegeris and McGeer, 1997). Activated microglia and astrocytes can produce large amounts of NO (nitric oxide), which can react with superoxide to form peroxynitrite, leaving nitrotyrosine as a marker of oxidative stress. Excess NO in AD is confirmed by increased amounts of nitrotyrosine modified proteins (Smith et al., 1997). In addition, myeloperoxidase (MPO) in AD microglia represents a free radical generating mechanism as well. MPO catalyzes a reaction between hydrogen peroxide and chloride to form hypochlorous acid which can further react with other molecules to generate different kinds of ROS. MPO can also catalyze the
formation of nitrotyrosine modified proteins and glycation end product modifications observed in AD. Also, there is evidence that MPO is present in activated microglia around amyloid plaques in AD brain, and that Aβ aggregates increase MPO mRNA expression in microglia in vitro, both of which support implicated role of MPO and microglia in the pathogenesis of AD (Reynolds et al., 1999).

1.4.9. Oxidative damage in the peripheral tissues of AD

Increased oxidative damage is a common feature in neurons and peripheral cells from both sporadic and familial AD cases (Cecchi et al., 2002). One way to detect oxidative damage to biomolecules in peripheral cells is to measure the end products of lipid peroxidation and protein oxidation (Migliore et al., 2005). It has been demonstrated that increased DNA damage due to oxidized purines and pyrimidines is present in peripheral cells of AD. Patients with mild cognitive impairment (MCI), which is considered to be an early stage of AD, have a higher level of oxidative DNA damage than healthy individuals (Migliore et al., 2005). It has been reported that AD lymphocytes have higher levels of 8-OHdG than controls as measured by HPLC, suggesting that oxidative stress in AD is detectable in peripheral cells as well (Mecocci et al., 2002). Fibroblasts from patients with familial or sporadic AD show an increase in lipoperoxidation products malondialdehyde and 4-HNE (Cecchi et al., 2002). Naderi et al. (2006) demonstrated that ROS levels in AD fibroblasts are markedly higher than in age-matched normal human fibroblast (NHF), which gives them typical senescence morphology and enhanced resistance to external oxidative stress. It has also been demonstrated that peripheral levels of both enzymatic and non-enzymatic antioxidants are depleted in both AD and MCI patients (Rinaldi et al., 2003). Furthermore, lymphoblasts carrying either PS-1 or APP mutation show significantly lower GSH content compared to age matched controls. This finding is in
accordance with the GSH levels in the central nervous system, strengthening the importance of oxidative stress in the pathogenesis of AD (Cecchi et al., 1999).

1.4.10. Neuroprotection

1.4.10.1. Iron chelators

Iron has been implicated in the pathogenesis of AD (look in the section 1.4.5.), so the role of iron chelators in the treatment of the disease needs to be investigated. Iron chelators are compounds that bind iron, rendering it inactive and unable to participate in chemical reactions such as those related to the onset of AD (Chaston and Richardson, 2003). Desferrioxamine (DFO), iron chelator used to treat the conditions of iron overload, has been recently suggested as a treatment option for AD (Whitnall and Richardson, 2006). Crapper-McLachlan et al. (1991) used DFO in a 2-year single blind study, and the treatment slowed down dementia progression associated with AD. The limitation of DFO as a possible treatment option is its large size and hydrophilic nature, which slows down gastro-intestinal absorption and penetration through the blood-brain barrier (Aouad et al., 2002). Furthermore, chelation of iron with EDTA has been shown to induce clinical improvement in patients with AD (Casdorph, 1983). Also, chronic administration of clioquinol, a chelating agent, to transgenic mice bearing the APP mutation (Tg 2576) lead to a decrease in Aβ build-up in the brain and it slowed down cognitive decline (Cherny et al., 2001). Another study showed that the lipophilic metal chelator DP-109, when applied to female Tg 2576 mice, resulted in a marked decrease of amyloid plaques (Lee et al., 2004). Moreover, House et al. (2004) demonstrated in their in vitro studies that both DFO and clioquinol prevent formation of β-pleated sheets in Aβ and Aβ aggregation, and are able to dissolve previously formed synthetic and AD brain-derived Aβ aggregates. The identification of
an IRE (iron-responsive-element) loop in 5’UTR of APP lead to the discovery of some new chelating drugs that target 5’UTR such as DFO (Fe (III) chelator), tetrathiomolibdate (Cu(II) chelator) and dimercaptopropanol (Pb(II) and Hg(II) chelator). These drugs suppress APP expression and lower Aβ secretion (Rogers et al., 2002)

1.4.10.2. Antioxidants and antioxidant drugs

Antioxidant defence compounds are based on their ability to inhibit free radical formation, scavenge generated free radicals, enzymatically detoxify accumulated free radicals, and initiate gene transcription responsible for long term support and induction of cellular self-defence. Direct antioxidants chemically interfere with the formed free radicals, and indirect antioxidants prevent formation of free radicals. Metabolic antioxidants limit the extent of damage to the cell by reducing the metabolic burden of increased level of ROS. A major group of direct antioxidants are chain braking antioxidants such as monophenolic (ie. Tocopherol (vitamin E), 17β-estradiol (estrogen), 5-hydroxytryptamine (serotonin), and derivatives of tyrosine) and polyphenolic compounds (ie. Flavonoids, stilbenes, and hydroquinones) (Behl and Moosmann, 2002). The hydroxyl group in vitamin E may donate an electron to inactivate the highly reactive single electron of free radicals. In AD, specifically, vitamin E prevents the accumulation of ROS induced by Aβ, which reduces the toxicity of Aβ in AD brain (Behl et al., 1992). Also, vitamin E prevents the oxidation of non-saturated carbohydrate side chains of the membrane lipids thus blocking lipid oxidation (Sano et al., 1997).

Estrogen and vitamin E share a phenolic radical scavenging structure. It is important to point out that the ROS scavenging ability of estrogen is based solely on the structure, and not on interaction with estrogen receptors (Moosmann and Behl, 1999). 17β-estradiol is capable of
preventing neuronal cell death caused by Aβ, glutamate, hydrogen peroxide, haloperidol, iron (II) sulphate, and ischemic damage (Blum-Degen et al., 1998; Dubal et al., 1998; Sagara, 1998). Furthermore, compounds such as serotonin, flavonoid quercetin, and trimethylphenol prevent lipid peroxidation and protect cells against oxidative stress in vitro (Moosmann et al., 1997; Skaper et al., 1997). Vitamin E has been a popular anti-oxidant ever since it was first found to be potent protective agent of neuronal cells against Aβ (Behl et al., 1992). Vitamin E reverses cognitive function impairment both in AD and other mild cognitive impairment cases (Sano et al., 1997; Grundman, 2000).

1.5. Rationale and Objectives

Part 1: Resistance of quiescent human diploid fibroblasts to a high dose of external oxidative stress and induction of senescence

It has been reported multiple times that chronic exposure of NHFs to sub-lethal doses of external oxidative stress (such as H₂O₂) leads to the development of premature senescence. In this part of my project, I report a novel approach for inducing premature senescence in NHFs by treating them in quiescent state with a single high dose of external oxidative stress (500μM H₂O₂). I will attempt to:

1. Investigate response of quiescent NHFs to a high dose of external oxidative stress
2. Investigate molecular mechanism of quiescent NHFs resistance to oxidative stress induced apoptosis
3. Determine the mechanism of senescence onset in quiescent NHFs post hydrogen peroxide treatment
Part 2: Presenilin-1 mutated fibroblasts have higher ROS levels and they withstand external oxidative stress better comparing to NHFs

Peripheral cells, including fibroblasts, are frequently used to study familial forms of neurodegenerative disorders. Fibroblasts are used due to the fairly uncomplicated culturing procedures and requirements, easy access, and the fact that they have identical genetic composition as neuronal cells. In the second part of my thesis, I will attempt to investigate possible differences between NHFs and AD fibroblasts, which might prove useful in the early diagnostics and susceptibility to AD. I will try to:

1. Determine the differences in protein expression and ROS production between NHFs and AD fibroblasts
2. Investigate reasons for higher ROS content in AD fibroblasts and their increased resistance to external oxidative stress
Chapter II: Materials and Methods

2.1. Chemicals and Supplies

2.1.1. Chemicals

The following chemicals were purchased from Sigma Chemical Company (Mississauga, Ontario): BSA (bovine serum albumin), DTT (dithiothreitol), EDTA (ethylenediaminetetraacetic acid), HEPES, hydrogen peroxide, MgCl₂, leupeptin trifluoroacetate salt, pepstatin A, PMSF (phenylmethanesulfonyl fluoride) glycine, PFA (paraformaldehyde), K₃Fe(CN)₆ (potassium ferricyanide), K₄Fe(CN)₆·3H₂O (potassium ferrocyanide) and Trypsin.

The following chemicals were obtained from Molecular Probes (Eugene, Oregon): DCFDA (dichlorofluoroscein diacetate), Hoechst 33342, Apo-BrdU™ TUNEL Assay kit, and JC-1.

The following chemicals were purchased from BDH Inc. (Toronto, Ontario): DMSO (dimethyl sulfoxide), NaOH (sodium hydroxide), NaCl (sodium chloride), NaHCO₃ (sodium bicarbonate), APS (ammonium persulfate), SDS (sodium dodecyl sulphate), citric acid and sucrose.

Glycine and agarose were obtained from EM Sciences (New Jersey, USA). Protein Assay reagent was purchased from Bio-Rad (Mississauga, Ontario). 30% Acrylamide Solution and TEMED were also purchased from Bio-Rad (Mississauga, Ontario). Gentamicin Reagent Solution was purchased from Invitrogen Canada Inc. (Burlington, Ontario). Tween 20 was purchased from EM Science Merck (Darmstadt, Germany). Trypan blue was purchased from Gibco (Toronto, Ontario). Triton X- 100 was purchased from VWR (Mississauga, Ontario). X-
Gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) was purchased from Fischer Scientific (Fair Lawn, New Jersey). Sodium phosphate (Na₃PO₄) was purchased from ACP (Montreal, Quebec).

2.1.2. Equipment

Mass measurements were performed using an Adventure OHAUS Balance (OHAUS Corp., Pine Brook, New Jersey). Fluorescent and phase contrast pictures were taken using a fluorescent microscope (Leica DM IRB, Germany). Zeiss Axiovert 200 inverted fluorescence microscope was also used for taking some fluorescent pictures. Fluorescent measurements were done using Spectra Max Gemini XS (Molecular Devices, Sunnyvale, California) plate reader. Absorbances were measured using Agilent 8543 UV-VIS Spectrophotometer (Agilent Technologies Canada Inc., Mississauga, Ontario). Mini-PROTEAN II Gel electrophoresis apparatus (BioRad Laboratories Ltd., Mississauga, Canada) was used for protein gel electrophoresis. BioTrace®NT, pure nitrocellulose membrane (PALL Corporation, Pensacola, Florida) was used for immunoblotting. Rocking platform model 200 (VWR Scientific products, Mississauga, Ontario) was used for gentle shaking of the protein blots. Alpha Innotech Corporation Imaging System (San Leonardo, CA) was used to visualize immunoblots. Corning stirrer/hot plate pc-240 series (Corning Life Sciences, Acton, MA) was used for stirring and heating various solutions. A standard heat block (VWR Scientific products, Mississauga, Ontario) was also used to heat protein solutions. pH/Temp/mV meter (VWR Scientific Products, Mississauga, Ontario) was used to measure the desired parameters of solutions.

Cells were grown in a Forma Scientific CO₂ incubator (Forma Scientific Inc., Marietta, Ohio). Sterile cell sub-culturing was enabled by NUAIRE Biological Safety Cabinet class II type A/B3 (Thermo Electron Corp., Burlington, Ontario). During cell harvesting, trypsinized cells
were centrifuged using Jouan Inc. CR3i centrifuge (Jouan Inc., Winchester, Virginia). Iso Temp 210 water bath (Fischer Scientific Ltd., Ottawa, Ontario) was used for warming up cell-culture related solutions, such as cell media. Cell counting was performed using hemocytometer (Reichert Co., Buffalo, New York). Tissue culture flasks and plates were obtained from Sarstedt Inc., Montreal, Quebec.

2.1.3. Antibodies

All the antibodies were purchased from Sigma (Mississauga, Ontario): anti-Bax antibody, monoclonal anti-p21WAF1/Cip1 antibody, anti-Bcl-2 antibody, anti-MnSOD, anti-non-Selenium Glutathione Peroxidase antibody, anti-actin antibody, anti-4-HNE antibody, anti-NT antibody, FITC-conjugated-anti-mouse antibody, goat-anti-rabbit IgG FITC conjugate, anti-mouse IgG peroxidase conjugate, and anti-rabbit IgG FITC conjugate.

2.2. Cell culture

Normal Human Fibroblasts (AG09309 and AG09319) and AD fibroblasts (AG09035 and AG04159) were obtained from Coriell Institute for Medical research (New Jersey, USA) and grown in Dulbecco’s Modified Eagle’s Medium supplemented with 15% FBS (Sigma, Mississauga) and 10μg/ml Gentamycin in 5% CO₂ in a humid incubator at 37°C.

2.3. H₂O₂ treatment of NHFs and AD fibroblasts

Cells were grown either to 50% or 100% confluence. The growth medium was replaced with fresh incomplete Minimum Essential Medium Eagle (Sigma, Mississauga) containing 500μM H₂O₂, and cells were incubated for 1 hour at 37°C. Then the media was replaced with
fresh complete Dulbecco's Modified Eagle Medium (Sigma, Mississauga) without H₂O₂, and cells were allowed to recover for different periods of time, as indicated by the figures.

*The concentration of H₂O₂ was previously tested using H₂O₂ electrode; it was found that H₂O₂ is stable for 6 months when stored at 4°C in dark.

2.4. SA-β-galactosidase Stain

Cells were treated as previously described by Dimri et al. (1995). Briefly, cells were washed in PBS, fixed for 3-5 min (room temperature) in 3% formaldehyde, washed, and incubated at 37°C (no CO₂) with fresh senescence associated β-Gal (SA-β-Gal) stain solution: 1mg of 5-bromo- 4-chloro-3-indolyl β-D-galactoside (X-Gal) per ml (stock = 20 mg of dimethylformamide per ml)/40 mM citric acid/sodium phosphate, pH 6.0/5 mM potassium ferrocyanide/5 mM potassium ferricyanide/150mM NaCl/ 2mM MgCl₂. Staining was maximal in 12-16 h.

2.5. Cellular Microscopy

Cells were grown and treated as described above and stained with Hoechst 33342, 10μM final concentration (Molecular Probes, Eugene, OR). Then, they were examined under a fluorescent microscope (Zeiss, Axiovert 200), and both phase contrast and fluorescence pictures were taken and processed using Northern Eclipse 6.0 software. Alternatively, cells were stained with Trypan Blue (Gibco, Toronto, Ontario) at various points following the treatment, and counted using a hemocytometer.

2.6. Measurement of Total Cell ROS
Cells were grown and treated as described above. Production of ROS at various post-treatment times was measured by membrane permeable dye 2'-7'-dichlorofluorescin diacetate (DCFDA) obtained from Molecular Probes (Eugene, OR) using a modification of a previously published procedure (Siraki et al., 2002). Briefly, at various time points cells were incubated with DCFDA to a final concentration of 10μM for 20 min at 37°C, and fluorescence (Ex. 495nm and Em. 530nm) was measured using a Spectra Max Gemini XS multi-well plate fluorescence reader (Molecular Device, Sunnyvale, California). Microsoft Excel version 6.0 software was used for data representation and statistical analysis.

2.7. Total Cell Lysate

Treated or untreated cultured cells were scraped using rubber policeman, washed three times in PBS, and lysed in a lysis buffer consisting of 1mM EDTA, 10mM HEPES, and 250mM sucrose. The buffer contained protease inhibitors: leupeptin, pepstatin (both at 1μM) and PMSF (0.1mM). The cell suspensions were homogenized in a glass homogenizer and incubated on ice for 20 min. The post-nuclear lysates were isolated by centrifugation (500g for 5 min).

2.8. Protein Estimation

Concentration of proteins in each post-nuclear lysate was determined using a protocol from BioRad Laboratories. Mixtures of cell lysates, BioRad reagent and water were vortexed, and incubated for 10min at room temperature. The absorbance of samples was measured at 595nm using a UV-Visible Spectrophotometer, and analyzed using a BSA standard curve.

2.9. Western Blots
Equal amount of proteins per lane (20\(\mu g\)) was resolved on a 12% SDS gel. Proteins were electrotransferred on to a nitrocellulose membrane. The membranes were blocked with 5% non-fat milk and probed with anti-Bax, anti-p21, anti-Bcl-2, anti-actin, anti Mn-SOD, and anti- non-Selenium Glutathione Peroxidase (Sigma, Mississauga). The blots were washed and exposed to HRP conjugated secondary antibodies (Sigma, Mississauga) and then developed using Chemiluminescence Peroxidase Substrate kit (Sigma, Mississauga, ON) and visualized with Alpha Innotech Corporation Imaging System (San Leonardo, CA). The molecular masses of the proteins were estimated relative to the Page Ruler Prestained Protein Ladder (Fermentas Life Sciences, Burlington, ON).

2.10. Mitochondrial Membrane Potential

NHF cells were grown and treated with H\(_2\)O\(_2\) as previously described. 24 h after H\(_2\)O\(_2\) treatment, the cells were incubated in culture medium containing 5\(\mu g/ml\) JC-1 for 20 min. The cells were observed under a fluorescent microscope (Leica DM IRB), and pictures were processed using Adobe Photoshop 7.0 software.

2.11. Oxidative Stress Markers Staining

NHF\(_s\) were grown on cover-slips and treated as previously described. 24h after the treatment, the cells were washed with PBS, and fixed in 2% paraformaldehyde. Following fixation, they were washed in PBS again, and incubated in 70% ethanol for 5 minutes. At this point they were washed again and incubated in blocking solution containing 10% FBS in PBS for 1 hour. The cells were then incubated with either anti-NT antibody (Sigma, Mississauga, ON) or anti-4-HNE (Sigma, Mississauga, ON) antibody, diluted 1:500 in Dako Antibody Diluents (DakoCytomation) for 1 hour. Following primary antibody incubation, the cells were washed
with PBS, and incubated with FITC conjugated anti-rabbit antibody (Sigma, Mississauga), diluted 1:200 in Dako Antibody Diluent (DakoCytomation), for 1 hour. Finally, NHFs were washed in PBS and the cover-slips were mounted onto slides using Ultra Cruz™ Mounting Medium with DAPI (Santa Cruz Biotechnology, California). The slides were observed and the pictures were taken using fluorescent microscope (Leica DM IRB).

2.12. TUNEL Assay

NHF cells were grown on cover-slips and treated with H2O2 as previously described. 24 hours after the treatment, the cells were tested for DNA damage using APO-BrdU™ TUNEL Assay kit (Invitrogen Molecular Probes) and manufacturer's recommended protocol. Following the TUNEL labelling, the cover-slips were mounted on slides using Ultra Cruz™ Mounting Medium with DAPI (Santa Cruz Biotechnology, California). The slides were observed and the pictures were taken using fluorescent microscope (Leica DM IRB).

2.13. γH2AX Staining for Double Stranded DNA Breaks

Immunofluorescent microscopy was performed as described by Young et al. (2002) but with minor modifications. Briefly, cells were seeded into 6-well culture plate containing a glass cover slip in each well to either 50% or 100% confluence. After the treatment described above, cells were allowed to recuperate for 1hr or 24hrs, after which they were fixed in 2% paraformaldehyde for 10 min, washed with PBS, and permeabilized in 70% ethanol. After blocking with blocking serum (10% FBS in PBS) for 1 h, slides were incubated with a mouse monoclonal anti-γH2AX antibody (Sigma, Mississauga) diluted in Dako Antibody Diluent (DakoCytomation), 1:200 dilution for 1h, followed with FITC-conjugated goat-anti-mouse secondary antibody (Sigma, Mississauga), diluted in Dako Antibody Diluent (DakoCytomation)
1:200 dilution for 1 h. Following the γH2AX labelling, the cover-slips were mounted on glass slides using Ultra Cruz™ Mounting Medium with DAPI (Santa Cruz Biotechnology, California). The slides were observed and the pictures were taken using fluorescent microscope (Leica DM IRB).
Chapter III: Results

Part 1: Resistance of quiescent human diploid fibroblasts to a high dose of external oxidative stress and induction of senescence

3.1. Response of dividing and quiescent NHFs to a high dose of external oxidative stress

In order to determine if young dividing and quiescent cells respond differently to a high dose of oxidative stress, we treated them with 500µM H₂O₂ for 1 hr as described in Materials and Methods section. Cells were stained and monitored 24hr following the treatment, for the indication of apoptosis using Hoechst staining and TUNEL labelling. Young dividing fibroblasts (50-70% confluence) are vulnerable to high levels of H₂O₂ and start undergoing apoptosis (Fig 1A) as indicated by chromatin condensation observable by Hoechst stain (Fig 2A) and TUNEL labelling (Fig 2B). Untreated cells show normal growth curve (Fig 1B), healthy nuclear morphology and no TUNEL labelling (Fig 2B). Interestingly, quiescent NHFs (100% confluence) resisted the external stress and did not show nuclear condensation or DNA fragmentation (Fig 2A and Fig 2B). Only treated young dividing NHFs were positively labelled by TUNEL assay, as indicated by bright green staining (Fig 2B). Similarly, Hoechst revealed chromatin condensation in treated young dividing NHFs only (Fig 2A), as indicated by bright nuclear stain and decreased nucleus size. The quiescent cells remained attached and retained a healthy nuclear and cellular morphology. The negative TUNEL labelling indicated that there was no DNA fragmentation. These results indicated that young dividing NHFs underwent apoptosis due to a high dose of external oxidative stress, while quiescent NHFs showed resistance. However, TUNEL labelling is too crude of a method to detect repairable DNA damage, so H2AXγ staining was performed.
Young dividing NHFs (PD26) were treated with 500 μM H₂O₂ and stained with Hoechst as described in Materials and Methods section. The number of apoptotic cells was monitored over a period of 2 days and represented as percentage of total cell population.
Young dividing NHFs (PD 26) were grown to approximately 80-90% confluence and passaged. The number of cells in the culture was monitored over a period of 6 days, starting at the time when they were seeded into new plates. Cells were stained with Trypan blue and counted using a hemocytometer.
Fig. 2. (A) Nuclear morphology of sub-confluent and quiescent NHFs before and after H$_2$O$_2$ treatment.

Young dividing NHFs and quiescent NHFs were treated with 500 μM H$_2$O$_2$ and stained with Hoechst as described in Materials and Methods section. Pictures were taken at 400x magnification.
Fig.2. (B) External oxidative stress induced DNA fragmentation and chromatin condensation in sub-confluent NHFs

Young dividing NHFs and quiescent NHFs were treated with 500 μM H₂O₂, subjected to TUNEL labeling, and stained with DAPI as described in Materials and Methods section. Pictures were taken at 400x magnification (the unit scale included into the pictures is 50μm). DNA fragmentation was indicated by positive TUNEL staining, and it was observed only in young dividing NHFs following oxidative stress. DAPI stained brightly only apoptotic collapsing nuclei, confirming the DNA damage in young dividing treated fibroblasts.
H2AXγ antibody targets phosphorylated H2AX histone, which is essential for DNA double strand break repair (Zhao et al, 2007). Our results indicate that quiescent NHFs once exposed to 500µM H2O2, undergo DNA double strand breaks and initiate an active DNA repair response as seen by very strong H2AXγ signal 1hr after the treatment (Fig. 3A and B). The H2AXγ signal is much weaker 24hrs after treatment, which is indicative of substantial DNA repair by that time. On the other hand, young dividing fibroblasts maintain a high level of phosphorylated of H2AX, indicative of a high level of DNA double stranded breakage (Fig. 3A and B). It seems that DNA damage is not successfully repaired by 24hrs (as seen by TUNEL labelling and Hoechst staining), which ultimately leads to apoptosis.

3.2. Induction of senescence by H2O2 treatment in quiescent NHFs

Although the quiescent NHFs did not show any sign of apoptosis following a high dose of oxidative stress, the question remained if they had experienced any biochemical changes under these circumstances. When these cells were sub-cultured 72 hrs following H2O2 treatment, we observed that they lost their ability to divide. Following sub-culturing, we continued to monitor cell growth of untreated quiescent cells as well as treated cells. The results shown in figures 4A and 4B clearly indicate that total cell number is effectively unchanged over a period of 6 days in quiescent cells treated with a high dose of external oxidative stress, whereas untreated quiescent NHFs resumed normal cell growth after sub-culturing. This observation indicated that treated quiescent cells may be undergoing senescence. To confirm the senescent state of the treated quiescent NHFs, we looked for the senescence specific marker Senescence-Associated (SA) β- galactosidase (Dimri et al., 1995). SA β-galactosidase assay was performed in untreated (Fig 5A) and H2O2 treated (Fig 5B) quiescent NHFs following sub-culturing, as described in Materials and Methods. Cells sub-cultured after H2O2 treatment stained positively.
Fig. 3. (A) External oxidative stress induced DNA double stranded breaks and DNA repair response

Young dividing NHFs and quiescent NHFs were treated with 500 μM H₂O₂, subjected to H2AX and DAPI staining, as described in Materials and Methods section. Positive H2AX staining indicated phosphorylated histone H2AX corresponding to DNA double stranded breaks repair. Pictures were taken at 400x magnification (the unit scale included into the pictures is 125μm).
Fig. 3. (B) External oxidative stress induced DNA double stranded breaks and DNA repair response.

Young dividing NHFs and quiescent NHFs were treated with 500 μM H₂O₂, subjected to H2AX and DAPI staining, as described in Materials and Methods section. Positive H2AX staining indicated phosphorylated histone H2AXγ corresponding to DNA double stranded breaks repair. Pictures show detailed view (2400x) of the nuclei, and the red arrow points to individual phosphorylated histone foci.
Quiescent NHFs were treated with 500 μM H₂O₂ for 1 hour, after which they were allowed to recover for 24 hours and then sub-cultured. The number of cells in the culture was monitored over a period of 6 days, starting at the time when they were seeded into new plates. Control cells (A) resumed normal cell growth, while the treated ones (B) did not grow nor change their total number significantly.
Fig. 5. Induction of Senescence-Associated β-galactosidase expression in quiescent NHFs following H$_2$O$_2$ treatment

Quiescent NHFs, untreated (A), and those treated with 500 µM H$_2$O$_2$ (B) were sub-cultured, fixed in 3% formaldehyde and stained with x-gal as described in Materials and Methods section. Both pictures were taken at 100x magnification; positive stain is observed only in senescent fibroblasts, which are also larger in size compared to untreated cells.
with SA-β-gal upon exposure to the enzyme’s substrate x-gal (Fig 5B), which is indicative of senescence phenotype, while the control cells were negative. These results indicate that a high dose oxidative stress induces premature senescence in quiescent cells.

3.3. Levels of ROS in young dividing and quiescent NHFs

In order to explain the differential behaviour of treated quiescent NHFs we determined the level of reactive oxygen species (ROS) in young dividing and quiescent NHFs both H₂O₂ treated and untreated. We measured total cellular ROS in young dividing and quiescent cells with or without H₂O₂ treatment using H₂DCFDA as described in Materials and Methods section. Untreated dividing NHFs had the lowest level of ROS, while H₂O₂ treated young dividing NHFs had the highest, which may sensitize them to apoptosis (Fig 6A). Untreated quiescent NHFs had higher ROS levels compared to young dividing NHFs and ROS levels did not change significantly following exposure to external oxidative stress (Fig 6A). This might lead to the conclusion that quiescent NHFs, by having a higher level of endogenous ROS, are better conditioned for dealing with oxidative stress and therefore do not enter the apoptotic pathway, but rather, become senescent. Higher endogenous ROS leads to lipid peroxidation and protein nitrosylation, and so we monitored the level of oxidative damage to lipids and proteins using anti-4-hydroxy-2-nonenal (4HNE) and anti-nitrotyrosine (NT) antibodies respectively, as described in the Materials and Methods section. There is a clear indication of lipid peroxidation and protein nitrosylation in NHFs undergoing apoptosis (Fig 6B, Fig 6C). Similarly, high level of ROS in quiescent NHFs, both treated and untreated, is correlated with increased oxidative damage to lipids and proteins (Fig 6B and 6C). These results indicate that quiescent NHFs, even though able to remain resistant to apoptosis, have undergone protein and lipid oxidative modification.
Fig. 6. (A) Production of ROS in sub-confluent and quiescent cells before and after \( \text{H}_2\text{O}_2 \) treatment

NHF (PD 26) cells were grown either to 50% (dividing) or to 100% (quiescent) confluence, treated with 500\( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) to induce oxidative stress and incubated with 10\( \mu \text{M} \) \( \text{H}_2\text{DCFDA} \) as described in Materials and Methods section.
Young dividing NHFs and quiescent NHFs were treated with 500 μM H₂O₂ for 1 hour, after which they were allowed to recover for 24 hours. They were then stained for 4-HNE and DAPI as described in Materials and Methods section. Pictures were taken at 400x magnification (the unit scale is 125μm). Cellular oxidative stress was indicated by positive 4-HNE staining and it was observed in young dividing NHFs following oxidative stress and quiescent NHFs both treated and non-treated. DAPI was used to simultaneously observe DNA damage. Only young dividing NHFs following the oxidative stress show significant chromatin condensation.
Young dividing NHFs, and quiescent NHFs were treated with 500 μM H₂O₂ for 1 hour, after which they were allowed to recover for 24 hours. They were then immunostained using anti-nitrotyrosine (NT) antibody as described in Materials and Methods section. Pictures were taken at 400x magnification (the unit scale is 125μm). Cellular oxidative stress was indicated by positive NT staining and it was observed in young dividing NHFs following oxidative stress and quiescent NHFs both treated and non-treated. DAPI was used to simultaneously observe DNA damage. Only young dividing NHFs following the oxidative stress show significant chromatin condensation.
3.4. H$_2$O$_2$ treatment of quiescent NHFs does not affect their mitochondria

Once we determined the differential ROS levels in young dividing and quiescent NHFs, we wanted to determine if mitochondrial membrane potential (MMP) is affected in these cells. Both dividing and quiescent NHFs were stained with the MMP-sensitive fluorescent JC-1 dye 24 hrs following H$_2$O$_2$ treatment, as described in Materials and Methods. Mitochondria that maintain MMP show red fluorescence due to accumulation and aggregation of JC-1 inside the mitochondria. Loss of MMP is evident only in treated young dividing NHFs (which were sensitive to oxidative stress and underwent apoptosis). To our surprise, quiescent NHFs maintained their MMP even after being exposed to such a high dose of H$_2$O$_2$ (Fig 7). Therefore, external oxidative stress does not lead to mitochondrial collapse in quiescent NHFs, indicating that these cells might have a mechanism to protect the mitochondria against oxidative stress.

3.5. Up-regulation of MnSOD and maintenance of non-Selenium glutathione peroxidase (GPx) expression in quiescent NHFs

In order to better understand the resistance of quiescent NHFs to oxidative stress induced apoptosis, and possibly reveal some mechanism involved in coping with excess ROS while maintaining healthy mitochondria, we investigated the protein levels of Manganese Superoxide Dismutase (MnSOD) and non-Selenium glutathione peroxidise (GPx). In young dividing cells, both treated and untreated, MnSOD is expressed at low level, whereas in quiescent NHFs it is upregulated and remains high even after treatment (Fig 8). High level of MnSOD might be responsible for efficient removal of superoxide in mitochondria, thus protecting it from excessive oxidative damage.
Fig. 7. Mitochondrial membrane potential (MMP) in dividing and quiescent cells before and after H\textsubscript{2}O\textsubscript{2} treatment.

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NHF cells were grown either to 50% (young dividing NHFs) or to 100% confluence (quiescent NHFs), treated with 500\textmu M H\textsubscript{2}O\textsubscript{2} to induce oxidative stress and stained with JC-1 as described in Materials and Methods section. Mitochondrial potential was evaluated using JC-1 staining and fluorescent microscopy; pictures were taken at 400x magnification. Young dividing NHFs at 50% confluence had healthy mitochondria as indicated by punctate red stain (JC-1 fluoresces red when in aggregated state). Mitochondria of young dividing NHFs post-treatment started to collapse, and JC-1 could no longer be retained in aggregated state within mitochondria, so red stain in absent. Green staining indicates JC-1 dye in non-aggregated monomer state. Quiescent NHFs, both untreated and treated, showed intact mitochondria.
NHF (PD 28 and PD 29) cells were grown either to 50% (young dividing NHFs) or to 100% confluence (quiescent NHFs), treated with 500μM H₂O₂ to induce oxidative stress, and harvested after 24hrs. Equal amount of protein (20μg) from post nuclear cell lysate of was used for immunoblotting as described in Materials and Methods section. A) Young dividing NHF-control; B) Young dividing NHF-treated; C) Quiescent NHF-control; D) Quiescent NHF-treated.
Non-selenium glutathione peroxidase is expressed at approximately the same level in young dividing and quiescent NHFs, and it continues to be expressed in quiescent NHFs after H$_2$O$_2$ treatment. However, this is not the case with young dividing treated NHFs (undergoing apoptosis), where non-selenium glutathione peroxidase is almost entirely absent. Therefore, it is possible that these enzymes play a role in protecting the mitochondria of quiescent NHFs.

3.6. Induction of p21 and down-regulation of Bax in quiescent NHFs following H$_2$O$_2$ treatment

In addition to specific enzymes responsible for anti-oxidative defence (as described above), we wanted to investigate the expression of other pro- and anti-apoptotic, as well as cell cycle-arrest-related proteins in young dividing and quiescent NHFs before and after H$_2$O$_2$ treatment. p21 is known to block cells in G1 stage of the cell cycle and as expected p21 is over-expressed in quiescent cells. Interestingly, we observed that p21 was further over-expressed in quiescent cells after H$_2$O$_2$ treatment (Fig 9), indicating a possible role in the induction of senescence (permanent cell cycle arrest). Dividing NHFs exposed to external oxidative stress undergo apoptosis, not senescence, which may be attributed to the involvement of Bcl-2 family proteins, such as Bax and Bcl-2. Bax is a pro-apoptotic protein and Bcl-2 is an anti-apoptotic protein; the ratio between these types of proteins dictates cell death or survival. As expected, treated dividing NHFs undergoing apoptosis show up-regulation of Bax and down-regulation of Bcl-2 (Fig 9). Interestingly, Bax is down-regulated in quiescent NHFs treated with H$_2$O$_2$, and Bcl-2 levels are unchanged after treatment (Fig 9). These observations indicate that quiescent cells have a protein expression profile consistent with heightened resistance to apoptosis and are more apt to become senescent.
Fig. 9. Expression of p21, Bax, and Bcl2 in dividing and quiescent NHFs before and after H$_2$O$_2$ treatment

NHF (PD 28 and PD 29) cells were grown either to 50% (young dividing NHFs) or to 100% confluence (quiescent NHFs), treated with 500μM H$_2$O$_2$ to induce oxidative stress, and harvested after 24hrs. Equal amount of protein (20μg) from post nuclear cell lysate of was used for immunoblotting as described in Materials and Methods section. A) Young dividing NHF-control; B) Young dividing NHF-treated; C) Quiescent NHF-control; D) Quiescent NHF-treated.
Part II: Preselin-1 mutated fibroblasts have higher ROS levels and they withstand external oxidative stress better comparing to NHFs

3.7. PS-1 mutated fibroblasts show signs of senescence

AD fibroblasts exhibit an increase in doubling time (Fig. 10) compared to NHFs (NHF growth curve is shown in Fig 1B), as well as an overall decrease in growth potential. Based on this observation, we suspected that AD fibroblasts might be undergoing early replicative senescence. To confirm this, we looked for the senescence specific marker Senescence-Associated (SA) β-galactosidase (Dimri et al., 1995) in an AD fibroblasts culture. SA β-galactosidase assay was performed in dividing NHFs and dividing AD fibroblasts, as described in Materials and Methods section. A high percentage of cells in the AD culture stained positive upon exposure to x-gal, which is indicative of a senescent phenotype, while the NHFs were negative (Fig. 11). Therefore, according to these results, AD fibroblasts enter senescence sooner than Normal Human Fibroblasts.

3.8. DNA double stranded breaks are present in untreated AD cells

Overall, the nuclei of AD fibroblasts have healthy morphology, as shown by DAPI stain (Fig. 12), but the more sensitive γH2AX stain made visible the minor DNA damage that is not visible by employing DAPI (Fig. 12). γH2AX antibody targets phosphorylated H2AX histone, which is essential for DNA double strand break repair (Zhao et al., 2007). Our results indicate that only AD fibroblasts, not NHFs, show double stranded DNA breaks even before being treated with \( \text{H}_2\text{O}_2 \) (Fig. 12 and Fig. 17). This DNA damage might be the reason why AD fibroblasts enter a state of senescence earlier than NHFs.
AD fibroblasts (PD 24) were grown to approximately 80-90% confluence and passaged. The number of cells in the culture was monitored over a period of 7 days, starting at the time when they were seeded into new plates. Cells were stained with Trypan blue and counted using a hemocytometer.
Fig.11. SA β-galactosidase expression in PS-1 mutated fibroblasts

Dividing NHFs (A) and AD fibroblasts (B) were washed in PBS, fixed in 3% formaldehyde and stained with x-gal as described in Materials and Methods section. Pictures were taken at 100x magnification; positive stain is observed in AD fibroblasts almost exclusively.
Dividing NHFs and AD fibroblasts were subjected to H2AXγ and DAPI staining (A), as described in Materials and Methods section. Positive staining indicated phosphorylated histone H2AX, corresponding to DNA double stranded breaks repair. Detailed view (approximately 2400x) of H2AXγ stained nuclei is provided in (B).
3.9. Levels of ROS in NHFs and AD fibroblasts

In order to explain the differential behaviour of AD fibroblasts with respect to cellular replicative life span, we tested the level of reactive oxygen species (ROS) in dividing NHFs and AD fibroblasts. We measured total cellular ROS in both cultures using H$_2$DCFDA as described in Materials and Methods section. Both untreated, AD fibroblasts had approximately twice as much endogenous ROS when compared to NHFs (Fig 13A). Higher endogenous ROS leads to lipid peroxidation and protein nitrosylation, and so we monitored the level of oxidative damage to lipids and proteins using anti-4-hydroxy-2-nonenal (4HNE) and anti-nitrotyrosine (NT) antibodies, as described in Materials and Methods section. There is a clear indication of lipid peroxidation and protein nitrosylation in NHFs and AD fibroblasts undergoing apoptosis following H$_2$O$_2$ treatment, but this was also observed in untreated AD fibroblasts, which is consistent with a higher level of endogenous ROS (Fig. 13B, and Fig. 13C). These results indicate that AD fibroblasts have undergone protein and lipid oxidative modification due to higher endogenous ROS content, which might explain their tendency to undergo premature senescence.

3.10. Differential expression of MnSOD and non-Selenium glutathione peroxidise in AD fibroblasts

In order to better understand differential ROS levels in AD fibroblasts, compared to NHFs, and their ability to better deal with external oxidative stress, as well as possibly reveal some mechanism involved in absorbing the excess ROS, we investigated the levels of Manganese Superoxide Dismutase (MnSOD) and non-Selenium glutathione peroxidase (Fig. 14).
Fig.13. (A) Endogenous ROS levels are higher in AD fibroblasts than in NHFs

NHF (PD 26) and AD fibroblasts (PD 24) were grown to approximately 50% (dividing) confluence, and incubated with 10μM H₂DCFDA as described in Materials and Methods section. Fluorescence is expressed as Relative Fluorescence Units (RFU) per 10 000 cells.
Dividing NHFs and AD fibroblasts were treated with 500 μM H₂O₂ for 1 hour, after which they were allowed to recover for 24 hours. They were then simultaneously stained for 4-HNE and DAPI as described in Materials and Methods section. Pictures were taken at 400x magnification (the included unit scale is 125μm). Cellular oxidative stress was indicated by positive 4-HNE staining and it was observed in young dividing NHFs following oxidative stress and AD fibroblasts both treated and un-treated. DAPI was used to observe DNA damage. Only young dividing NHFs following the oxidative stress show significant chromatin condensation. The nuclei of AD fibroblasts seem to be less affected.
Fig.13(C) Protein nitrosylation in dividing NHFs and AD fibroblasts before and after H$_2$O$_2$ treatment.

Dividing NHFs, and AD fibroblasts were treated with 500 μM H$_2$O$_2$ for 1 hour, after which they were allowed to recover for 24 hours. They were then immunostained using anti-nitrotyrosine (NT) antibody as described in Materials and Methods section. DAPI was used to simultaneously observe DNA damage. Pictures were taken at 400x magnification (the unit scale of 125μm is included with the pictures). Cellular oxidative stress was indicated by positive NT staining and it was observed in young dividing NHFs following oxidative stress AD fibroblasts both treated and non-treated. Only young dividing NHFs following the oxidative stress show significant chromatin condensation. AD fibroblasts’ nuclei are not as affected by external oxidative stress as that of treated NHFs.
Fig. 14. Expression of MnSOD and non-selenium Glutathione Peroxidase in NHFs and AD fibroblasts

NHF cells and AD fibroblasts were grown to approximately 50-75% confluence, scraped using rubber policeman, and lysed as described in Materials and Methods. An equal amount of protein (20μg) from the post-nuclear cell lysate was used for immunoblotting as described in Materials and Methods section. There is a marked decrease in the level of non-selenium GPx in AD fibroblasts compared to NHFs, while MnSOD level is up-regulated.
MnSOD is up-regulated in AD fibroblasts and a higher level of MnSOD might be responsible for efficient removal of superoxide from the mitochondria, thus protecting it from oxidative damage. Non-selenium glutathione peroxidase is down-regulated in AD fibroblasts, so those cells are not as efficient in degrading endogenous H$_2$O$_2$, which results in overall higher ROS levels. Therefore, it becomes clear that MnSOD is crucial for protecting AD mitochondria against increased internal oxidative stress, as well as providing some protection against high level of external oxidative stress.

3.11. AD fibroblasts exhibit some resistance to H$_2$O$_2$ treatment compared to age-matched NHFs

Differential endogenous ROS levels and an increase in the number of cells with a senescent phenotype might determine how a cell responds to an external oxidative insult. Our data indicates that AD fibroblasts are somewhat resistant to oxidative stress induced apoptosis: interestingly, the rate of apoptosis is lower compared to NHFs (Fig. 15). Due to this finding, we wanted to determine the state of the mitochondria in AD fibroblasts, both treated with 500μM H$_2$O$_2$ and untreated, through JC-1 staining (Fig. 16). The cells were stained with the MMP-sensitive fluorescent JC-1 dye before and 24 hrs after H$_2$O$_2$ treatment, as described in Materials and Methods. Mitochondria that maintain MMP show red fluorescence due to accumulation and aggregation of JC-1 inside the mitochondria. Both cell lines have healthy, functional mitochondria before treatment. Loss of MMP is clearly evident in dividing treated NHFs, while dividing AD fibroblasts do not show complete collapse of MMP. It seems that a proportion of cells within the actively dividing AD fibroblast culture are able to maintain healthy mitochondria even after being challenged with a high dose of external oxidative stress. Therefore, external
Fig. 15. PS-1 mutated fibroblasts have lower rate of oxidative stress induced apoptosis than NHFs.

Dividing AD fibroblasts and NHFs were treated with 500 μM H₂O₂ and stained with Hoechst as described in Materials and Methods section. The number of apoptotic cells was monitored over a period of 2 days and represented as percentage of total cell population. Amount of apoptosis is lower by approximately 20% in AD fibroblasts.
**Fig. 16.** PS-1 mutated fibroblasts are able to partially maintain mitochondrial membrane potential after being exposed to high dose of external oxidative stress

NHF and AD fibroblasts were grown to approximately 50-75% confluence, treated with 500µM H\textsubscript{2}O\textsubscript{2} to induce oxidative stress and stained with JC-1 as described in Materials and Methods section. Mitochondrial potential was evaluated using JC-1 staining and fluorescent microscopy; pictures were taken at 400x magnification (the unit scale is 125µm). JC-1 dye gets aggregated in healthy mitochondria, and as aggregate, it fluoresces red. JC-1 cannot aggregate inside damaged mitochondria, and as monomer, it fluoresces green. Young dividing NHFs and AD fibroblasts pre-treatment had healthy mitochondria as indicated by the red stain. Mitochondria of young dividing NHFs post treatment started collapsing as indicated by the absence of red staining. AD fibroblasts are able to partially maintain MMP after H\textsubscript{2}O\textsubscript{2} treatment.

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oxidative stress does not completely damage the mitochondria of AD fibroblasts, indicating that PS-1 mutated fibroblasts might be equipped with a mechanism for dealing with increased oxidative stress, which agrees with our findings of higher endogenous ROS levels in these cells.

Furthermore, to understand the state of AD nuclei following H$_2$O$_2$ treatment and their ability to repair oxidative stress induced DNA damage, we subjected both NHFs and AD fibroblasts to $\gamma$H2AX labelling. $\gamma$H2AX antibody targets phosphorylated H2AX histone that is present at double stranded DNA break sites. Our results indicate that 500µM H$_2$O$_2$ exposure causes DNA double strand breaks and initiates an active DNA repair response in both AD fibroblasts and NHFs, visualized as bright foci after 1hr (Fig. 17). However, it seems that 24hrs following treatment, AD fibroblasts managed to repair some of the double stranded DNA breaks, as seen by the decrease of $\gamma$H2AX signal intensity (Fig. 17); this was not the case with NHFs.
Young dividing NHFs and AD fibroblasts were treated with 500 μM H₂O₂ and subjected to H2AXγ staining, as described in Materials and Methods section. Positive H2AXγ staining indicated phosphorylated histone H2AX corresponding to DNA double stranded breaks repair. AD fibroblasts are capable of better repairing oxidative stress induced DNA double stranded breaks, as seen by the decrease in H2AX stain intensity by 24hrs after the treatment. Pictures were taken at 400x magnification (the unit scale is 125μm).
Chapter IV: Discussion

4.1. Resistance of quiescent human diploid fibroblasts to a high dose of external oxidative stress and induction of senescence

Quiescent fibroblasts are temporarily withdrawn from the cell cycle due to contact inhibition, and are able to resume normal cell division upon sub-culturing. On the other hand, senescent fibroblasts are permanently withdrawn from the cell cycle and cannot resume normal cell division. It has been previously reported that repeated exposure of normal human fibroblasts to sub-cytotoxic oxidative stress induces premature cellular senescence (Caldini et al., 1998; Frippiat et al., 2001), but our study demonstrates that premature senescence can be induced by a single high dose of H₂O₂ when fibroblasts are in a quiescent state. We report for the first time that quiescent normal human fibroblasts enter premature senescence upon brief exposure (1 hr) to 500μM H₂O₂. Unlike dividing NHFs, which undergo apoptosis following a high dose of oxidative stress, quiescent NHFs exhibit resistance to this dose and become senescent. We have previously reported that only dividing NHFs (not quiescent) exhibit p38MAPK activation following H₂O₂ treatment and became apoptotic while quiescent cells were unaffected (Naderi et al., 2003). Induction of apoptosis by H₂O₂ treatment in dividing cells was evident by chromatin condensation and DNA fragmentation. Quiescent cells on the other hand, did not show chromatin condensation or DNA fragmentation following the same treatment, while they showed successful, but incomplete, repair of double stranded DNA breaks. Furthermore, there is a clear difference in the expression of pro- and anti-apoptotic proteins in dividing and quiescent fibroblasts following H₂O₂ treatment. While dividing NHFs showed up-regulation of Bax protein and down-regulation of anti-apoptotic Bcl-2, quiescent cells exhibited down-regulation of Bax
and no change in Bcl-2 level. Interestingly, p21, a cell cycle arrest protein, which was already present in higher amounts in contact-inhibited quiescent cells, was further up-regulated after H$_2$O$_2$ treatment. This result implicates the importance of p21 in the onset of senescence. Indeed, H$_2$O$_2$ treated quiescent NHFs had a senescent phenotype and tested positive for SA-β-galactosidase, a characteristic marker of senescence (Dimri et al., 1995). The question now becomes: why are quiescent fibroblasts resistant to oxidative stress-induced apoptosis and why do they enter senescence after H$_2$O$_2$ treatment?

Oxidative stress-induced DNA damage, depending on the extent, can lead to either cell death or senescence through a common p53 mechanism (Itahana et al., 2001). If the DNA damage is not severe enough to induce apoptosis, then cellular senescence occurs (Beckman and Ames, 1998). We showed via γH2AX staining that the DNA in quiescent cells gets damaged, but is for the most part successfully repaired 24hrs following treatment. Our observations indicate that a high dose of external oxidative stress actually caused a substantial amount of DNA double stranded breaks in quiescent cells, but an active DNA repair response was initiated. However, the DNA was never entirely repaired, which is why those cells did not continue through normal cell cycle; even though the DNA damage was insufficient to cause apoptosis, it was indeed enough to cause a senescent response. On the other hand, in dividing treated NHFs, there was substantial H2AX phosphorylation throughout the 24hr post-treatment period. H2AX gets phosphorylated by ATM kinase (Zhao et al., 2007), and according to our data, histone phosphorylation remains active throughout this 24hr time period, but the DNA does not get completely repaired. γH2AX signal remains relatively strong; these cells are unable to repair, and thus undergo apoptosis.

In addition to DNA damage, p21 protein is implicated in the onset of senescence. The cell cycle inhibitor p21 is responsible for exerting G1 cell cycle arrest after excessive DNA
damage (Waldman et al., 1995). It has been also reported that p21 protects HST116 colon carcinoma cells against hyperoxia (Helt et al., 2001), which supports our finding that H$_2$O$_2$ treated quiescent NHF cells are resistant to oxidative stress-induced apoptosis. Furthermore, down-regulation of p21 transcription promotes a p53-dependant apoptotic response to genomic stress (Seoane et al., 2002). Therefore, p21 promotes cell survival by limiting p53-dependent apoptosis. It has also been reported by Sohn et al. (2006) that p21 is able to protect cells from IR-induced apoptosis by suppressing CDK activity, which seems to be needed to activate the caspase-cascade downstream of the mitochondria. Our observation that p21 is up-regulated in quiescent fibroblasts and is further up-regulated post H$_2$O$_2$ treatment in these cells supports the pro-survival role of p21. Up-regulation of p21 in quiescent NHFs before and after exposure to oxidative stress may therefore protect cells from oxidative stress-induced apoptosis directly, not merely block their division by locking them in G1.

Bcl-2 expression was maintained in quiescent cells following H$_2$O$_2$ treatment, and thus they were protected from apoptosis. Bcl-2 has been found to decrease hyperoxia-induced apoptotic cell death by preventing caspase activation and the release of the pro-apoptotic factors cytochrome c and AIF from the mitochondria (López-Diazguerrero et al., 2006). Increased levels of ROS do not cause significant damage in quiescent H$_2$O$_2$ treated fibroblasts; these cells may be able to activate cellular anti-oxidant defence mechanisms against major oxidative insults, which might be the reason why quiescent NHFs resist apoptosis. Bcl-2 over-expression may lead to protection against oxidative stress and cell cycle arrest that could promote senescence (López-Diazguerrero et al., 2006). Quiescent NHFs retained Bcl-2 expression levels even after H$_2$O$_2$ treatment, while young dividing NHFs exhibited null Bcl-2 expression; quiescent NHFs became senescent after H$_2$O$_2$ treatment, highlighting the importance of Bcl-2 in both pro-survival and
senescence-promoting cellular responses. Dividing fibroblasts treated with H\textsubscript{2}O\textsubscript{2} showed the expected marked increase in Bax levels, which coincides with other apoptotic events observed in these cells following treatment.

Endogenous H\textsubscript{2}O\textsubscript{2} in quiescent cells did not change significantly after treatment with a high dose of oxidative stress, which suggests an enhanced anti-oxidative cellular mechanism. We have observed that MnSOD is up-regulated in quiescent fibroblasts and continues to be up-regulated even after H\textsubscript{2}O\textsubscript{2} treatment (Fig 8). We then suspected involvement of MnSOD in resistance of quiescent fibroblasts to high levels of external oxidative stress. MnSOD is a mitochondrial enzyme and is considered to be the front-line of defence against external oxidative stress. It has been previously reported that MnSOD up-regulation induced by Resveratrol leads to oxidative stress resistance in the human lung fibroblast cell line MRC-5 (Robb et al., 2008). We postulate that up-regulated MnSOD in quiescent fibroblasts might be responsible for superoxide being sequestered from the mitochondria, thus keeping the mitochondria viable even after exposure to a high dose of H\textsubscript{2}O\textsubscript{2} (Fig 7). Indeed, our results indicate that mitochondrial membrane potential is maintained following H\textsubscript{2}O\textsubscript{2} treatment in quiescent cells. Higher levels of endogenous H\textsubscript{2}O\textsubscript{2} recorded in quiescent fibroblasts both pre- and post-treatment agree with our hypothesis as H\textsubscript{2}O\textsubscript{2} is a product of superoxide radical conversion by MnSOD. Glutathione peroxidase levels were not significantly changed in quiescent NHFs when compared to the NHFs still undergoing cell division, indicating insufficient removal of generated H\textsubscript{2}O\textsubscript{2} by MnSOD (Fig 8). Excess H\textsubscript{2}O\textsubscript{2} caused some oxidative damage to cellular macromolecules, which was indicated by lipid peroxidation and protein nitrosylation; however, it is important to emphasize that no additional oxidative damage was observed in the treated quiescent fibroblasts compared to
untreated controls. Maintaining healthy mitochondria by up-regulation of MnSOD seems to be a crucial event in resistance to H$_2$O$_2$ induced apoptosis.

Quiescent fibroblasts, which have up-regulated MnSOD and p21, are able to withstand a high dose of external oxidative stress by entering into permanent cell cycle arrest or senescence. MnSOD and p21 up-regulation, Bax down-regulation and maintenance of Bcl-2 are largely responsible for quiescent cell survival after H$_2$O$_2$ treatment. More importantly, even though there was substantial DNA damage in those cells after treatment, they were able to activate an efficient DNA repair response. However, DNA repair was not complete and even though able to avoid apoptosis, the cells entered senescence due to a lack of a "threshold level" of DNA damage. As proposed before, cells with DNA damage that are unable to undergo apoptosis, enter senescence in order to prevent tumorigenesis (Campisi et al., 2001). Apoptosis, stress induced premature senescence and replicative senescence seem to share a common pathway; however, it remains largely unclear as to how a cell's fate is determined.

4.2. Preselin-1 mutated fibroblasts have higher ROS levels and they withstand external oxidative stress better than NHFs

Increased oxidative damage is a common feature in neurons and peripheral cells from both sporadic and familial AD cases (Cecchi et al., 2002). Here we confirm that peripheral cells from AD patients, specifically fibroblasts, have higher levels of endogenous ROS than age-matched NHFs (Fig 13A). Due to this, some oxidative damage to proteins and lipids is observable in AD fibroblasts (Fig.13B and 13C), even before exposure to external oxidative stress. Also, MnSOD was found to be up-regulated in these cells, while non-selenium glutathione peroxidise was down-regulated, which could explain higher intracellular H$_2$O$_2$ levels. AD
fibroblasts reach senescence earlier than NHFs, which might be due to higher endogenous ROS levels. In addition, these cells exhibit increased resistance to external oxidative stress when compared to their age matched NHF controls. It seems that increased ROS content and senescent phenotype in AD fibroblasts provide some protection against oxidative stress induced apoptosis.

We have previously reported that p21 over-expression in AD fibroblasts might lead to early senescence (Naderi et al., 2006). p21 is a well known cell cycle inhibitor responsible for arresting the cell in the G1 phase of cell cycle upon DNA damage (Waldman et al., 1995). It follows then that p21 over-expression is crucial for the onset of senescence, and early senescence in AD fibroblasts is a consequence of p21 over-expression. Untreated AD fibroblasts did not exhibit damage to the nucleus when stained with DAPI, however γH2AX labelling revealed a higher content of DNA double stranded breaks in AD fibroblasts compared to NHFs (Fig. 12 and Fig. 17). AD fibroblasts were found to have approximately twice as much endogenous H2O2 than age-matched NHFs, which resulted in oxidative damage to proteins and lipids and, according to γH2AX labelling, DNA damage as well. According to Itahana et al. (2001), the extent of DNA damage determines if a cell undergoes apoptosis or senescence through a common p53 pathway. DNA damage that is not severe enough to initiate programmed cell death will initiate senescence as a defence mechanism against carcinogenesis. Therefore, oxidative DNA damage in AD fibroblasts due to higher endogenous ROS seems to be a major event leading to premature senescence through the p21/p53 pathway.

However, even though ROS was substantially increased in these cells, mitochondria still retained a healthy mitochondrial membrane potential (MMP) as observed by JC-1 stain. The MMP of AD fibroblasts did not differ from NHFs’ MMP even though ROS levels in AD fibroblasts were twice as high as those in NHFs. This is probably due to over-expression of
MnSOD, a mitochondrial superoxide dismutase that converts superoxide radical into H$_2$O$_2$ (Dröge, 2002). MnSOD up-regulation could be keeping the mitochondria healthy by removing hazardous superoxide radical from mitochondria. However, by removing superoxide from mitochondria and converting it to H$_2$O$_2$, it contributed to an increase in cytosolic H$_2$O$_2$, and oxidative damage to cellular macromolecules. H$_2$O$_2$ levels were also higher due to down-regulation of non-selenium glutathione peroxidise, an enzyme responsible for converting H$_2$O$_2$ to water while glutathione is oxidised to glutathione disulfide (Dröge, 2002). This down-regulation of cellular anti-oxidant enzymes has been observed and is a well established phenomenon in all neurodegenerative disorders including AD. However, MnSOD over-expression in AD fibroblasts has not been reported before. This result successfully explains the healthy state of mitochondria even when overall cellular oxidative stress has increased, and other cytosolic anti-oxidant enzymes fail to neutralize excess ROS.

High internal ROS in AD fibroblasts might pre-condition them to withstand external oxidative stress insults, primarily via up-regulation of the anti-oxidant enzyme MnSOD. It has been previously reported that MnSOD up-regulation induced by Resveratrol leads to oxidative stress resistance in the human cell line (MRC-5) (Robb et al., 2008), so it is very plausible that MnSOD is one of the most important players in providing resistance to high level oxidative stress insults in AD fibroblasts. When NHFs are exposed to high exogenous oxidative stress (500μM H$_2$O$_2$), their mitochondria completely collapse, whereas AD fibroblasts manage to retain some healthy functioning mitochondria. This finding agrees with a lower rate of apoptosis in AD fibroblasts. AD fibroblasts seem to be more resistant to external oxidative stress, and MnSOD up-regulation might be the most important variable in neutralizing excess ROS thus keeping the mitochondria healthy.
Higher oxidative stress in peripheral tissues and neurons of Alzheimer’s patients has been well documented. Migliore et al. (2005) reported that there is an increase in DNA damage due to oxidized purines and pyrimidines in the peripheral cells of AD patients, as well as MCI (mild cognitive impairment) patients. Some studies suggest that oxidative stress in AD occurs early in disease onset, even before the development of amyloid plaques and neurofibrillary tangles (Nunomura et al., 2001). MCI is considered to be a beginning stage of AD, and increased oxidative damage in MCI patients confirms that increased oxidative stress is one of the earliest events in the onset of AD, and measuring ROS levels in peripheral cells might become a new diagnostic tool in early diagnosis of AD. We confirmed that ROS levels, as well as oxidative damage to lipids and proteins, are much higher in AD fibroblasts than in NHFs. Analysis of peripheral cells may therefore provide a valuable insight into onset and risk of development of AD.

4.3. Summary

In the first part of my project I reported a novel approach of inducing premature senescence in NHFs by treating them in quiescent state with a single high dose of external oxidative stress (500µM \( \text{H}_2\text{O}_2 \)). Quiescent NHFs were resistant to oxidative stress induced apoptosis in this case, but they developed senescence post-treatment. It seems that MnSOD expression in quiescent NHFs might be responsible for mitochondrial membrane potential retention, and therefore resistance to apoptosis. Due to increased expression of this anti-oxidant enzyme, quiescent NHFs are able to maintain healthy MMP, and endogenous ROS levels remain approximately the same following the treatment with high dose of external oxidative stress.

Second part of the project involved the study with PS-1 mutated fibroblasts. PS-1 mutation is highly correlated with early AD onset, and investigating the differences between
NHFss and AD fibroblasts with this mutation may provide a valuable insight into the mechanism of onset of early AD, as well as early diagnostics of it. I found that AD fibroblasts have higher endogenous ROS levels than age matched NHFs, as well as higher expression of MnSOD, providing us with a possible peripheral marker for AD.

4.4. Future Prospects

Even though I have investigated some differences in expression profiles of NHFs in dividing and quiescent states, as well as the differences in expression profiles of AD fibroblasts and NHFs, it is imperative to achieve complete protein expression signatures. This would lead us to the mechanism of premature senescence onset, as well as details of AD onset. In addition to protein expression profile, specific oxidatively modified proteins would need to be identified using oxy-blot, which would potentially reveal individual targets of ROS that might be involved in either senescence onset, or AD development. In both parts of the project, it is MnSOD that has the central spot in cellular anti-oxidant defence and mitochondrial survival, so siRNA studies involving knocking-out of MnSOD gene would help in either strengthening the hypothesis of MnSOD as the front-line defence against external ROS, or in potentially refuting our findings. If not MnSOD, then perhaps another protein is responsible for sensitizing quiescent NHFs and AD fibroblasts to ROS and their consequent resistance to external oxidative stress.
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VITA AUCTORIS

CURRICULUM VITAE

Danijela Domazet-Damjanov
University of Windsor
401 Sunset Avenue, Windsor, ON N9B 3P4
(519) 253-3000 ex. 3593
domazet@uwindsor.ca

Education

2006- Present
M. Sc. Candidate, Department of Chemistry & Biochemistry, University of Windsor, Windsor, Ontario, Canada
Thesis: Implications of oxidative stress on premature cellular senescence and development of Alzheimer’s disease

2002- 2006
B. Sc. (Hons) with thesis, Department of Chemistry & Biochemistry, University of Windsor, Windsor, Ontario, Canada
Thesis: Production of protein disulfide isomerase and thioredoxin columns for one step reduction of proteins and small peptides

Significant Academic Accomplishments

Refereed Publications In Preparation:

Somayajulu-Nitu, M., Domazet-Damjanov, D., Matei, A., Schwartzenger, E., Pandey, S. Role of environmental and inflammatory toxicity in neuronal cell death (Manuscript submitted to Open Toxicology Journal and accepted)


Domazet-Damjanov, D., and Pandey, S. Presenilin-1 mutated fibroblasts have higher ROS levels and they withstand external oxidative stress better than NHFs. Submitted to Aging Cell on July 15, 2008.
M. Sc. Thesis:

Honours Thesis:

Presentations and Conferences:

Honours and Awards

2007- Present OGS-ST, University of Windsor
November 2007 SFRBM (Society for Free Radical Biology and Medicine) Conference Travel Award, Washington DC
2006-2007 Graduate Tuition Scholarship, University of Windsor
2002-2006 Dean’s List, University of Windsor
2002-2006 President’s Renewable Entrance Award, University of Windsor
Summer 2004 NSERC-USRA
Summer 2003 NSERC-USRA
2001-2002 Governor General’s Academic Medal
- Awarded to the high school graduate with the highest average