Aging and Oxidative Stress

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Aging and the Accumulation of Oxidative Damage to DNA

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Summary The oxidative stress hypothesis of aging is currently one of the most popular explanations for the cause of aging. Oxidative stress, including that due to reactive oxygen species, causes lesions to biomolecules such as DNA, protein and lipids. 8-Oxo-2'-deoxyguanosine (8-oxodG) is a major oxidative product of DNA and can be detected using a variety of assay methods. For an accurate measurement of 8-oxodG, it is important to minimize as much as possible the oxidation of intact DNA during its isolation from cells or tissues and during the hydrolysis of DNA to nucleosides. The most popular analytical technique is high-performance liquid chromatography with electrochemical detection. The levels of oxidative damage to DNA are likely to increase in mammalian tissues during aging, although it is not clear whether the levels increase linearly during aging or exponentially in aged animals. The accumulation of oxidative damage to DNA is recognized as inducing mutations and dysfunction during aging. The steady state levels of oxidative damage to DNA are proposed to depend on the balance between the formation and repair of damage. The repair activity appears to be weak in aged animals compared with young animals, but antioxidative activity has not been shown to undergo a consistent changing pattern. Thus, it is assumed that the decline in repair activity in aged animals leads to the accumulation of oxidative damage in aged tissues.

Key Words: aging, oxidative stress, DNA damage, 8-oxo-2'-deoxyguanosine

Introduction

During the aging process, the functional capacities of a variety of physiological systems undergo progressive impairment after the maturational phase, decaying the ability to maintain homeostasis and resulting in death. Many hypotheses have been offered to explain the mechanism of the aging pro-

cess. The oxidative stress hypothesis of aging, which was first proposed as the free radical hypothesis of aging [1], is currently one of the most popular explanations for the cause of aging. In this hypothesis, the progressive and irreversible accumulation of molecular oxidative damage, induced by oxidative stress, is a main causal factor underlying the age-related losses of physiological functions. This hypothesis has been widely accepted because numerous studies have shown a correlation between increasing in age and the accumulation of oxidative damage to cellular biomolecules [2, 3]. Since not only free radicals but also non-radical species attack biomolecules oxidatively,

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“reactive oxygen species (ROS)” is a preferred term to characterize the oxidants involved in such biomolecular modifications. The most frequently generated ROS are the superoxide anion radical, hydrogen peroxide, and hydroxyl radicals, and the main biological targets of ROS are proteins, lipids, and DNA. Oxidative damage is observed as products derived from the oxidative modification of these biomolecules such as proteins and protein-associated carbonyls, advanced glycation end products (AGEs), lipid peroxidation products, lipofuscin, 8-oxo-2'-deoxyguanosine (8-oxodG), *etc.* ROS are formed continuously in cells during normal cellular metabolism and by exogenous agents such as carcinogenic compounds and ionizing radiation [4]. Thus, such damage is detectable under normal physiological conditions, even in healthy young animals. Although mammalian cells have numerous antioxidant defenses to limit the generation and attack of ROS, such as catalase, superoxide dismutase and glutathione peroxidase, ROS can escape these defense systems and injure biomolecules, suggesting that the efficiency of the antioxidative and repair mechanisms is not complete. The amount of oxidative damage to various biomolecules has been shown to increase during aging in a variety of tissues from different species [3]. Further-

more, it has been shown that certain types of pathological lesions arisen with age are associated with increased levels of oxidative damage to cellular biomolecules [5, 6]. Thus, the rate of ROS formation appears to be a determining factor in the aging process.

Oxidative DNA Damage

Oxidative damage to DNA is also implicated in mutagenesis and carcinogenesis as well as in aging [7, 8]. Numerous lesions have been characterized as arising from oxidative damage to DNA. Hydroxyl radicals cause many types of DNA damage including the oxidative modification of bases and sugars, strand breaks, and cross-linking to proteins [9, 10]. The structures of main modified DNA bases are shown in Fig. 1. The 8-oxodG lesion has been studied most extensively because it is the major oxidative lesion and because it can be detected relatively easily using a variety of assay methods [11]. 8-OxodG has been reported to be induced by the treatment of DNA with Fenton-type reagents [12], carcinogens [13] and X ray-irradiation [14]. These treatments are known to generate ROS during the reaction. Metal ions, such as iron and copper ions, both of which are

Fig. 1. Molecular structures of the main modified bases produced from the reaction of DNA with hydroxyl radicals.

Fig. 2. Mispairing of 8-oxodG in the *syn*-conformation (keto form) with adenine.

known to bind to DNA because of its overall negative charge, are believed to produce 8-oxodG through metal-catalyzed oxidation reactions. The C-8 hydroxyl group of 8-oxodG tautomerizes to the C-8 keto form (*syn*-conformation) in neutral aqueous solutions [15]. Since the keto form is more stable than the enol form (8-hydroxyguanine) [16], 8-oxodG adopts the *syn*-conformation. Because 8-oxodG pairs preferentially with adenine rather than cytosine, its presence can generate G→T transversions as shown in Fig. 2 [17, 18], which are likely to be involved in carcinogenesis [7, 8]. Indeed, 8-oxodG has been detected at higher levels in cancer cells [19, 20], and, further, the formation of 8-oxodG has been demonstrated in target organ DNA in a wide variety of *in vivo* experimental carcinogenesis models [21, 22].

Mitochondrial DNA (mtDNA) is not protected by nucleosomes, being different from nuclear DNA (nDNA), and it lies in close proximity to the free radical producing processes of oxidative phosphorylation. Therefore, mtDNA is thought to be highly prone to oxidative damage. Richter *et al.* first reported that the levels of 8-oxodG were 16-fold higher in mtDNA than nDNA in rat liver [23]. Subsequently, many investigators have reported significantly higher

levels of 8-oxodG in mtDNA than in nDNA [24–26]. However, Higuchi and Linn reported that the levels of 8-oxodG measured in mtDNA isolated by cesium chloride density gradient centrifugation were lower than those reported for nDNA [27]. Anson *et al.* measured the levels of oxidative damage in mtDNA from rat liver using an Fpg/EndoIII/southern blot assay and concluded that endogenous oxidative damage to mtDNA had been overestimated [28].

Measurement of Oxidative Damage to DNA

Because the levels of oxidative damage to DNA are low (on the order of fmole range per μg DNA), it is not easy to detect and quantify these lesions with accuracy. Techniques used to measure oxidative damage to DNA include high-performance liquid chromatography with electrochemical detection (HPLC/ECD), gas chromatography-mass spectroscopy (GC/MS), immunochemical techniques, post-labeling assays, and the comet assay for DNA break measurement, not for oxidative damage. The most frequently used technique is HPLC/ECD among these analytical techniques. By the HPLC/ECD following the mild enzymic hydrolysis of DNA, modified nucleosides such as 8-oxodG can be detected in a fmole range [29]. Because of the availability of this technique and of the mutagenic properties of 8-oxodG [17], most studies using HPLC/ECD have concentrated on this compound. On the other hand, GC/MS in combination with a selective-ion monitoring (SIM) technique can detect a large number of DNA lesions simultaneously. Recently, liquid chromatography/tandem mass spectrometry (LC/MS/MS) [30] and liquid chromatography/mass spectrometry (LC/MS) [31] have emerged as new techniques for the measurement of modified nucleosides in DNA.

It has become apparent that a significant amount of DNA oxidation can occur during the DNA isolation and sample preparation for assay. The oxidation of intact bases during DNA isolation and/or under subsequent procedures is recognized to depend on analytical techniques used for the measurement of DNA damage. Indeed, it has been shown that DNA is oxidized during isolation, and that precautions must be taken to prevent the oxidation of guanine [32]. The oxidation of DNA has been discussed extensively in recent reviews [33, 34]. Such oxidation has raised important questions about the accuracy of various assays used to measure *in vivo* levels of DNA

oxidation. For example, 8-oxodG levels obtained by GC/MS are considerably higher than those obtained using HPLC/EC [35]. Artifacts may occur during the derivatization of DNA acid-hydrolysates at high temperature prior to GC/MS analysis [36]. Guanine is the most prone to oxidation among DNA bases [37], and is oxidized to yield 8-oxoguanine (8-oxoG). Several modified bases, such as 5-hydroxycytosine, 8-hydroxyadenine, 5-hydroxymethyluracil, and 5-formyluracil, as well as 8-oxoG, have been reported to be formed during the derivatization procedures for GC/MS [38]. It has been suggested that in the previous works, the derivatization under such improper conditions as the insufficient removal of oxygen might have been made [35, 36, 39]. Because LC/MS/MS and LC/MS do not require sample derivatization for assay, the oxidation of DNA is suppressed [34]. On the other hand, in the case of HPLC/ECD, a significant amount of oxidative damage can be induced during the isolation of DNA, especially in the presence of phenol [40]. Phenol, a reducing agent, is thought to reduce metal ions (*e.g.*, iron ions) present in biological extracts. Reduced metal ions can participate in Haber-Weiss/Fenton reactions and generate hydroxyl radicals, which can oxidize DNA [41]. Because various tissues contain relatively large amounts of iron ions (2–5 nmol/mg protein), these reactions could play very important roles in generating oxidative damage during DNA isolation [42]. Nakae *et al.* reported very low 8-oxodG levels in nDNA isolated from rat liver using sodium iodide (NaI) [43]. In this method, NaI, a chaotropic salt, is used to precipitate proteins from DNA solutions, instead of phenol. With the NaI method, 8-oxodG levels are very low compared with those obtained by other methods, including the classic phenol method [25, 32]. Recent trials by the European Standards Committee for Oxidative DNA Damage (ESCODD) have shown that the levels of 8-oxoG or 8-oxodG in the same DNA samples measured in various laboratories are inconsistent [46–49]. The results strongly suggest that the measurement of 8-oxoG or 8-oxodG may depend on the laboratories and the techniques used.

Repair of DNA Damage

Oxidative damage to DNA can be repaired by cellular repair systems. The majority of non-bulky modifications, such as damage to DNA bases, are repaired by base-excision repair (BER) [48]. DNA

Fig. 3. Possible repair pathway of 8-oxoG in DNA. BER, base excision repair, TCR, transcription-coupled repair.

glycosylases recognize damaged DNA bases and remove the aberrant base by hydrolysis of the *N*-glycosil bond linking the base to the sugar. The resulting abasic site (AP site) is then recognized and cleaved by an AP endonuclease. Finally, a DNA polymerase fills the one-nucleotide gap, and the nick is sealed by DNA ligase. Each modified base is repaired by a specific DNA glycosylase. In the case of 8-oxoG as shown in Fig. 3, in humans, hOgg1 has been identified as a glycosylase with the ability to remove 8-oxoG that is paired opposite cytosine [49]. The mammalian enzyme MYH excises an adenine residue misincorporated opposite an 8-oxoG by mismatch repair [50]. Furthermore, 8-oxoG in DNA has been reported to be removed by transcription-coupled repair (TCR) [51]. When DNA containing 8-oxoG is replicated, DNA polymerase η can insert a cytosine residue efficiently and accurately across from the lesion [52]. Thus, the 8-oxodG lesion is constantly present at low levels in all cells, although 8-oxoG in DNA can be repaired by some repair enzymes as mentioned above.

Nucleotide excision repair (NER) appears to have a minor function in the repair of oxidative lesions [53]. Through NER of oxidative lesions, 8-oxoG is likely to result in the excision of a lesion-containing oligomer approximately 25 nucleotides in length. Further, an endonuclease has been reported to cleave the phosphodiester bonds 5' and 3' to the 8-oxodG lesion, generating a gap of one nucleotide and releasing 3',5'-8-oxodGDP [54].

Oxidative Damage to DNA and Aging

Fraga *et al.* have first observed a significant increase in 8-oxodG levels in nDNA isolated from the liver, kidney, and intestine of male rats 2 to 24 months of age during normal aging, but no increase

in brain and testes [55]. They suggested that 8-oxodG levels increase linearly with age. Sai *et al.* have also reported that the 8-oxodG levels in the liver and kidney DNA of both male and female rats increase significantly with age, but do not change in the brain, lung, and spleen of male rats, and increase moderately in the brain, lung, and spleen of female rats [56]. Furthermore, the levels of 8-oxodG in mtDNA isolated from male rat liver increased 2- to 3-fold with age [5]. Thereafter, a number of research groups have studied age-related changes in the level of 8-oxodG in both nDNA and mtDNA in a variety of animal tissues [24]. However, some groups have reported an inability to detect a significant increase in oxidative damage to DNA in rodent tissues with age [57, 58]. We tried to clarify whether or not the levels of 8-oxodG in nDNA increase, and whether 8-oxodG levels increase linearly during aging even if antioxidant defense systems comprising of antioxidant enzymes and low molecular weight antioxidants can act in cells. The levels of 8-oxodG in nDNA from male F344 rats 2 to 30 months of age were measured. As shown in Fig. 4, we observed a significant increase in 8-oxodG levels in nDNA in all tissues studied, *i.e.* liver, brain, kidney, and heart, at 24–30 months of age [59]. The age-related increase in 8-oxodG from 2 to 30 months of age ranged from 65% for brain, to 100% for heart, to 140% for liver and kidney. The age-associated changes in 8-oxodG levels in mammalian tissues exhibited a biphasic increasing pattern. That is, the steady-state levels of 8-oxodG increase slightly during young and middle ages, because the capacity of antioxidant defense systems could fully provide against daily oxidative stress. However, the defense capacity to maintain low 8-oxodG levels may not be retained in old age. Recently, Hamilton *et al.* conducted a comprehensive study of the effects of age on DNA oxidation and measured the levels of 8-oxodG in DNA isolated from the tissues of young, middle, and old age rodents [60]. They also observed a significant increase in 8-oxodG levels in nDNA in all tissues, brain, heart, liver, kidney, and muscle of all strains of old rodents studied.

The production rates of superoxide anion and hydrogen peroxide in mitochondria increase during aging in parallel with increases in biomolecular oxidative damage [3]. These relationships suggest that mitochondria play an important role in the aging process. Previous studies have reported an age-related increase in 8-oxodG levels in mtDNA isolated

Fig. 4. Changes in 8-oxodG levels of nuclear DNA from tissues of male Fischer 344 rats during aging. *Significantly different from 2, 6, 12, and 24 month-old rats at $p < 0.01$. **Significantly different from 2, 6, 12, 24, and 27 month-old rats at $p < 0.01$. ***Significantly different from 6 month-old rats at $p < 0.05$ and from 2 and 12 month-old rats at $p < 0.01$. ****Significantly different from 2 and 12 month-old rats at $p < 0.05$.

from the liver [5, 24], brain [61], and heart [62] of rodents. However, there is a report that 8-oxodG in mtDNA has been overestimated [28]. Hamilton *et al.* [60] used large amounts of liver tissues to increase the yield of mtDNA because the levels of 8-oxodG in DNA are apparently elevated in measurements of samples containing small amounts of mtDNA [63]. They observed that the levels of 8-oxodG in the mtDNA of the livers were 1.3- to 10-fold higher than those in the nDNA, and that the levels of 8-oxodG in mtDNA from the livers of old rodents were 1.5- to 2.3-fold higher than those observed in mtDNA from the livers of young rodents. The accumulation of DNA damage and mutations in the genome of old animals is predicted to lead to cellular dysfunction and consequent cell loss.

To understand the mechanism responsible for the age-related increase in 8-oxodG in tissues in more detail, we examined the time-dependent changes in 8-oxodG levels and the activities of 8-oxodG-releasing enzymes in the livers of young (6 months of age) and old (27 months of age) male F344 rats exposed to γ -irradiation [64]. Young and old rats were irradiated with 5 Gy of γ -rays, and 8-oxodG levels and the activities of 8-oxodG-releasing enzymes were measured at different time intervals after irradiation. The 8-oxodG levels in young and old rats increased immediately and significantly after irradiation as

Fig. 5. Changes in DNA polymerase α ($\circ$), β (Δ), and γ ($\square$) activities in the livers of male Fischer 344 rats 6 months (A) and 27 months (B) following γ -ray irradiation. DNA polymerase activities were estimated from the area of the peak from the counts of incorporated [^3H]dTMP in each fraction eluted from the phosphocellulose column.

Fig. 6. Changes in the 8-oxodG level of nuclear DNA in Fischer 344 rat liver after γ -ray irradiation. DNA was extracted from the livers of rats 6 months ($\bullet$) and 27 months ($\blacktriangle$) of age exposed to 5 Gy of γ -rays. *Significantly different from non-irradiated liver at $p < 0.001$. **Significantly different from non-irradiated liver at $p < 0.01$. ***Significantly different from non-irradiated liver at $p < 0.05$.

shown in Fig. 5 (1.7- and 2.7-fold, respectively). The level of 8-oxodG in the nDNA of livers of young rats decreased smoothly within a half-life of 6 h and returned to the basal level by 24 h, while the level in old rats decreased with a half-time of 17 h and did not return to the basal level even 1 week after irradiation. It has been observed that the γ -irradiation-induced increase in 8-oxodG levels in nDNA of livers of young rodents returned rapidly to the basal level [25, 65, 66]. The activity of 8-oxodG-releasing enzymes in the livers of young rats was found to increase 7-fold 2 h after γ -irradiation and to return

to steady-state levels at 24 h after irradiation, whereas the activity in old rats reached 2-fold 6 h after irradiation and returned to the steady-state level at 72 h after irradiation. Furthermore, DNA polymerase activities were examined in the livers of young and old rats exposed to γ -irradiation [67]. DNA polymerases β and γ were induced from 2 to 6 h after irradiation in both young and old rats. However, the extent of enzyme induction was less in old rats than in young rats (Fig. 6). These results suggest that the ability to repair damaged DNA is lower in aged rats, and that the decline in the repair activity of aged rats is likely to lead to the age-dependent accumulation of oxidative damage and DNA mutations in tissues. The tissues of old animals appear to be prone to the generation of oxidative DNA damage when exposed to oxidative stress, e.g., γ -irradiation. For example, Zahn *et al.* have reported that DNA strand breaks were induced twice as numerous in tissues from old mice compared with tissues from young mice, when tissues, such as liver or heart, were incubated with nitroquinolin-*N*-oxide *in vitro* [68]. Guo *et al.* have found that after UV-irradiation, more cyclobutane pyrimidine dimers in nDNA are induced by UV-irradiation in primary cultures of hepatocytes isolated from old rats than from young rats [69].

Effect of Caloric Restriction

Caloric (dietary) restriction is the only effective manipulation known to extend the life span of rodents and to decrease the incidence of various age-related diseases [3, 70]. If the increase in oxidative damage to DNA with age is important in the aging process, the increase should be retarded and/or reduced by dietary restriction. Several reports have indicated that caloric restriction reduces the level of DNA oxidation in rodent tissues [71, 72]. We examined whether or not dietary restriction alters the age-related accumulation of 8-oxodG in nDNA from tissues of male F344 rats [73]. Under dietary restriction, they were fed 3 days a week (Monday, Wednesday, and Friday) from 4 weeks of age to 33 months of age. The 50% survival age of the dietary-restricted rats was 35.2 months, while that of *ad libitum*-fed rats was 28.2 months. There were no differences between the dietary-restricted and *ad libitum*-fed rats in the 8-oxodG levels of any organ until 24 months of age. However, the levels at 30 months of age were significantly lower in the dietary-restricted rats than

Fig. 7. Comparison of age-related changes in 8-oxodG content of nuclear DNA from tissues of *ad libitum*-fed and dietary-restricted male Fischer 344 rats. *Ad libitum*-fed rats (○), dietary-restricted rats (●). *Significantly different from *ad libitum*-fed rats at $p < 0.05$.

in *ad libitum*-fed rats (Fig. 7). These results indicate that dietary restriction can retard the onset of the age-related accumulation of oxidative damage in tissues. Recently, it was observed that the 8-oxodG levels in the nDNA from the brain, heart, and skeletal muscle of old caloric-restricted rats were significantly decreased compared with old *ad libitum*-fed rats; however, dietary restriction did not decrease the 8-oxodG levels in nDNA from rat liver and kidney [60]. Thus, dietary restriction is not likely always to reduce oxidative damage to nDNA in every tissue and every time. It has also been reported that caloric restriction completely prevents the age-related increase in 8-oxodG levels in liver mtDNA in male F344 rats [60]. Caloric restriction may have a greater effect on the age-related accumulation of oxidative damage in mtDNA than in nDNA.

Conclusion

Mammalian cells have evolved multiple lines of defense, which include antioxidant enzymes, antioxidants, and DNA repair enzymes, against the accumulation of oxidative damage. The steady state level of modified DNA should be determined by the balance between its formation and removal. The idea that aging may be due to a decrease in antioxidant capacity in old age is not strongly supported, because it has been observed that antioxidant capacity does not change according to a consistent pattern as a

function of age; that is, it decreases, increases, or does not change in old animals, depending on the type of antioxidants, tissues, or animal strains. Indeed, Hamilton *et al.* have found no changes in the activities of Mn-SOD, CuZn-SOD, catalase, and glutathione peroxidase between tissues from young and old mice [60]. In addition, antioxidative defenses are not clearly affected by environmental manipulations that increased the longevity of experimental animals, such as caloric restriction in mice. ROS may be produced at specific sites and attack nearby sensitive targets, to which access by antioxidants may be difficult.

The accumulation of oxidative damage to DNA may be a result of deficiencies in DNA damage repair. Indeed, it has been found that the ability to repair damaged DNA is lower in aged rats than in young rats [64, 67]. Furthermore, it has also been observed that 8-oxodG-incision activity in mouse liver increases with age in mitochondria but not in nuclei [74]. These results support the above-mentioned concept that the decline in repair activity in aged animals leads to the accumulation of oxidative damage in their tissues. Furthermore, the difference between the antioxidant abilities of young and old animals may not be clear under normal conditions. The redundancy of antioxidant activities may decrease in old animals and hence their antioxidants defense may be insufficient to prevent oxidative damage under stressful conditions.

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