ANTIOXIDANTS & REDOX SIGNALING
Volume 8, Numbers 5 & 6, 2006

Forum Review

Urinary Measurement of 8-OxodG, 8-OxoGua, and 5HMUra: A Noninvasive Assessment of Oxidative Damage to DNA

RYSZARD OLINSKI,[1] RAFAL ROZALSKI,[1] DANIEL GACKOWSKI,[1]
MAREK FOKSINSKI,[1] AGNIESZKA SIOMEK,[1] and MARCUS S. COOKE[2]

ABSTRACT

Numerous DNA repair pathways exist to prevent the persistence of damage, and are integral to the maintenance of genome stability, and hence prevention of disease. Excised lesions arising from repair may ultimately appear in the urine where their measurement has been acknowledged to be reflective of overall oxidative stress. The development of reliable assays to measure urinary DNA lesions, such as HPLC prepurification followed by gas chromatography/mass spectrometry, offers the potential to assess whole body oxidative DNA damage. However, some studies suggest a possibility that confounding factors may contribute to urinary levels of 7,8-dihydro-8-oxoguanine (8-oxoGua) and 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxodG). This article considers several possible sources of urinary lesions: (a) the repair of oxidatively damaged DNA; (b) a possible dietary influence; and (c) cell death. The authors conclude that data from their laboratories, along with a number of literature reports, form an argument against a contribution from cell death and diet. In the absence of these confounding factors, urinary measurements may be attributed entirely to the repair of DNA damage and suggests their possible use in studying associations between DNA repair and disease. *Antioxid. Redox Signal.* 8, 1011–1019.

INTRODUCTION

The background level of 8-oxo-7,8 dihydroguanine (8-oxoGua) in cellular DNA represents a dynamic equilibrium between the rate of oxidative DNA damage formation, and the rate of repair in the specific tissue/cells studied. Numerous highly redundant repair processes have evolved precisely to prevent 8-oxoGua persisting in DNA, a clear indicator of the importance this lesion has in disrupting genome stability. Normal metabolic processes can give rise to 8-oxoGua, and as a consequence, levels of this lesion can be detected in cells (so-called background levels). However, controversy surrounds the issue of exactly how much damage is present, not least due to the potential for damage to be formed during extraction of DNA from cells. The European Standards Committee on Oxidative DNA Damage (ESCODD) was formed to resolve the problems associated with the measurement of background levels of oxidative damage to DNA (in particular, 8-oxoGua) in human cells. As a result of these endeavors, assays for this damage have become more precise and accurate (1, 2). Instead of measuring damage in specific cells, with concomitant problems such as artefact formation, a whole body burden of oxidative stress may be assessed by the measurement of urinary excretion of 8-oxoGua and its deoxynucleoside equivalent 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) (10, 41, 57, 62).

The analysis of 8-oxoGua in urine presents particular difficulties (i.e., poor solubility can caused a loss of the analite) (33) and until recently there has been no reliable assay for its detection. New techniques, based on mass spectrometric detection (MS), have been developed that allowed for the simultaneous determination of 8-oxodG, 8-

[1]Department of Clinical Biochemistry, Nicolaus Copernicus University, Collegium Medicum in Bydgoszcz, Poland.
[2]Radiation and Oxidative Stress Group, Department of Cancer Studies and Molecular Medicine, and Department of Genetics. University of Leicester, Leicester Royal Infirmary, University Hospitals of Leicester NHS Trust, Leicester, United Kingdom.

oxoGua, and 5-methylhydroxyuracil (5HMUra) in the same urine sample (27, 55, 69). One such method involves HPLC prepurification, followed by gas chromatography with isotope dilution MS detection (27). In addition to unequivocal identification of the analyzed compounds and high sensitivity, the use of isotopically-labeled internal standards compensate for potential losses of the analytes during sample work-up.

It is understood that, following excision from DNA, the oxidatively modified lesions are released into the bloodstream and consequently appear in the urine. There is a common belief that the presence of 8-oxodG in urine represents the primary repair product of the oxidative DNA damage *in vivo* and that this compound may reflect the involvement of the nucleotide excision repair pathway (NER) (10, 41). However, oxidatively damaged DNA bases are primarily repaired by the base excision repair pathway (BER), although NER may also play a role in the repair of some oxidized bases in DNA (17), particularly under certain cellular conditions (23). Moreover, several glycosylases, which specifically recognize and remove 8-oxoGua in human cells, have been recently described (32, 54), whereas the route by which 8-oxodG arises in extracellular matrices is less clear. Therefore, urinary assays that measure 8-oxoGua reflect glycosylase activities, and those that measure 8-oxodG reflect other, as yet undefined, activities.

SOURCES OF URINARY LESIONS

To fully understand the results derived from analyses of urinary lesions there is a need to answer a key question "from where do these lesions originate?" In this article we will consider several possible sources of urinary lesions: (a) the repair of oxidatively damaged DNA; (b) a possible dietary influence; and (c) cell death (Fig. 1).

DNA repair as a source of urinary 8-oxoGua

Eukaryotic cells use a specific DNA glycosylase, the product of the *ogg1* gene, to remove 8-oxoGua from cellular DNA. Recently homozygous *ogg1 −/− null* mice were generated (38). To assess an involvement of the enzyme in generation of urinary 8-oxoGua, and to further clarify the issue concerning the origin of this DNA modification, 8-oxoGua levels was determined in *ogg1*-deficient mice and compared to the wild-type strain. If OGG1 were the only enzyme to remove 8-oxoGua, one would expect no excretion of 8-oxoGua. On the other hand, if there were efficient backup glycosylase(s), no change in urinary excretion of the modified base should be observed between *ogg1*-defective and wild-type strains (assuming that the repair process is the main source of the compound). Our results indicated that there was about a 26% reduction in levels of urinary 8-oxoGua in the deficient

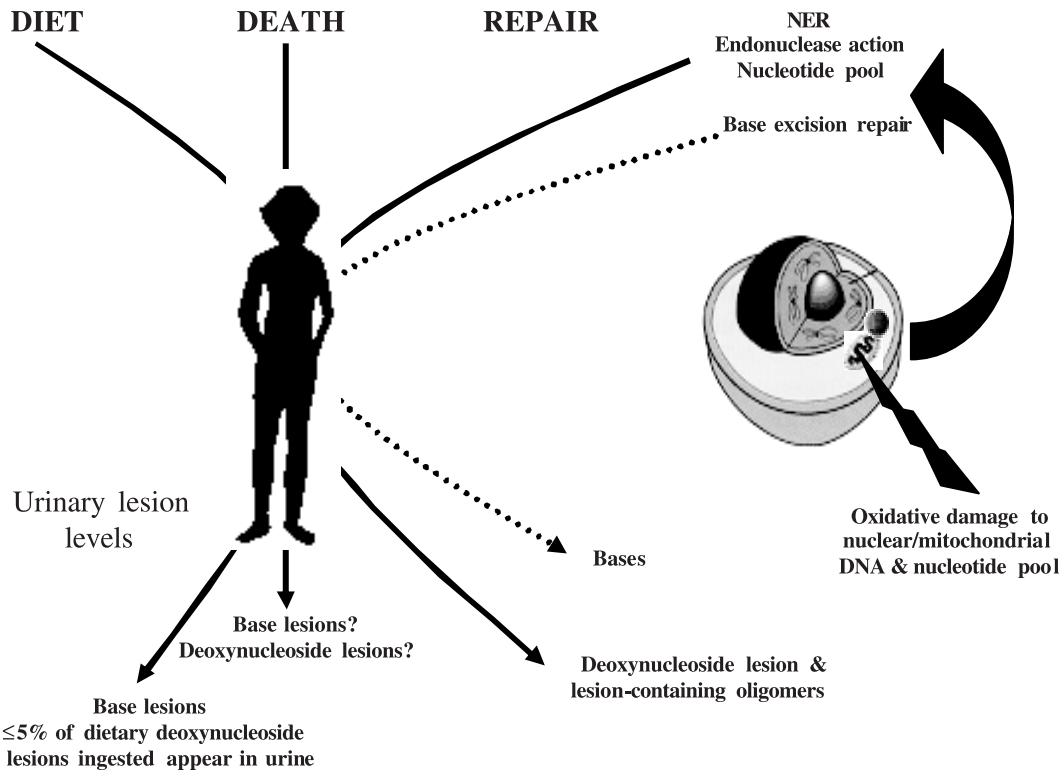

FIG. 1. Scheme representing the possible sources of diet, cell death/cell turnover, and DNA repair to urinary levels of deoxynucleoside and base lesions. (NER, nucleotide excision repair). Reprinted from *Free Radic Biol Med* 33, Cooke, MS, Lunec, J, and Evans, MD. Progress in analysis of urinary oxidative DNA damage, 1601–1614, 2002, with permission from Elsevier.

strain, compared to wild type (59). This indicates that mouse OGG1 glycosylase is a significant, but not unique, source of urinary 8-oxoGua.

Our results clearly suggest the existence of backup DNA glycosylase(s), which operate with a lower efficiency than OGG1. The reversion to a less efficient backup system should result not only in reduction of 8-oxoGua in urine, but also in the simultaneous gradual increase in the background level of 8-oxoGua in cellular DNA. Indeed, it has been demonstrated that nuclear DNA 8-oxoGua levels in the liver of *ogg1*-deficient mice increase during aging (38, 45, 51). At 14 weeks of age, a sevenfold increase is observed in *ogg1*-deficient mice rising to 40-fold at 50 weeks of age (45, 47). The same is also true for the mitochondrial genome where a 20-fold increase in the 8-oxoGua level was observed in the *ogg1*-defective mice (64). The recent finding of Seeberg′s group supports these data. This group described a backup glycosylase (Nei-like glycosylase 1) that, depending upon sequence context, is approximately 10-fold less effective than hOGG1 for 8-oxoGua removal (46).

However, this reasoning alone cannot fully explain our findings since the buildup of 8-oxoGua appears to be limited to certain organs, such as liver and possibly skeletal muscles, and urinary excretion of the modified base should represent the average rate of DNA damage in the whole body (50). This suggests the existence of another repair pathway, the product of which is not 8-oxoGua that maintains lowered 8-oxoGua levels, in the absence of OGG1, in most tissues.

DNA repair as a source of urinary 8-oxodG

It is worth stressing that 8-oxodG cannot be a product of glycosylase action since the glycosidic bond is cleaved, some other DNA repair activity must be responsible for generating 8-oxodG, an activity that may maintain genomic stability in the absence of OGG1, described above. An 8-oxodG endonuclease has been described that can produce 8-oxodG, but is reported to be unstable and difficult to purify (8). The activity of an 8-oxo-2′-deoxyguanosine 5′-triphosphate pyrophosphohydrolase (hMTH1; human MutT homologue) has been proposed to also contribute to 8-oxodG in urine (10). This enzyme sanitizes the nucleotide pool, degrading 8-oxodGTP to 8-oxodGMP, and preventing misincorporation of this modified precursor into DNA (31). 8-oxodGMP derived from the activity of hMTH1 is proposed to be subject to further degradation by 5′(3′)-nucleotidase activity, yielding 8-oxodG, which is then excreted. In MTH1-deficient mouse cells, an 8-oxo-dGTP-degrading activity has been observed, suggesting that similar, as yet uncharacterized enzyme(s), have the potential to produce 8-oxodG (39).

Nucleotide excision repair (NER) is generally associated with the repair of helix distorting, bulky, adducts. However, there is evidence that this repair system may also act upon common, nonbulky, oxidative DNA damage such as 8-oxoGua and thymine glycol (37, 40, 56), presumably to yield, in the first instance, lesion-containing oligonucleotide products. The ability of NER to process these small lesions is apparently not surprising since NER is known to have a diverse range of substrates. Lesion-containing oligomers from NER may be subject to intra-/extracellular 5′–3′ exonucleolytic digestion to ultimately produce oligomers 6–7 nucleotides long. The latter may however be degraded further and such poorly characterized postexcision processing may ultimately yield 8-oxodG. There is some evidence that lesion-containing oligomers may be present in urine and it is feasible that these may in part be derived from NER (11), although the presence of 8-oxodG-containing oligomers in urine is controversial (70). Studies examining the role of NER in the repair of small oxidative DNA lesions have also used cell lines. Repair of several small oxidative DNA lesions is defective in cells derived from patients with xeroderma pigmentosum complementation group A (XP-A), which possess <2% of normal NER activity, providing further evidence for a role for NER in the repair of these lesions, or that XP-A plays an as yet poorly defined role in BER (40, 56). Whether NER is a major pathway for the repair of 8-oxoGua under all circumstances is debatable, it may be the case that NER functions preferentially in certain cell types and under specific conditions, perhaps when some BER mechanisms are unavailable or compromised.

Diet as a potential source of urinary 8-oxoGua and 8-oxodG

The prevailing thought is that base lesions can be influenced by diet, but deoxynucleoside lesions cannot, which is why reports of 8-oxodG measurement dominate 8-oxoGua measurement. To confirm that such a conclusion is entirely legitimate, we have undertaken a number of studies to specifically address the question of dietary influence upon urinary 8-oxoGua/8-oxodG.

We have applied HPLC prepurification, followed by gas chromatography with isotope dilution MS detection methodology to the determination of 8-oxodG and 8-oxoGua in human urine (27). Since a previous study demonstrated that diet could influence the level of 8-oxoGua in rat urine (52), it was our intention to investigate whether a similar phenomenon could be observed in humans. The previous study revealed that the amount of 8-oxoGua in urine reached the lowest value 2–3 days after switching to a nucleic acid-free diet (52). Therefore, in our study the urine samples (24 h output) were collected after 3 days that a nucleic acid-free diet was completed, and from the same group of 24 individuals 3–5 days after returning to a normal unrestricted diet (for detailed characteristics of the subjects and the treatments, see Ref. 27). The mean levels of 8-oxoGua and 8-oxodG in the urine samples of the subjects on unrestricted diet were 1.87 and 0.83 nmol/kg 24 h, respectively, and in the case of the studied groups we concluded that they did not depend on the applied diet (Figs. 2 and 3) (27).

We have also used ^{15}N-labeled DNA as a probe for the influence of diet upon urinary lesions level. Various amounts (as much as 25 mg) of oxidatively modified ^{15}N-labeled DNA were ingested by volunteers, and blood and urine samples collected over a 2-week period. No ^{15}N-labeled 8-oxoGua or 8-oxodG was detected in urine, or the DNA of peripheral blood mononuclear cells, collected from these individuals (9), confirming the lack of dietary influence upon these lesions.

5-Hydroxymethyluracil (5-HMUra) is a free radical derivative of thymine (16), and its presence in urine should also re-

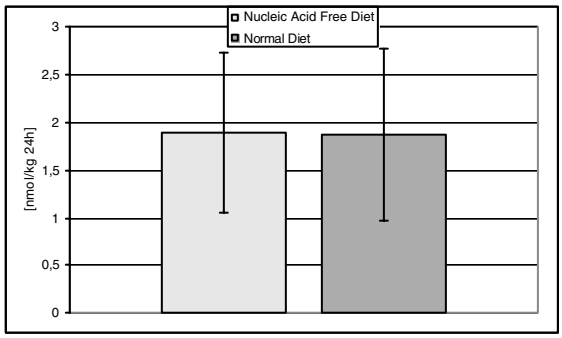

FIG. 2. Urinary excretion of 8-oxoGua for humans fed with normal- and nucleic acid-free diet (data from Ref. 27).

flect whole body oxidative DNA damage. Moreover, in humans and other vertebrates, an interesting glycosylase has been described that specifically excised 5-HMUra from DNA. It appears to be unique to higher eukaryotes, reportedly absent from prokaryotes (60). To further clarify the controversial issue of potential dietary influence upon urinary DNA lesion levels, experimental animals were fed with normal and nucleic acid-free diet, and the LC–GC/MS technique was applied that allowed for simultaneous determination of 8-oxoG, 8-oxodG, and 5-HMUra in the same urine sample (58). Our results clearly point out that irrespective of the diet the excretion rates were similar. Taken together and using a number of approaches, our results clearly indicate that diet does not contribute to urinary 8-oxoGua, 8-oxodG, or 5-HMUra in mice or humans.

Cell death as a source of urinary 8-oxoG and 8-oxodG

It has been suggested that urinary 8-oxodG represents the degradation, and subsequent oxidation, of DNA from dead cells. We have examined a number of literature reports of urinary 8-oxodG in cancer patients. The purpose of the cytotoxic agents used in chemotherapy is to cause cell death. No increases in urinary 8-oxodG were noted in the studies of both Faure *et al*. (24) and Erhola *et al*. (20), despite evidence of extensive cell death: significant increases ($p < 0.01$) in urinary uric acid (a biochemical index of cell turnover); and reduction in tumor mass, respectively. Furthermore, any reported increases in urinary 8-oxodG following chemo- or radiotherapy have been attributed entirely to increases in DNA repair (7, 28). Hematological cell counts allow for precise calculation of "nadir days", days when the most distinct cell death can be observed. If cell death contributed to an increase in the modified base/nucleoside, one should observe the most distinct increase in urinary lesion levels during the "nadir days". However, a study from our group, involving cisplatin, revealed that a statistically significant ($p = 0.0003$) increase (60%) in 8-oxoGua was observed 24 h after infusion of the drug, a period when no loss of blood cells was reported. In the "nadir days" urinary excretion of 8-oxoGua dropped significantly in comparison with aforementioned time point. No statistically significant differences we noted between samples collected during different time points was observed regarding 8-oxodG values (A. Siomek *et al*. submitted). These results clearly indicate that cell death does not contribute to urinary 8-oxoGua and 8-oxodG in humans.

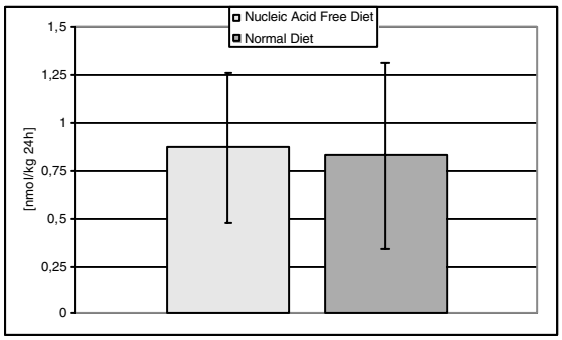

FIG. 3. Urinary excretion of 8-oxodG for humans fed with normal- and nucleic acid-free diet (data from Ref. 27).

Further argument against a contribution from cell death derives from patients with the autoimmune disease systemic lupus erythematosus, who have been reported to possess both elevated rates of apoptosis and levels of DNA in their systemic circulation (4, 12, 19). Irrespective of this, mean urinary 8-oxodG levels have been reported to be equal to, or actually less than, those seen in corresponding control subjects (22, 42). Such findings are supported by *in vitro* data from our group, in which we have demonstrated the potential to measure 8-oxodG in cell culture supernatants, in the absence of any cell death (43).

Furthermore, we have argued previously that were cell death to be the primary source of urinary lesions, the ratio between native and modified deoxynucleosides and bases would be similar to that seen in cells, reportedly anywhere between one 8-oxodG/10^5 and one 8-oxodG/10^7 dG. In fact the ratio appears to be very close for the deoxynucleoside, 28 ± 2; 8-oxodG, 12 ± 2 dG nmol/24 h, although not as close for the base, 136 ± 12; 8-oxoGua, 1931 ± 182 Gua nmol/24 h (69). Neither ratio even approaches that seen in cells. This argument is supported by further work of Weimann *et al*. (70), in which urinary oligonucleotides were examined. A conclusion of the authors was that the limited excretion of oligonucleotides into urine argues against oligonucleotides, or indeed nucleosides, originating from cell death.

We propose that the above findings combined, exonerate urinary 8-oxoGua and 8-oxodG levels from the influences of diet and cell death, although we suggest caution in making generalizations that this applies to all oxidatively modified DNA lesions in urine. Nevertheless, in the absence of these confounding factors, urinary 8-oxoGua and 8-oxodG measurements may be attributed entirely to DNA repair, although the exact contribution of the putative processes remain to be established.

URINARY LESION LEVELS CORRELATE WITH MAMMALIAN LONGEVITY

DNA damage is considered of prime importance in aging (29, 30), with free radical-induced oxidative DNA damage

being a major contributor (3, 13). Since oxidatively modified DNA bases have a broad range of potential effects upon cellular function (21), their accumulation with time might be a major cause of the physiological changes associated with aging. To investigate possible involvement of oxidative DNA damage in aging, we analyzed lesion levels in several mammalian species including mice, rats, rabbits, dogs, pigs, and humans.

Formation of oxidative DNA damage, and hence measurement of these lesions in urine, should depend on oxygen consumption and metabolic rate. In turn, the metabolic rate may be described by specific metabolic rate (SMR) values (36, 63). In agreement with these assumptions, we have found good positive correlations between SMRs of different species and their excretion rates of all analysed modifications (Fig. 4). It is also noteworthy that the ratio of the urinary excretion of 8-oxoGua in mice (18.8 nmol/kg/24 h) and humans (1.8 nmol/kg/24 h) is very similar to the ratio of the reported oxygen consumption between these species (2.7 and 0.25 ml/g/h, respectively) (59). This agrees well with previous investigations that employed HPLC/EC technique, and found that the rate of urinary excretion rates of 8-oxodG of rats, mice, and humans correlated well with oxygen consumption of these species (62), although at the time of these studies it was not possible to measure accurately the urinary levels 8-oxoGua. Since metabolic rate may be associated with maximum life span (MLSP) (63), we also attempted to determine whether there is a relationship between the excretion rates of all analyzed modifications and life span. It was found that only 8-oxoGua levels inversely correlate significantly with MLSP (Fig. 5). 8-OxodG and 5-HMUra appeared to be inversely correlated with MLSP, although these relationships were not statistically significant.

The correlation of the excretion rate of 8-oxoGua with MLSP is in good agreement with previous studies that demonstrated that oxidative damage to DNA is inversely related to MLSP of different mammals (25). However, in these studies no humans were included, and the assessment of DNA damage was restricted to certain organs (6, 13, 14). In contrast, the analyses of the urinary base/nucleoside products, from our group, are reflective of oxidative DNA damage on the level of the whole organism. Our results demonstrate that ROS continually damage DNA and that this damage is lower in long-lived species than in short-lived species (25). Incomplete repair of such damage leads to its accumulation over time and eventually results in age-related deterioration.

Despite substantial differences between humans and pigs in MLSP (120 and 27 years, respectively), the excretion rates of all measured modifications were very similar. One possible explanation for this inconsistency may be related to the differences in the life span energy potential of different species (LEP; i.e., amount of energy expended per unit body mass during adult life). The difference in LEP value for humans and other mammals used in this study is exactly fourfold (67). There is also a possibility that maximum life span in the case of humans differs three to four times from the "expected life span" under the conditions in which our species evolved (65). In other words, it is likely that in the case of humans when environmental hazard was significantly reduced many organisms survived far beyond the survival time expected in the environment in which they evolved. It is also noteworthy that current aging hypotheses widely accept that aging is due to more than one single cause, and that maximum life span may be genetically determined and, hence, characteristic for the species (5).

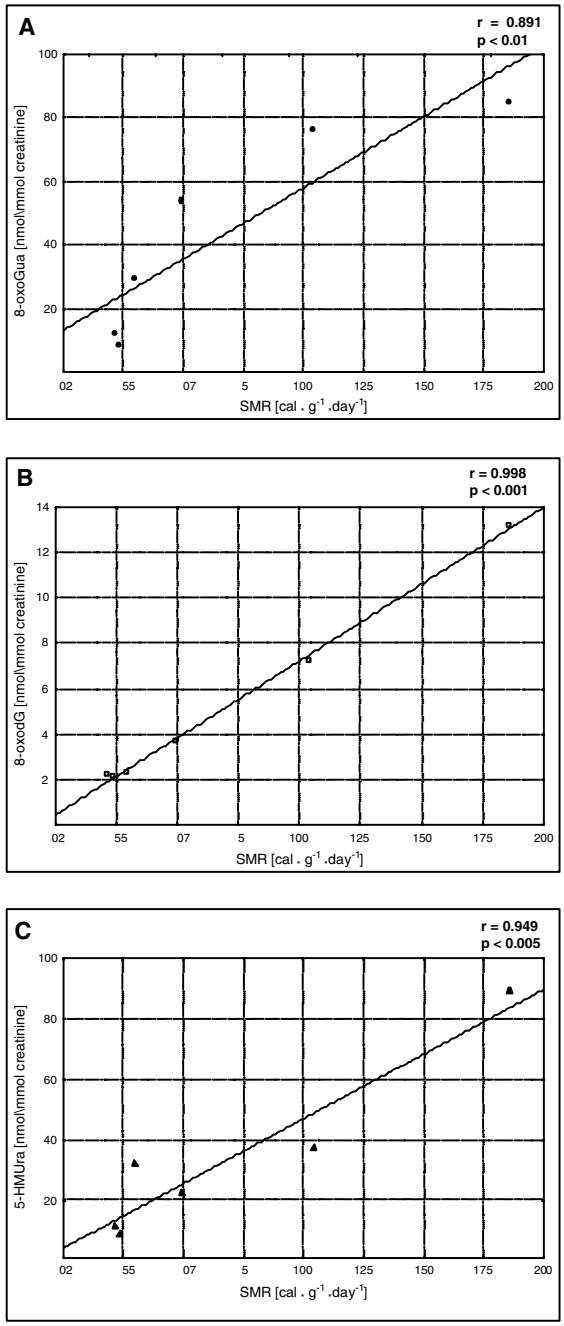

FIG. 4. Relationship between the urinary excretion rates of the analyzed modifications and specific metabolic rates (SMR) of six different mammalian species. The 0.05 level was selected as the point of minimal statistical significance.[2] Reprint from ref. 25 with permission from Elsevier.

Expression of the urinary excretion rates in nmol/kg/24 h enables the estimation of the number of the repaired lesions, per

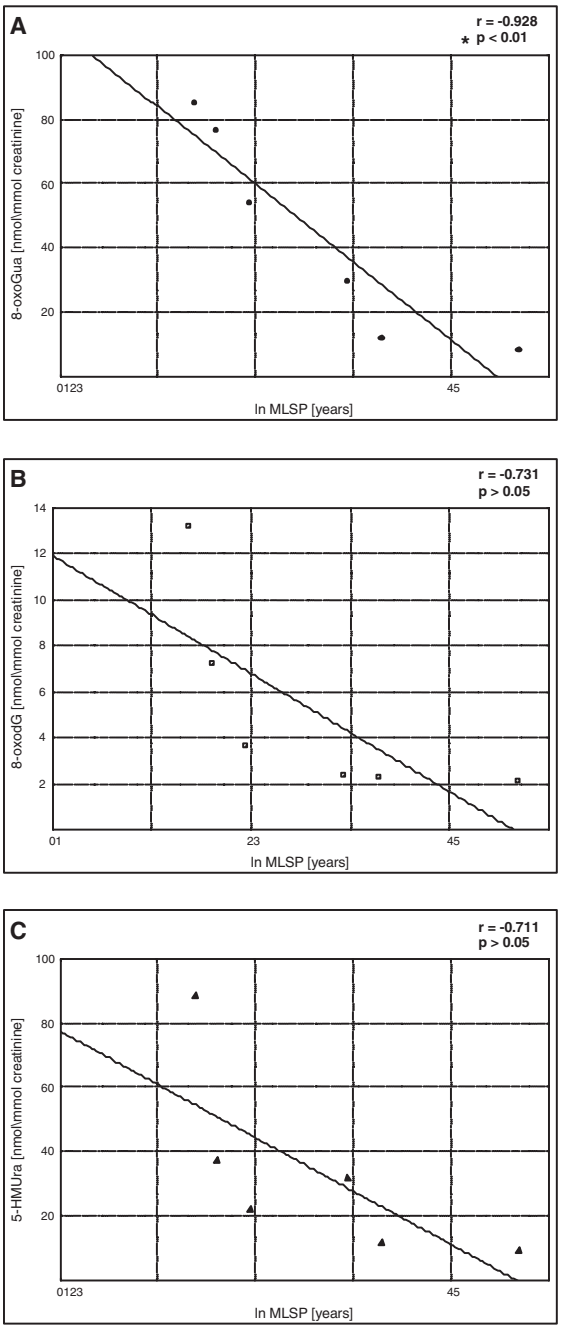

FIG. 5. Relationship between the urinary excretion rates of the analyzed modifications and natural logarithm from maximum lifespan (MLSP) of six different mammalian species. The 0.05 level was selected as the point of minimal statistical significance. Reprint from Ref. 25 with permission from Elsevier.

day, per cell (33). Interestingly, urinary level of all measured modifications, found in our study accounted for about 29,350 repaired events per average cell of the mouse per day (58). In contrast, the number of all lesions analyzed in our work, in humans accounts for about 2,800 repair events in the average cell per day. It is therefore possible that high metabolic rate in mouse (or other short-lived animal) may be responsible for severe everyday oxidative DNA insult that may be accumulated faster than in long-lived species and it may be the 'ticking' of the clock of programmed senescence. To our best knowledge our values are the first estimates based on the analyses of urinary excretion of several lesions, and are in good agreement with the values calculated for total oxidative DNA lesions (estimated 20 different oxidative base modifications) (33).

In summary, the results presented here show that urinary 8-oxoGua levels, as well as the other modifications, are higher in short-lived mammalian species. Furthermore, these parameters correlate well with both MLSP and SMR. Taken together, our results, that for the first time include humans, are consistent with the idea that DNA damage induced by oxygen-derived free radicals is one of the substantial factors of aging of mammals, including humans.

URINARY EXCRETION OF 8-oxoGUA AND 8-oxodG IN CANCER PATIENTS

Since the level of the modified nucleosides/bases in urine may be an indicator of oxidative insult to DNA, a general marker of oxidative stress, or perhaps reflective of DNA repair, we examined the amount of 8-oxoGua and 8-oxodG in urine from cancer patients. We found that the amount of the modified base, but not the deoxynucleoside, excreted into urine was approximately 50% higher in cancer patients than in the control group (57). The level of the lesions in urine can depend on oxidative DNA insult. Therefore, the higher level of 8-oxoGua in urine of cancer patients may be explained, at least partially, by the reported oxidative stress in cancer tissue (34, 44, 49, 68). However, the amount of the modified base/nucleoside excreted into urine should represent the average rate of DNA damage in whole body (10, 41). Therefore, it is doubtful that the elevated level of the base product in cancerous cells alone could account for the observed 50% increase of 8-oxoGua in urine. Our results suggest that, rather than representing increased oxidative stress in just the tumor, cancer patients have subtly raised levels of oxidative stress in other tissues (or the whole body). Equally, treatment may contribute to increasing oxidative stress. Whilst the precise mechanism(s) involved are currently unknown, some factors may be considered:

(a) It has been recently documented that cancer patients show signs of extensive granulocyte activation with a release of reactive oxygen species, followed by a dramatic increase of 8-isoprostane, one of the biomarkers of oxidative stress (61);
(b) Malignant cells can produce hydrogen peroxide at levels as large as those characteristic for stimulated polymorphonuclear leukocytes (66). Therefore, one of the reasons for the observed oxidative stress in advanced stages of cancer may be a release of the large number of cancer cells into the blood stream (15) and their penetration into other tissues. Interestingly, it has been demonstrated that exposure to the activated leukocytes causes oxidative DNA base modifications (among them 8-oxoGua) in target cells (18);

(c) Still another reason for the observed phenomenon could be that some tumors may stimulate the defence systems of the body so that they react against the tumor to produce cytokines (26). Some of the cytokines can be responsible for ROS production (35, 48). It has been shown that elevated plasma level of tumor necrosis factor-alpha is responsible for increased oxidative DNA damage of CD 34^+ cells (53).

It is also possible that prooxidant environment is characteristic for advanced stages of cancer and that oxidative stress is rather a result of the disease development.

CONCLUSIONS

For meaningful interpretation of results derived from different assays of urinary oxidatively modified base/nucleoside, there is a need to answer a key question "from where do these lesions originate?" Data from our laboratories, supported by literature reports, have been largely responsible for exonerating urinary 8-oxoGua and 8-oxodG levels from the influences of diet cell, death/turnover, and artifactual formation. In the absence of these confounding factors, urinary 8-oxoGua and 8-oxodG measurements may be attributed entirely to DNA repair, although the exact contribution of the putative processes, glycosylases, NER, endonuclease(s), and 8-oxodGTPase activity, plus any undiscovered activities, remains to be established. The removal of these impediments to the interpretation of urinary data presents new and exciting challenges, not only determining the relative contributions of the repair pathways to urinary lesion levels, but also investigating DNA repair modulation and associations between repair and disease.

ACKNOWLEDGMENT

RR, DG, and RO were supported by a Foundation for Polish Science fellowship. The authors also acknowledge financial support from the EU NoE "ECNIS" Grant #513943.

ABBREVIATIONS

5HMUra, 5-methylhydroxyuracil; 8-oxodG, 7,8-dihydro-8-oxo-2′-deoxyguanosine; 8-oxoGua, 7,8-dihydro-8-oxoguanine; BER, base excision repair; dG, 2′-deoxyguanosine; Gua, guanine; HPLC, high-performance liquid chromatography; LEP, life span energy potential; MLSP, maximum life span; MTH1, MutT homologue 1; NER, nucleotide excision repair; OGG1, 7,8-dihydro-8-oxoguanine DNA glycosylase; ROS, reactive oxygen species; SD, standard deviation; SMR, specific metabolic rate.

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Address reprint requests to:
Professor Ryszard Olinski
Department of Clinical Biochemistry
Karlowicza 24
85–092 Bydgoszcz, Poland

E-mail: ryszardo@cm.umk.pl

Date of first submission to ARS Central, December 1, 2005; date of acceptance, December 19, 2005.

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