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Review

Oxidative damage to DNA and antioxidant status in aging and age-related diseases★

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Aging is a complex process involving morphologic and biochemical changes in single cells and in the whole organism. One of the most popular explanations of how aging occurs at the molecular level is the oxidative stress hypothesis. Oxidative stress leads in many cases to an age-dependent increase in the cellular level of oxidatively modified macromolecules including DNA, and it is this increase which has been linked to various pathological conditions, such as aging, carcinogenesis, neurodegenerative and cardiovascular diseases. It is, however, possible that a number of short-comings associated with gaps in our knowledge may be responsible for the failure to produce definite results when applied to understanding the role of DNA damage in aging and age-related diseases.

Keywords: oxidative DNA damage, aging, age-related diseases, BER, GO system, ROS

INTRODUCTION

The oxidative stress hypothesis of aging (or free radical hypothesis) is one of the most popular explanations of how aging occurs at the molecular level. Harman with his "free radical theory of aging" (Harman, 1956) was the first to propose that the age-related decline is due to an accumulation of damage to cellular macromolecules by the by-products of oxidative respiration, namely reactive oxygen species (ROS). ROS can be produced both during the biochemical utilization (e.g. there are more than 60 enzymatic reactions that utilize O_2 as a substrate where ROS may be formed, such as oxidase-catalyzed reactions, autooxidation of reduced forms of NAD^+/NADP-dehydrogenases, macrophage-catalyzed reactions and cytochrom P450 proteins, to name a few (Vanderkooi *et al.*, 1991) and as a by-product of O_2 metabolism in mitochondria. Oxidative stress may occur as a result of increased production of ROS or by deterioration of antioxidant mechanisms, or both. Whatever the reason, oxidative stress leads in many cases to an age-dependent increase in the cellular level of oxidatively modified macromolecules, including DNA (Sohal, 2002). Damage to DNA is particularly harmful since it may be fixed into mutation, if not repaired in a proper time, and passed onto daughter cells. Moreover, unlike proteins and lipids, entire new molecules of DNA cannot be synthesized to replace those containing damage. Although background levels of oxidatively damaged DNA always exist, oxidative stress can lead to an

★R. Olinski dedicates this work to the memory of Professor M. Gniazdowski and Dr. E. Ciesielska from Department of Molecular Pharmacology, Lodz Medical University, Poland.

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Abbreviations: BER, base excision repair; dG, 2′-deoxyguanosine; GC/MS, gas chromatography with isotope dilution mass spectrometric detection; Gua, guanine; hMTH1, human MutT homologue 1; hOGG1, human 8-oxo-7, 8-dihydroguanine glycosylase; HPLC, high performance liquid chromatography; MLSP, maximum life span; NER, nucleotide excision repair; ODD, oxidative DNA damage; ROS, reactive oxygen species; SMR, specific metabolic rate; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; 8-oxoGua, 8-oxo-7,8-dihydroguanine.

increase in the damage, and it is this increase which has been linked to various pathological conditions, such as aging, carcinogenesis, neurodegenerative and cardiovascular disease (reviewed by Cooke *et al.*, 2006).

This review presents recent advances from our laboratories, along with other data that suggest an involvement of oxidative DNA damage in the development and/or progression of aging and the aging-related diseases.

AGING

Age-related changes in oxidative DNA damage in humans

A number of research groups have reported on the effect of aging on DNA oxidation in animal models (Fraga *et al.*, 1990; Hamilton *et al.*, 2001b). However, a summary of these kinds of studies shows no clear effect. It is difficult to explain why in some studies age-related increase in oxidative DNA damage was observed (Hudson *et al.*, 1998), whereas in others no effect was seen (Anson *et al.*, 1999). It is possible that one reason for the discrepancies may be the reliability of the biomarkers used. Moreover, to date no comprehensive studies concerning age-related oxidative DNA damage in humans have been conducted.

Therefore, the purpose of our recently published work (Siomek *et al.*, 2007) was to assess the age-related changes in oxidative DNA damage in humans. For the first time, a broad spectrum of oxidative DNA damage biomarkers was analysed; urinary excretion of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) and 8-oxo-7,8-dihydroguanine (8-oxoGua) as well as the level of oxidative DNA damage in leukocytes.

Antioxidant vitamins (A, C and E) and uric acid are effective free radical scavengers, therefore they should protect biomolecules such as DNA. In addition to the aforementioned analyses the concentration of the antioxidant vitamins A, C, E and uric acid was determined in blood serum.

There was a highly significant rise in the background level of 8-oxodG in leukocyte DNA in the elderly (group D; mean age 67 years) and middle age group (group C; mean age 50 years) in comparison with adults (group B; mean age 31years) (Fig. 1) and a statistically significant, positive correlation between age and 8-oxodG levels in leukocytes' DNA. However, a steady increase of 8-oxodG levels in DNA isolated from leukocytes with age was seen only when the youngest group was excluded (Fig. 2), since the level was significantly elevated in

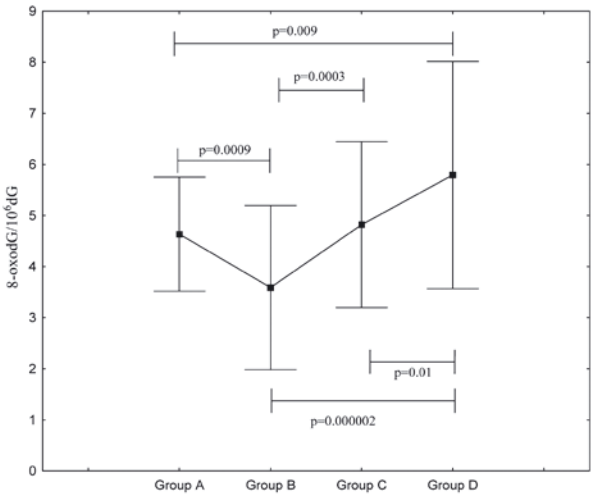

Figure 1. Mean level of 8-oxodG in leukocyte DNA in different age groups.
Group A–D mean age 13, 31, 50, 67 years. Data from (Siomek *et al.*, 2007) with permission from Mary Ann Liebert Inc., Publisher.

the group of youngest subjects (group A, mean age 13 years) when compared with the group representing "adults" (group B). It is likely that the unexpected high level of oxidative DNA damage in group A may reflect the higher metabolic rate of children. Children, who grow fast, have a higher metabolic rate than adults. The high metabolic rate, in turn, requires a high level of mitochondrial respiration and subsequent elevated production of ROS, which are responsible for the formation of the DNA modifications analyzed in our work. Indeed, in our previous study highly significant, positive correlations between specific metabolic rates and urinary excretion rates for 8-oxodG and 8-oxoGua were found (Foksinski *et al.*, 2004) (see also below — chapter: Why do different mammalian species age at different rates?).

The obvious question is, why does oxidative DNA damage increase with age? The background

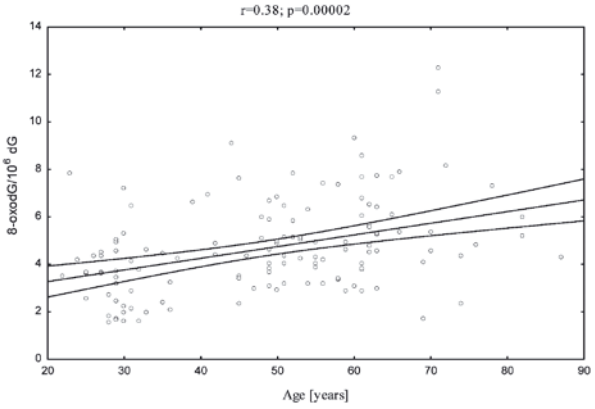

Figure 2. Correlation between the level of 8-oxodG in leukocyte DNA and age with exclusion of the youngest group of mean age 13 years.
Data as in Fig. 1.

level of 8-oxoGua in cellular DNA represents a dynamic equilibrium between the rate of oxidative DNA damage formation and the rate of repair of the damage. Therefore, the observed age-related increase may be a result of a deficiency in the ability of the cells from older subjects to remove the damage or it may mirror an intensification of processes responsible for the damage formation, or both.

An age-related decrease in DNA repair capacity has been demonstrated mostly for nucleotide excision repair (NER) (Hart & Setlow, 1974; Goukassian *et al.*, 2000). However, base excision repair (BER) is primarily responsible for the removal of oxidative DNA base damage, and age-dependent reduction of hOGG1, the major enzyme involved in the removal of 8-oxoguanine, was also reported (Chen *et al.*, 2003).

Urinary excretion rate, especially that measuring the level of 8-oxoGua, is the most sensitive marker of the average oxidative stress to DNA of all body cells (Shigenaga *et al.*, 1989; Cooke *et al.*, 2000). Therefore, besides analyses of the background level of 8-oxodG in leukocytes' DNA also urinary excretion of the modified base and nucleosides was determined. Since both parameters showed a similar age-related pattern it is likely that their changes reflected, at least in part, an age-dependent intensification of oxidative stress which resulted in DNA damage. However, since urinary excretion rates may also represent repair processes (see Cooke *et al.*, 2005), we cannot entirely exclude the possibility that the observed changes, in age-dependent urinary excretion rates less distinct than these of the background level of 8-oxodG in DNA (compare Fig. 1 and Fig. 3A, 3B), may also reflect some deterioration of the repair mechanism(s).

Impaired mitochondrial function is a factor which may be responsible for increased ROS production and therefore predispose to oxidative stress and DNA damage in the aged subjects. Indeed, several studies of the mitochondrial respiratory chain function in humans and animals have demonstrated an age-related decrease in respiration and an increased production of ROS during aging (Takasawa *et al.*, 1993; Martin & Loeb, 2004). Another support for the age-related decline in mitochondrial function is provided by the demonstration that the amount of COX-deficient muscle fibers increases in healthy aging humans (Kopsidas *et al.*, 1998).

Another source of age-related increase of oxidative stress may be a decline of antioxidant defense, and an age-dependent decline in the concentration of vitamin C in plasma was also observed (Fig. 4). Vitamin C is a major aqueous-phase antioxidant. It should also be remembered that vitamin C acts in synergy with tocopherol by regenerating tocopheroxyl radical to tocopherol. One of the plausible explanations of the changes in vitamin concentrations is the sequential consumption of these antioxidants as a result of age-dependent intensification of oxidative stress. It has been shown that during free radical-mediated oxidation a decrease in vitamin E concentration in plasma can only be seen after the complete consumption of vitamin C. The sequential consumption of these antioxidants was also shown by the use of electron spin resonance (ESR) spectroscopy (Sharma & Buettner, 1993).

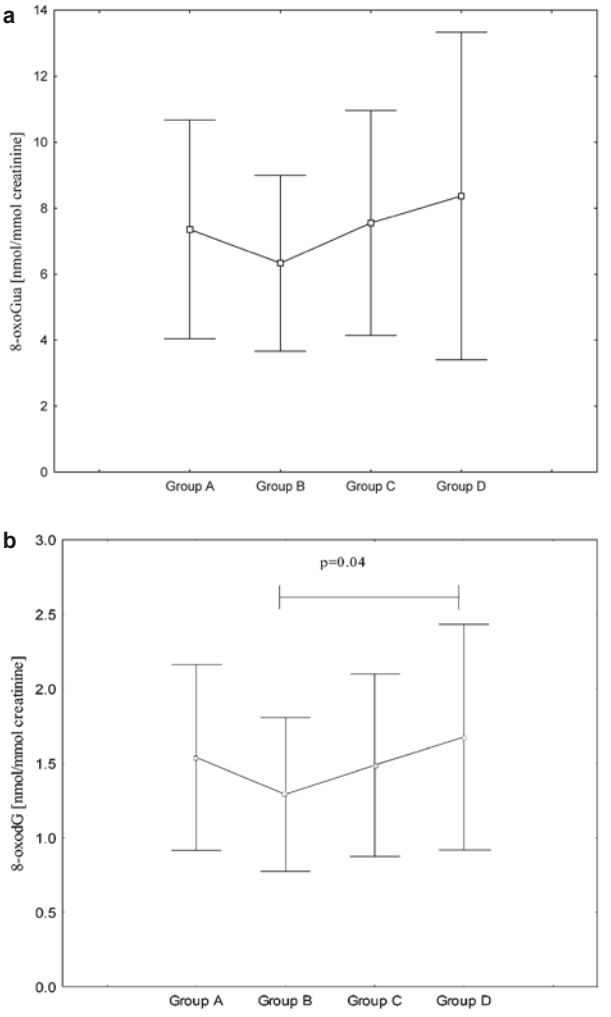

Figure 3. Mean levels of 8-oxoGua (a) and 8-oxodG (b) in urine in different age groups.
Group A–D mean age 13, 31, 50, 67 years. Data as in Fig. 1.

As can be seen in Fig. 1 the "adult" group B exhibits the lowest values of oxidative DNA damage in leukocytes. Evolution theory assumes that organisms are not programmed to age, instead evolution selects for survival and reproduction (Kirkwood & Austad, 2000). Therefore, it is possible that the lowest values of this harmful, potentially mutagenic, oxidative DNA modification in the aforementioned group may constitute a proof of "a specific concern" of evolution for humans of reproductive age. Indi-

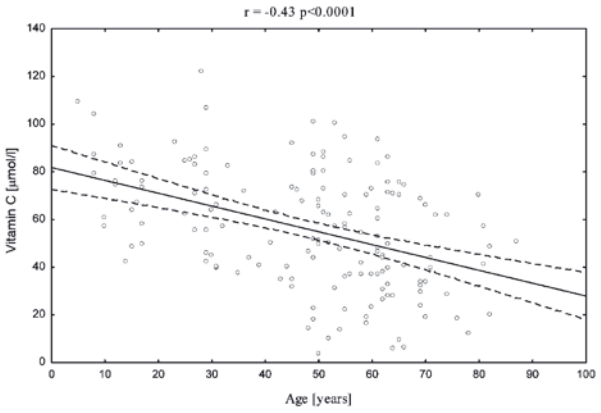

Figure 4. Relationship between the level of vitamin C and age in all studied subjects.
Data as in Fig. 1.

viduals differ greatly in their rate of aging. There are also quite substantial inter-individual differences in the level of 8-oxodG in DNA (Fig. 2). These differences can also be seen within the "adult" group, with a subgroup where the values are around 2 modifications per 10^6 unmodified bases and a second subpopulation where the values are much higher than the mean level (Fig. 2). It has been postulated that different factors which may influence the genome in adult life may influence the rate of subsequent functional decline of the organism (McCarroll *et al.*, 2004). Therefore, it is possible that one of these factors is oxidative DNA damage with genome destabilizing properties.

Why do different mammalian species age at different rates?

One of the intriguing issues concerning aging is why different mammalian species age at different rates. One hypothesis that has attempted to explain these differences is once again the free radical theory of aging (Harman, 1956; Sohal *et al.*, 2002). All aerobic organisms utilize oxygen, which is linked to the production of reactive oxygen species (ROS). The above-mentioned differences may be explained, at least partially, by different metabolic rates that in turn are connected with oxygen consumption and ATP production during oxidative phosphorylation. The more ATP is required, the more oxygen must pass through the mitochondria and the more oxygen radicals are likely to be generated. The more oxygen radicals are produced the greater will be the damage to cellular components, including DNA. Oxidative DNA damage is removed *via* different repair pathways. Following excision from DNA, the oxidatively induced lesions are released into the blood stream and consequently into the urine, where their measurement has been shown to be reflective of the overall oxidative stress (Loft & Poulsen, 1998).

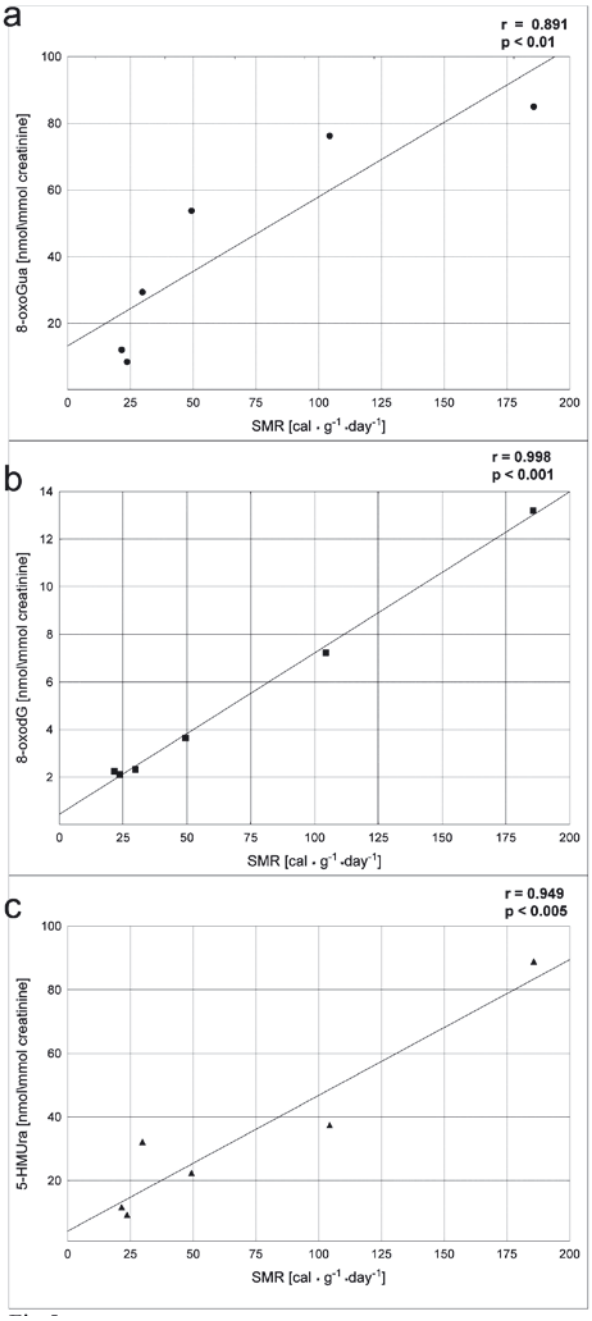

Figure 5. Relationship between the urinary excretion rates of the analyzed modifications and specific metabolic rates (SMR) of six different mammalian species: mice, rats, rabbits, dogs, pigs and humans.
Data from (Foksinski *et al.*, 2004) with permission from Elsevier.

In our study we decided to analyze urinary excretion of possible repair products of oxidative DNA damage: 8-oxoGua, 8-oxodG and 5-(hydroxymethyl)uracil (5-HMUra), in mammalian species that substantially differ in the metabolic rates and longevity, namely mice, rats, rabbits, dogs, pigs and humans (Foksinski *et al.*, 2004).

The analyzed excretion rates should depend on oxygen consumption and the metabolic rate. The

metabolic rate, may be, in turn described by specific metabolic rate (SMR) values (Kleiber, 1961; Sohal *et al.*, 2002). In agreement with these assumptions we have found good positive correlations between the SMRs of different species and their excretion rates of all analyzed modifications (Fig. 5).

Since the metabolic rate may be associated with maximum life span (MLSP) (Sohal *et al.*, 2002) we also determined whether there is some relationship between the excretion rates of all analyzed modifications and the life span. It was found that only 8-oxoGua excretion rate significantly correlates with MLSP. 8-OxodG and 5-HMUra were inversely correlated with MLSP, but these relationships were not statistically significant (Fig. 6). This in turn suggests that urinary excretion of 8-oxoGua reflects oxidative DNA damage better than the other two modifications. Likewise, in the case of cancer patients only urinary 8-oxoGua reflects oxidative stress associated with the disease (Rozalski *et al.*, 2002; Gackowski *et al.*, 2003).

The correlation of the excretion rate of 8-oxoGua with the MLSP, found in our work, is in good agreement with previous studies which have demonstrated that oxidative damage to DNA is inversely related to MLSP of different mammals. However, in those studies no humans were included and the assessment of DNA damage was restricted to certain organs (Cutler, 1991a; 1991b; Barja & Herrero, 2000). In contrast, the urinary base/nucleoside products analyzed in our work are reflective of oxidative DNA damage on the level of the whole organism. Our results demonstrated that ROS continually damage DNA and that, in normal conditions *in vivo*, this damage is lower in long-lived species than in short-lived ones. Incomplete repair of such damage would lead to its accumulation over time and eventually result in age-related deterioration.

Expression of the urinary excretion rates in nmol/kg per 24 h enables measurement of the number of the repaired lesions per day per cell (Helbock *et al.*, 1998). Interestingly, the urinary level of all measured modifications found in our study accounted for about 28200 repaired events per average cell of the mouse per day and fit well the estimation of Hamilton and co-workers (2001a) who calculated that the DNA of the liver cell in mouse is exposed to about 47000 8-oxoGua lesions in a 24 h period (taking into consideration that the liver is a high metabolic rate organ and that our values are an average for the whole organism). In contrast, the number of all lesions analyzed in our work, in humans accounts for about 2800 repair events in the average cell per day. It is therefore possible that the high metabolic rate of the mouse (or other short-lived animals) may be responsible for the severe everyday oxidative DNA insult that may be accumulated faster than in long-lived species. It is also noteworthy that the difference in urinary excretion of 8-oxoGua between mice and humans (10 fold) is very similar to the difference in the reported oxygen consumption between these species (11 fold) (Shigenaga *et al.*, 1989).

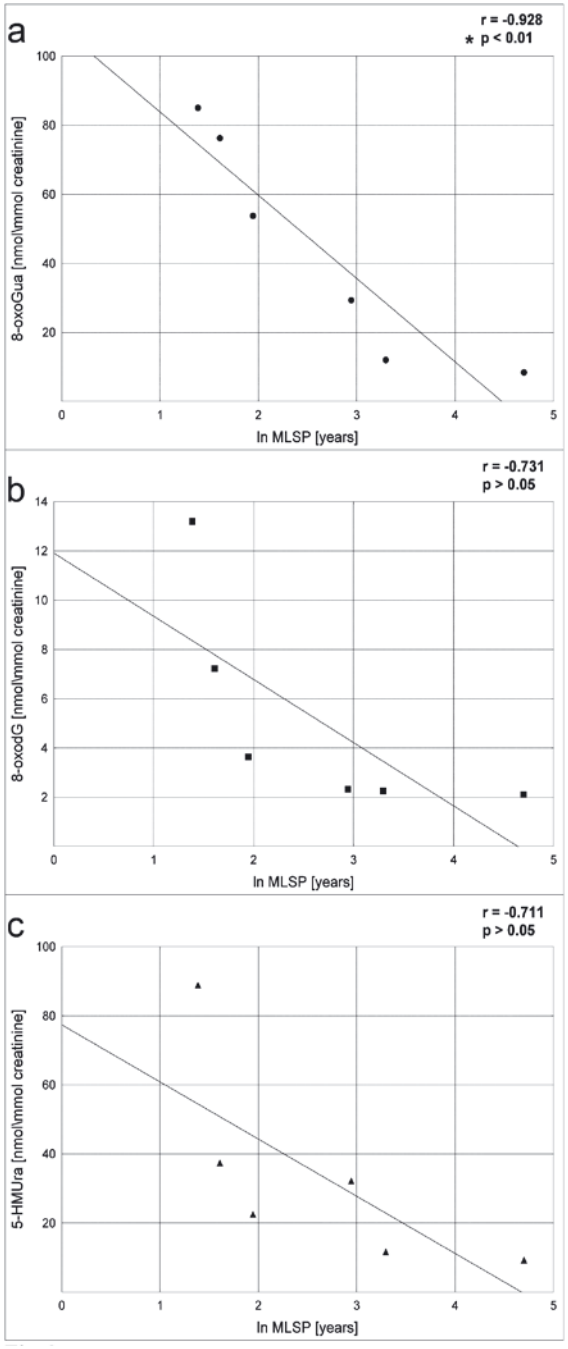

Figure 6. Relationship between urinary excretion rates of the analyzed modifications and natural logarithm of maximum life span (MLSP) of six different mammalian species: mice, rats, rabbits, dogs, pigs and humans.
Data as in Fig. 5.

To conclude, on the basis of the results presented above showing that urinary excretion of 8-oxoGua and the other modifications are higher in

rapidly aging mammalian species and the presented correlative association between oxidative DNA damage parameters and age in humans it seems reasonable that this damage may be one of the substantial factors in mammalian (including human) aging.

ROS/OXIDATIVE DNA DAMAGE AND AGE-RELATED DISEASES

Cancer

There is a dramatic age-dependent escalation in cancer risk and sequential accumulation of somatic mutations over a life time may be responsible for this phenomenon.

Cancer is a disease of genes. In view of the importance of DNA damage in carcinogenesis, it is conceivable that any agent capable of reacting with DNA and chemically modifying it could be carcinogenic. It is very likely that reactive oxygen species (ROS) have mutagenic/carcinogenic potential. An increase in somatic mutations, which are the main factor responsible for cancer development, has been documented in aged cells and tissues (DePinho, 2000). This accumulation of mutations presumably relates to cumulative lifetime exposure to endogenous and exogenous DNA damaging agents. Although many carcinogens can be found in food and drink, generated during their processing (e.g. cooking of meet) or as a result of the activities of the chemical industry, it is likely that the most important human carcinogens may be metabolites of atmospheric oxygen (ROS). Free radical attack upon DNA generates a whole series of DNA damage, among them modified DNA bases. Hydroxyl radical causes the formation of a large number of pyrimidine- and purine-derived lesions in DNA (reviewed in Dizdaroglu, 1992). Some of these modified DNA bases have considerable potential to damage the integrity of the genome (reviewed in Floyd, 1990; Jackson & Loeb, 2001). 8-oxoGua is one of the most widely studied lesions. The presence of 8-oxoGua residues in DNA leads to GC→TA transversions unless repaired prior to DNA replication (Cheng *et al.*, 1992). Therefore, the presence of 8-oxoGua in cells may lead to point mutations.

Many observations indicate a direct correlation between the formation of 8-oxoGua and other oxidative stress-derived DNA lesions and carcinogenesis *in vivo* (Floyd, 1990; Feig *et al.*, 1994). Oxygen-derived radicals are known to induce mutagenesis in hot spot codons of the human *TP53* and *Haras* genes (Du *et al.*, 1994; Yu *et al.*, 2002). Also a lipid peroxidation product, 4-hydroxy-nonenal, tends to react selectively with a guanine residue in *TP53*

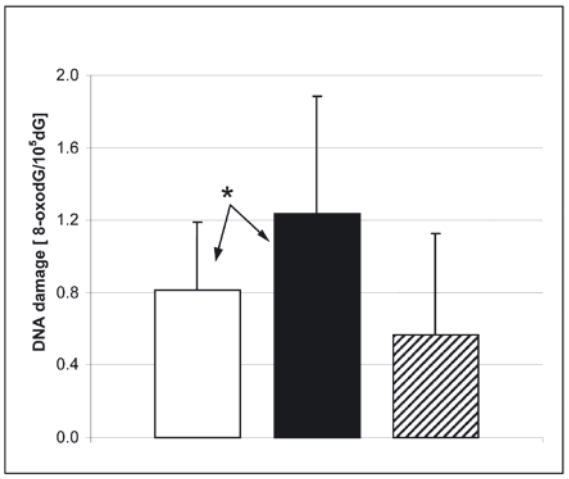

Figure 7. Level of 8-oxodG in DNA samples isolated from:
□, control tissues; ■, myoma tissues and ⊠, lymphocytes of myoma patients. Statistically significant differences ($P<0.01$) between the isolates are depicted by star. Data from Foksinski *et al.*, 2000, with permission from Elsevier.

codon 249 sequence GAGG*C/A (Hu *et al.*, 2002), which is the mutation hot spot in hepatocellular carcinoma, and other types of human cancer.

Our recent investigations of benign tumors showed that oxidative DNA damage may be a causative factor in cancer development. A higher endogenous level of 8-oxodG in uterine myoma tissues was observed when compared to their respective tumor-free tissues (Fig. 7) (Foksinski *et al.*, 2000). Uterine myomas are among the most common gynaecological tumors. They are monoclonal, benign tumors derived from a single mutated myometrial cell. One of the factors that may predispose toward malignant transformation is a greater size of the tumor (Schwartz *et al.*, 1993). The positive correlation found in our work between the size of the tumor and the amount of 8-oxodG (Fig. 8) (Foksinski *et al.*, 2000) suggests that the higher level of 8-oxodG in benign tumors may be a risk factor that may determine the transformation of benign tumors to malignant ones. Conversely, the increased levels of modified DNA bases may contribute to the genetic instability and metastatic potential of tumor cells in fully developed cancer. Indeed, it has been demonstrated that severe oxidative stress is associated with several types of adenocarcinomas (Schmielau & Finn, 2001).

Since the level of modified nucleosides/bases in urine may be an indicator of the oxidative insult on DNA and a general marker of oxidative stress, we investigated whether the amount of 8-oxoGua and 8-oxodG excreted into urine was higher in lung and breast cancer patients than in a control group. It was found that the amount of the modified base (but not the nucleoside) excreted into urine was approximately 50% higher in cancer patients than in

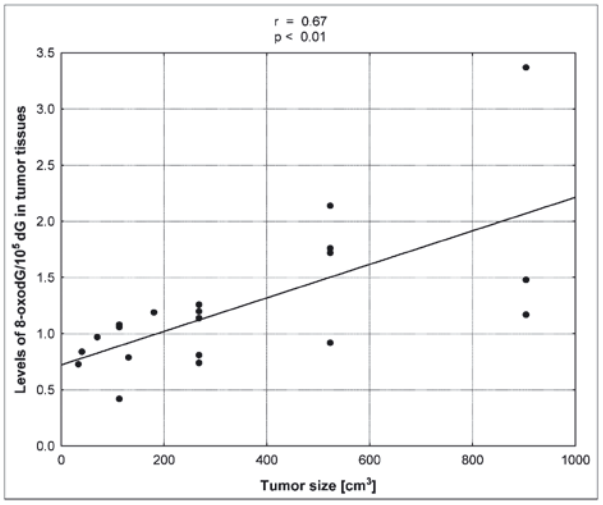

Figure 8. Correlation coefficient between the level of 8-oxodG in tumor tissues and the uterine myoma tumor size.
Data from (Foksinski *et al.,* 2000) with permission from Elsevier.

the control group (Fig. 9) (Rozalski *et al.,* 2002). Since the modified base present in urine may represent the primary repair product of oxidative DNA damage *in vivo*, our results suggest an important role of DNA glycosylases in carcinogenesis.

Elevated levels of oxidative DNA damage may arise as a consequence of (i) an environment in the tumors low in antioxidant enzymes and high in ROS (Toyokuni *et al.*, 1995) or (ii) reduced DNA repair.

Increased ROS generation

It has been reported that at least some tumor cell lines can produce significant levels of H_2O_2 without exogenous stimulation, perhaps accounting for the elevated levels of oxidative DNA damage seen. It appears that the presence of the labile iron pool (LIP) in cells, which comprises low molecular weight iron, together with H_2O_2 can result in the production of ROS. In a study of Gackowski *et al.* (2002) a significant correlation was determined between LIP and the endogenous level of 8-oxodG in human lymphocytes (r = 0.57, p = 0.000066). This in turn highlighted the possibility that under physiological conditions a LIP is available for catalysing Fenton-type reactions in close proximity to cellular DNA. There are also experimental data which demonstrate the existence of a free iron pool in the sera of patients with haemochromatosis (de Valk *et al.*, 2000). This disease predisposes to cancer, and there are growing epidemiological data to suggest that elevation of the body iron level may increase the risk of cancer (Nelson *et al.*, 1994; Stevens *et al.*, 1994). Results from our laboratory (Gackowski *et al.*, 2002) suggest a mechanism that may directly link iron overload with carcinogenesis. Specifically, iron overload may favour the persistence of harmful LIP, which may catalyse the generation of potentially carcinogenic oxidative DNA damage (ODD) in cellular DNA. As a result of elevated ROS, some transcription factors and their target genes, may be permanently activated which, coupled with the increased DNA damage, creates a selection pressure for the malignant phenotype seen in cancer.

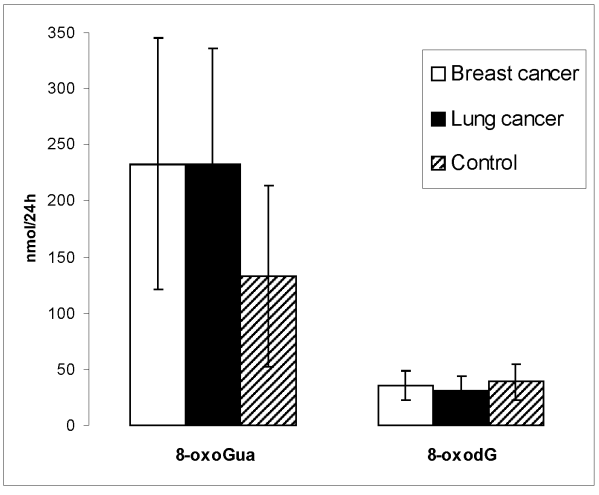

Figure 9. Urinary excretion of 8-oxoGua and 8-oxodG in cancer patients and in control group.
Control group (n=33), lung cancer patients (n=19), breast cancer patients (n=14). Data from (Olinski *et al.,* 2002) with permission from Elsevier.

Alterations in DNA repair

To combat the deleterious biological effects of the presence of 8-oxodG, cells have developed specific mechanisms to remove this lesion from DNA. From bacteria to mammals three enzymes form "GO" systems that are involved in protecting cellular DNA from the mutagenic effect of 8-oxodG (Michaels & Miller, 1992). The first level of this protection is MTH, a protein with pyrophosphohydrolase (8-oxodGTPase) activity. This enzyme is responsible for hydrolysis of 8-oxodGTP, thereby eliminating it from the nucleotide pool and preventing its incorporation into DNA by DNA polymerases (Fig. 10). The second level of defense is/are specific DNA-glycosylase(s) that initiate the base excision repair pathway (BER). The major 8-oxoGua glycosylase in *E. coli* is the Fpg protein (MutM), and more recently endonuclease VIII (Nei), originally discovered as a damaged-pyrimidine-specific DNA glycosylase, was also found to have this activity (Hazra *et al.*, 2000). MutM preferentially excises 8-oxoGua when it is paired with C, T or G, while the Nei glycosylase is more specific to the 8-oxoGua:A mispair.

In humans two 8-oxoGua glycosylases have been identified, OGG1, a functional but not structural analog of the Fpg protein specific to the 8-ox-

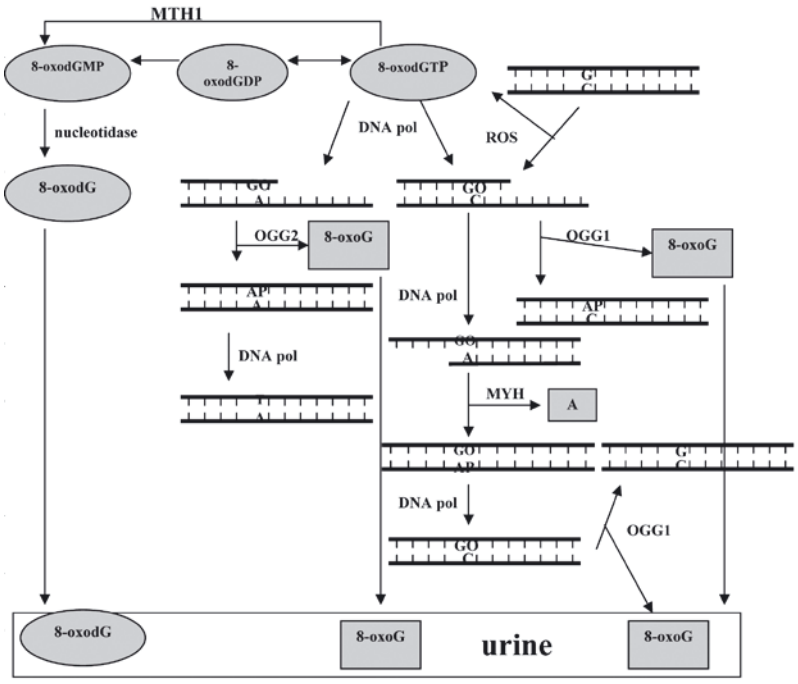

Figure 10. Involvement of different repair pathways (enzymes) in oxidative DNA damage removal.

oGua:C mispair (Radicella *et al.*, 1997) and OGG2, specific to 8-oxoGua mispaired with G or A (Hazra *et al.*, 1998). It was postulated that the OGG1 type glycosylase may be a housekeeping enzyme which removes 8-oxoGua from DNA of non-dividing cells whereas OGG2 may be more specific for the repair of 8-oxoGua in nascent or transcriptionally active DNA (Hazra *et al.*, 2001). Two Nei-like human glycosylases, NEIL1 and NEIL2, specific to oxidized pyrimidines but excising also 8-oxoGua, have been characterized. These glycosylases exhibit an unusual ability to excise oxidized bases both from ds and ssDNA (Hazra *et al.*, 2002; Dou *et al.*, 2003). However, the major 8-oxoGua DNA glycosylase in human cells is probably OGG1.

Finally, the third level is realized by MYH proteins that remove adenine mispaired with 8-oxoGua (Fig. 10).

There are numerous experimental data which suggest that a decreased activity of the enzymes comprising the "GO" system may be linked with cancer development. It has recently been demonstrated that many types of DNA repair pathways are reduced in cancer patients (Rajaee-Behbahani *et al.*, 2001). The precise mechanism(s) of the reduced repair capacity in cancer patients is/are still unknown. However, some mechanisms may be suggested.

Firstly, it has recently been documented that lung and other cancer patients show loss of heterozygosity at the *hOGG1* gene locus (Hardie *et al.*, 2000; Wikman *et al.*, 2000), and those patients have a higher level of 8-oxodG adducts (Hardie *et al.*, 2000).

Secondly, it has been suggested that polymorphism in DNA repair genes may be associated with differences in the repair efficiency of DNA damage (Butkiewicz *et al.*, 2001). Some studies suggest an involvement of hOGG1 polymorphism in lung cancer development (Le Marchand *et al.*, 2002). Few *hOGG1* polymorphisms have been described, Ser326Cys being the most common, which is characterized by a lower enzyme activity (Kohno *et al.*, 1998). It was suggested that the presence of two hOGG1 326Cys alleles confers a 2-fold increased risk of lung cancer (Sugimura *et al.*, 1999; Le Marchand *et al.*, 2002), and also an elevated risk of prostate cancer and nasopharyngeal carcinoma (Goode *et al.*, 2002). Inherited variants of hMYH at conserved amino acids are associated with somatic GC→TA transversions in colorectal tumors (Al-Tassan *et al.*, 2002).

In our study (Gackowski *et al.*, 2003), of lung cancer patients and matched control groups all the parameters, which may represent oxidative DNA base damage were measured. Besides urinary excretion of the modified base and nucleosides also the level of 8-oxodG in leukocyte DNA and the capacity for 8-oxoGua repair were analyzed. The level of 8-oxodG in DNA isolated from leukocytes of cancer patients was significantly higher than in DNA isolated from the two control groups of smokers and ex-smokers. Since the oxidative DNA insult represented by urinary excretion of oxidative DNA lesions was similar in cancer patients and the control group with similar smoking status, it appears likely that the higher rate of generation of oxidative damage in cellular DNA of lung cancer patients is a result of a deficiency of repair mechanism(s) (most likely the BER pathway) in this group.

This suggestion was confirmed with the measurement of 8-oxoGua repair activity in leukocytes

of the both smoker groups. In the smoking cancer patients this activity was significantly lower than in healthy smokers. Similarly, Paz-Elizur and coworkers (2003) observed a lower 8-oxoGua excising activity in blood leukocytes of lung cancer patients than in healthy controls, pointing to the important role of 8-oxoGua repair as a risk factor for developing lung cancer. It has also been shown that *OGG1* knockout mice are predisposed to develop lung carcinoma and 8-oxoGua was found to accumulate in their DNA (Sakumi *et al*., 2003).

In our recent study (Speina *et al*., 2005) we have found that 8-oxoGua level in human DNA is determined not only by its excision rate, but also by the frequency of its incorporation from the nucleotide pool into DNA by DNA polymerases, and the latter may be the most important contributor. We studied 8-oxoGua level in DNA, OGG1 repair activity, and hMTH1 activity in tumors and in surrounding lung tissue without histological changes (normal lung) of lung cancer patients. We found that 8-oxoGua level was lower in tumor than in normal lung tissue, OGG1 activity was also lower in tumor, but hMTH1 activity was higher in tumor than in normal lung. The activity of hMTH1 was three orders of magnitude higher than that of hOGG1 (nanomoles *versus* picomoles per hour per milligram of protein). This tremendous difference can be attributed mostly to differences in the turnover rates of these enzymes, since the expression of hMTH1 and OGG1 mRNAs is similar (Kennedy *et al*., 2003). The k_{cat} values are 211 min^{-1} and 0.1 min^{-1} for hMTH1 (Speina *et al*., 2005) and hOGG1 (Hill *et al*., 2001), respectively. The role of hMTH1 protein is further highlighted by observation that over expression of hMTH1 protein in mismatch repair deficient cell lines decreased the mutation rates to normal and reduced microsatellites instability which was accompanied by reduction of the 8-oxodG level in DNA (Russo *et al*., 2004). Also expression levels of hMTH1 mRNA were inversely proportional to the levels of 8-oxodG in DNA in 11 human lung cancer cell lines and SV-transformed non-tumorigenic human bronchial epithelial cells (Kennedy *et al*., 1998). The higher activity of 8-oxodGTPase also coincided with lower background levels of 8-oxodG in DNA of foetal compared with maternal mouse organs (Bialkowski *et al*., 1999).

Surprisingly we observed decreased 8-oxoGua excision activity in tumor lung tissue in comparison with non-affected surrounding areas. The mechanism of this decrease is not known, but probably is not related to mutations in *hOGG1* gene in tumor, since they have been found in only 4% of human kidney cancers, and were also sporadic in lung cancers (Chevillard *et al*., 1998). Some studies have shown frequent allelic loss in cancer tissue of chromosome fragments in the position locating *hOGG1* gene. Accordingly, the decrease of *hOGG1* expression was observed, e.g. in head and neck squamous cancer cases (Fan *et al*., 2001). However, loss of heterozygosity in *hOGG1* locus may vary between cancers types. No differences in OGG1 expression were observed between tumor and non-affected surroundings in human lung and kidney cancers (Chevillard *et al*., 1998). In model systems OGG1 activity is stimulated by at least two proteins, HAP1 and XRCC1 (Hill *et al*., 2001; Marsin *et al*., 2003). One cannot exclude deregulation in tumor tissue of OGG1 cooperation with downstream partners of the BER pathway, although HAP1 expression was shown to increase in tumors (Fritz *et al*., 2003). The decrease of OGG1 activity may also be tumor-specific, driven by a loss of OGG1 activators in tumor tissue. One such effector may be a tumor suppressor protein–tuberin. In tuberin-deficient Eker rats, which spontaneously develop renal cancers, OGG1 expression and activity was reduced 3-fold (Habib *et al*., 2003).

It is also possible that the increased oxidative stress in tumor tissue may directly inactivate some repair enzymes. Exogenous nitric oxide and peroxynitrite have been shown to inhibit hOGG1 (Jaiswal *et al*., 2001), DNA ligase (Graziewicz *et al*., 1996), formamidopyrimidine-DNA-glycosylase and O^6-alkylguanine-DNA-alkyltransferase by direct nitrosylation (Laval & Wink, 1994; Wink & Laval, 1994). However, there are conflicting data concerning production of nitric oxide in human lung adenocarcinoma. Fujimoto *et al.* (1997) reported higher activities of nitric oxide synthase isoforms in adenocarcinoma than in other types of lung cancer and in normal lung, while Ambs *et al.* (1998) did not find any up-regulation of the synthase isoforms during non-small cell lung carcinoma (NSCLC) progression.

We also assessed the role of oxidative stress-driven lipid peroxidation (LPO) in the pathogenesis of lung cancer (Speina *et al*., 2003). We measured the levels of 1,N^6-ethenoadenine (εA) and 3,N^4-ethenocytosine (εC) in DNA by immunoaffinity/^{32}P postlabeling, as well as the repair capacity for εA and εC (by the nicking assay) in normal and tumor lung tissues, as well as in blood leukocytes of lung cancer patients and healthy volunteers, matched with cancer patients for age, sex and smoking habit.

In humans εA is eliminated from DNA by alkylpurine-DNA-*N*-glycosylase (ANPG) (Saparbaev *et al*., 1995), and εC by mismatch-specific thymine-DNA-glycosylase (TDG) (Saparbaev & Laval, 1998). Both enzymes are monofunctional DNA-glycosylases and require AP-endonuclease to incise DNA at the site of the removed base. Moreover, *in vitro* the activity of TDG is stimulated several fold by human AP-endonuclease (HAP-1), which increases the turnover of the enzyme on damaged DNA (Privezentzev *et al*., 2001). Thus, the tissue repair capacity for εC

and εA may depend on the availability of DNA-glycosylases and AP-endonuclease.

In contrast to 8-oxoGua, no difference in εA and εC levels between tumor and non-affected lung tissues was recorded. The repair activities for εA and εC were significantly higher in tumor than in normal lung tissue. No significant differences in εA and εC repair activities were associated with age, sex or smoking habit. However, a significant difference in the repair capacity was observed between two histological types of NSCLC, squamous cell carcinoma (SQ), which is related to the sensitivity to tobacco smoke components, and adenocarcinoma (AC), linked to chronic infections and healing of scars. In individuals suffering from lung AC, the εA and εC repair activities in normal lung and blood leukocytes were significantly lower than in SQ patients. Differences were also found between the εA and εC repair activities of cancer patients and healthy volunteers. The repair capacity for εA was significantly lower in blood leukocytes of lung cancer patients than in leukocytes of healthy volunteers. This difference was even higher between healthy volunteers and patients developing inflammation-related adenocarcinoma. In contrast, the repair activities for εC were the same in leukocytes of healthy controls, all lung cancer patients, and SQ patients. However, individuals with adenocarcinomas had revealed a significantly lower εC repair activity.

These results suggest that oxidative stress-mediated lipid peroxidation might contribute to the induction and progression of lung cancer. A decreased activity of the base excision repair pathway for εA and εC is associated particularly with inflammation-related lung adenocarcinoma. Lung adenocarcinoma has also been linked with defective repair of 8-oxoGua. *OGG1* gene polymorphism (Sugimura *et al.*, 1999; Le Marchand *et al.*, 2002) and down-regulation of hMTH1 expression have been demonstrated in AC in comparison to SQ types of lung cancer (Kennedy *et al.*, 2003). Thus, the development of histological types of NSCLC may be related to different causative factors, and among them is the deficiency of different repair pathways of oxidative stress-induced DNA damage.

Oxidative DNA damage: cause or consequence of cancer development?

Numerous studies have demonstrated that a prooxidant environment is characteristic for advanced stages of cancer. Some mechanisms responsible for the oxidative stress characteristic for cancer patients have been suggested:

i) It has been recently documented that cancer patients show signs of extensive granulocyte activation with a release of reactive oxygen species (Schmielau & Finn, 2001).

ii) Another reason for the observed phenomenon may be that some tumors may stimulate the defence systems of the body so that they react against the tumor to produce cytokines (Franks & Teich, 1997). Some of the cytokines can produce large amounts of ROS (Kayanoki *et al.*, 1994; Ohba *et al.*, 1994). It has been shown that elevated plasma level of TNF is responsible for increased oxidative DNA damage in CD^+ cells (Peddie *et al.*, 1997).

iii) It has been shown that malignant cells can produce hydrogen peroxide at levels as high as those characteristic for stimulated polymorphonuclear leukocytes (Szatrowski & Nathan, 1991). Therefore, one of the reasons for the observed oxidative stress in advanced stages of cancer may be the release of a large number of cancer cells into the blood stream (De Vita *et al.*, 2001) and their penetration into other tissues. In this context it is noteworthy that we have demonstrated that exposure to activated leukocytes causes oxidative DNA base modifications (among them 8-oxoguanine) in target cells (Dizdaroglu *et al.*, 1993).

The conditions outlined above are characteristic for advanced stages of cancer development. Therefore, if they are solely responsible for oxidative DNA damage and decreased concentration of antioxidants, one should not observe similar changes in patients with benign colon tumors. However, our results demonstrate that the 8-oxodG level in leukocyte DNA and the urinary excretion rate of 8-oxodG is significantly higher in patients with polyps in comparison with a control group, while no further statistically significant increase is observed in carcinoma patients (Fig. 11). Moreover, we found strong support for a role of oxidative DNA damage in the development of adenoma comparing subjects with the highest level of 8-oxodG in cellular DNA with these with the lowest level (OR=5.75; 95% CI: 2.26–14.62). Similar, the OR value of urinary 8-oxodG, for a risk of polyps was 7.60 (95% CI: 2.57–22.49), while no significant OR value was found for carcinoma. Interestingly, a recent population-based prospective study has demonstrated that high excretion of 8-oxodG is significantly associated with an increased risk of developing lung cancer (Loft *et al.*, 2006). The urinary excretion rate of the modified base (8-oxoGua) was significantly elevated only in carcinoma patients. Our results indicate that oxidative DNA damage may be involved in the early stages of colon cancer development (i.e. adenoma) as well as accumulate further during subsequent stages of colon cancer development.

It has been estimated that thousands of individual DNA mutations exist in a single carcinoma cell and a fraction of these mutations may arise during early development of the disease (Loeb, 2001). This in turn suggests that they are generated con-

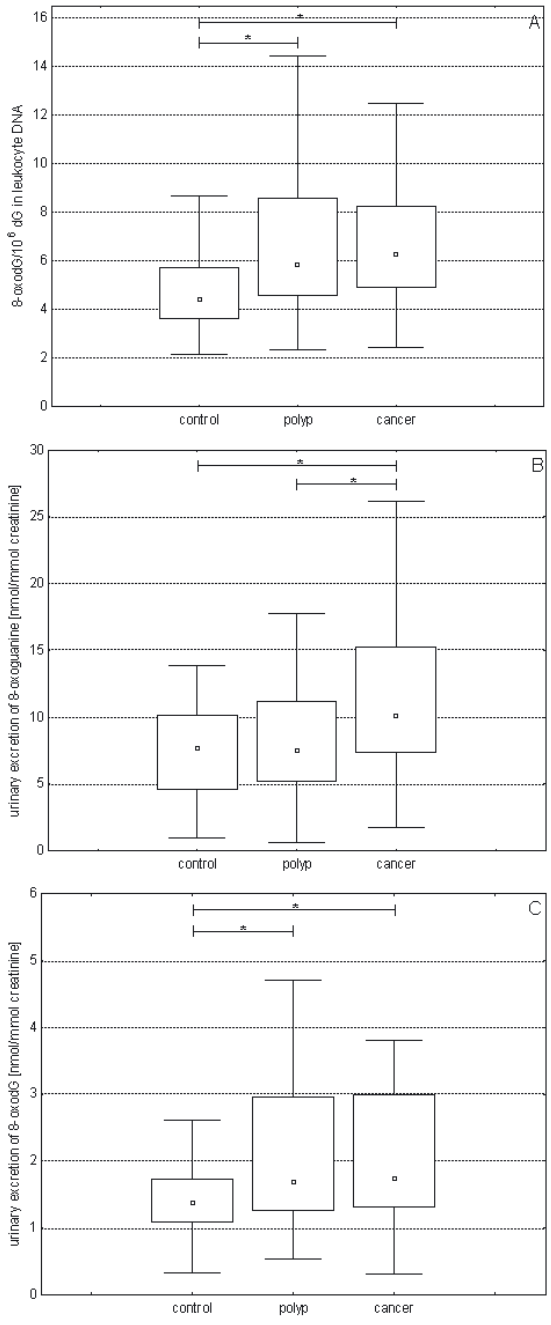

Figure 11. 8-OxodG level in leukocyte DNA (A), urinary excretion of 8-oxoguanine (B) and 8-oxodG (C) in the study groups.

tinuously during tumor progression. Endogenous cellular processes (oxidative phosphorylation, peroxisomal fatty acid metabolism, cytochrome P450 reactions or "respiratory burst" of phagocytic cells) are efficient sources of harmful ROS that may be responsible for oxidative DNA base modifications and may serve as a source of mutations that initiate carcinogenesis. In order to contribute to mutations, oxidative DNA damage would need to occur at a sufficiently high frequency to exceed the capacity of the cell for DNA repair. There are more than 20 different oxidative DNA base modifications (Dizdaroglu, 1998). In this context it is also noteworthy that in our earlier study it was demonstrated that the combined amount of 8-oxoGua and 8-oxodG excreted into the urine of healthy subjects was 2.5 nmol per kg per day, corresponding to about 2000 oxidative modifications of guanine per cell per day (Olinski *et al.*, 2002). Therefore, the extent of oxidative DNA base modifications appears to be very high. As a consequence these modifications might contain the lesions that initiate carcinogenesis as well as possibly contributing to ongoing tumor progression.

Atherosclerosis

Several studies suggest that DNA alterations are present in atherosclerotic tissues and can play a fundamental role in the pathogenesis of this disease (De Flora *et al.*, 1997; Lee & Blair, 2001). Interestingly, DNA samples extracted from cells of atherosclerotic lesions show a transforming capability (Penn *et al.*, 1986). These data support the view that at least some of atherogenic lesions may be initiated by mutational events in the same manner as a benign tumor is (Trosko & Chang, 1980; Penn *et al.*, 1986). Moreover, some experimental data strongly suggest that the elevated level of 8-oxodG found in lesions of the aorta wall in atherosclerotic patients may be one of causes of the disease (De Flora *et al.*, 1997). We showed that the level of 8-oxodG in lymphocytes of atherosclerotic patients was significantly higher than in the control group (12.78±5.14 and 9.80±4.13 lesions/10^6dG, respectively) (Gackowski *et al.*, 2001). The plaques of the arterial walls contain, among other components, lymphocytes (Ross, 1993) and, since 8-oxoGua has mutagenic properties, it is possible that lymphocytes with elevated levels of this modified base trapped in the plaque can more easily be involved in the initiation and/or promotion processes. There are suggestions that the processes may be responsible for the formation of atherosclerotic lesions (Ross, 1993). Thus, a higher number of trapped cells could lead to more advanced lesions.

Oxidized low-density lipoprotein (LDL) might play an important role in the development of atherosclerotic lesions (Ross, 1993). Interestingly, it has recently been found that oxidized LDL (but not the unmodified molecule) down-regulates enzymes which take part in the base excision repair of 8-oxoGua from cellular DNA (Dianov *et al.*, 1998; Chen *et al.*, 2000). Therefore, it is possible that oxidized LDL that contributes directly to the development of atherosclerosis (*via* formation of foam cells), may also be responsible for the high level of 8-oxodG observed by our laboratory in blood lymphocytes. Moreover, it has also been shown that vitamin C

(together with α-tocopherol) prevents the down-regulation of BER by oxidized LDL (Chen *et al.*, 2000).

Neurodegenerative diseases

The high rate of oxygen consumption per unit mass of tissue renders the brain especially vulnerably to the deleterious effects of oxidative stress, which can arise from the overproduction of ROS and/or from a deficiency of the antioxidant defense systems. It is possible that oxidative stress is an important factor that may be involved in pathogenesis of Alzheimer's disease (AD). This theory derives from the fact that ROS are implicated in the neurotoxicity of amyloid beta peptides. It has been demonstrated that the peptides themselves spontaneously generate free radicals. There is considerable evidence that oxidative stress occurs in AD (Huang *et al.*, 2004; Leutner *et al.*, 2005), and increased 8-oxodG levels have been found in DNA isolated from brain tissues as well as from leukocytes of AD patients (Markesbery & Carney, 1999; Migliore *et al.*, 2005). A decreased repair of DNA damage could be involved in this process and it has been found that the OGG1 level is significantly lower in patients with AD than in control cases.

An interesting hypothesis was recently put forward that explains the massive death of neurons in AD. It has been shown that neurons that are about to die (like in the case of AD) are those that have been attempting to re-enter the cell cycle and synthesize DNA. The accumulation of DNA damage in neurons, however, may result in triggering cell death. Moreover, it has been demonstrated in experiments with a mouse model of AD that all neurons that re-entered the S-phase had oxidized bases in their DNA (Klein *et al.*, 2002).

CONCLUSIONS

It is becoming increasingly apparent that oxidative damage plays a role in numerous pathological conditions (Halliwell & Gutteridge, 1999). However, knowing of whether oxidative DNA damage initiates the disease process or whether it is merely a by-product of the disease development is of critical importance. On the basis of the presented data and literature reports it seems reasonable to postulate that oxidative DNA damage/oxidative stress is probably a contributing factor in aging. Thus, oxidative stress may contribute to a limited extent to the aging of some individuals and could be a major factor in others. It should be also remembered that the association between oxidative stress and aging is complicated by there being no general agreement as to what aging is, when it begins and what triggers its onset, and also by the fact that oxidative stress occurs by multiple mechanisms.

Although there is evidence that oxidative DNA damage may play a causative role in atherosclerosis, it is also clear that the presence of the damage in the atherosclerotic plaque need not necessarily be the direct cause of the disease development. It may simply mirror the prooxidative conditions of a patient's blood.

There is no doubt that the toxicity of amyloid peptides and tau protein can be directly responsible for the AD development. It is still unclear whether oxidative stress/oxidative DNA damage is the primary initiating event of the disease. However, a growing body of evidence implicates it as being involved in apoptotic cell death that leads to neurodegeneration in AD patients and might influence the progression of the disease.

While many details regarding the role of ROS-induced DNA damage in the etiology of complex multifactoral diseases like cancer are yet to be discovered, it is evident that oxidants act at several stages in the malignant transformation since they can induce permanent DNA sequence changes (Jackson & Loeb, 2001). In the light of the presented data it is likely that severe oxidative stress is a consequence of the development of many types of cancer. However, it is impossible at present to answer directly the question concerning the involvement of oxidative stress in cancer origin since full development of the disease in response to carcinogen exposure takes 20–40 years. Therefore, it is very difficult to prove directly that the DNA lesion responsible for the carcinogenic process is the lesion present in tumors many cell generations later. Nevertheless, it should be remembered that DNA damage, altered gene expression and mutations are necessary participants in carcinogenesis. Although these events may be derived by different mechanisms, an involvement of oxidants is a common feature of all these phenomena.

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