Acta Biochimica Polonica

Vol. 45 No. 1/1998

133–144

QUARTERLY

Minireview

Estimation of oxidative DNA damage in man from urinary excretion of repair products*

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Key words: biomarkers, oxidative DNA damage, 8-oxo-2′-deoxyguanosine, cancer, aging, antioxidants, human

DNA is constantly damaged and repaired in living cells. The repair products of the oxidative DNA lesions, i.e. oxidised nucleosides and bases, are poor substrates for the enzymes involved in nucleotide synthesis, are fairly water soluble, and generally excreted into the urine without further metabolism. Among the possible products, 8-oxo-2′-deoxyguanosine, 8-oxoguanine, thymine glycol, thymidine glycol and, 5-hydroxymethyluracil have so far been identified in urine. It should be emphasised that the excretion of the repair products in urine represents the average rate of damage in the total body whereas the level of oxidised bases in nuclear DNA is a concentration measurement in that specific tissue/cells in the moment of sampling.

The rate of oxidative DNA modifications has been studied in humans by means of the repair products as urinary biomarkers, particularly with respect to 8-oxo-2′-deoxyguanosine. The data obtained so far indicate that the important determinants of the oxidative damage rate include tobacco smoking, oxygen consumption and some inflammatory diseases whereas diet composition, energy restriction and antioxidant supplements have but a minimal influence, possibly with the exception of yet unidentified phytochemicals, e.g. from cruciferous vegetables.

The data are consistent with the experimentally based notion that oxidative DNA damage is an important mutagenic and apparently carcinogenic factor. However, the proof of a causal relationship in humans is still warranted. In the future the use of biomarkers may provide this evidence and allow further investigations on the qualitative

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*Presented at the Conference on "Mechanisms of DNA Repair and Mutagenesis" Commemorating the 100th Anniversary of the Discovery of Polonium and Radium, October, 1997, Warsaw, Poland.

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Abbreviations: 8-oxodG, 8-oxo-2′-deoxyguanosine; 8-oxoGua, 8-oxoguanine; 8-oxoG, 8-oxoguanosine; Tg, thymine glycol; dTg, thymidine glycol; 5-OHmU, 5-hydroxymethyluracil; HPLC, high performance liquid chromatography; GC/MS-SIM, gas chromatography/mass spectrometry-selective ion monitoring; ELISA, enzyme linked immuno sorbent assay; ROS, reactive oxygen species.

and quantitative importance of oxidative DNA modification and carcinogenesis in man, as well as elucidate possible preventive measures.

Oxidative damage to DNA has been proposed to be an important factor in carcinogenesis, a suggestion supported by experimental studies in animals and *in vitro* [1-10]. DNA is constantly damaged and repaired in the cells in the body. The products of repair of these lesions are excreted into the urine. The rate of excretion of the repair products in terms of oxidised nucleosides and bases reflects the average rate of oxidative DNA in the body. Indeed, the excretion rates correspond to a damage rate of up to 10^4 oxidative DNA modifications in each cell every day. This paper will review the use of these urinary excretion products as biomarkers of oxidative DNA damage.

URINARY BIOMAKERS OF DNA DAMAGE

The repair products from oxidative DNA damage, i.e. oxidised bases and nucleosides, are poor substrates for the enzymes involved in nucleotide synthesis, are fairly water soluble, and generally are excreted into the urine without further metabolism [11, 12]. Among the possible repair products from oxidative DNA modifications 8-oxo-2'-deoxyguanosine (8-oxodG), 8-oxoguanine (8-oxoGua), thymine glycol (Tg), thymidine glycol (dTg) and 5-hydroxymethyluracil (5-OHmU) have so far been identified in urine (Fig. 1) [10, 11, 13-17] (Table 1). Of these 8-oxodG and the thymine derivatives have been the most intensively studied ones.

The assays for the urinary DNA repair products include HPLC with detection by electrochemistry for 8-oxodG and 8-oxoGua and by UV absorbance for dTg and Tg, whereas all the repair products can potentially be measured by GC/MS [16, 17]. The major problem with all these assays involves separation of the very small amounts of these products from urine which is a very complicated matter. Thus, although several of the products are electrochemically active and high sensitivity is achievable, the HPLC methods require extensive clean up procedures such as multiple solid phase extractions, HPLC column switching techniques or immunoaffinity columns [11, 13, 18-23]. The complicated extraction procedures cause recovery problems and both HPLC and GC/MS-SIM methods may require labelled internal standards. Moreover, the complicated procedures limit the analytical capacity. An ELISA assay based on monoclonal antibodies has been developed for estimation of 8-oxodG in urine samples [24]. However, the values obtained in rat urine were 3-5 times higher than other published values. Similarly, in four smokers studied before and after smoking cessation the urinary 8-oxodG excretion values estimated by the ELISA method were 8 times higher than, and showed only a weak correlation ($r = 0.42$) with the values obtained by HPLC [25].

The repair pathway of 8-oxoGua in DNA may be discussed in relation to the use of the base or the nucleoside as urinary biomarkers [20]. Thus, two different DNA repair enzymes, one with glycosylase activity and one excising single 8-oxodG as a nucleotide, have been isolated from nuclear extracts of a human cell line [26]. Moreover, the 8-oxodG phosphatase and 8-oxodGMP nucleotidase will selectively and rapidly convert the liberated oxidised nucleotide to a nucleoside ready for excretion [27, 28]. These enzymes will also sanitise oxidised dGTP from the cellular pool and allow its excretion as 8-oxodG. In addition, digestion of damaged DNA derived from cell renewal and mitochondrial turnover will liberate 8-oxodG. Unpublished data from studies on rats indicate that the induction of 8-oxodG in nuclear DNA from target organs corresponds to the increase in urinary excretion after administration of the carcinogen 2-nitropropane, supporting the view that 8-oxodG is the primary repair product *in vivo*.

Experiments on animals have shown that injected 8-oxodG is readily excreted unchanged into the urine [11, 12]. Furthermore, after administration of radiolabelled 8-oxodG in the gut and dG intravenously to rats no labelled 8-oxodG was found in the urine, indicating that 8-oxodG present in the diet or oxidation of dG during excretion does not contribute to the urinary excretion of 8-oxodG [11]. In rats at least, dietary purines are an important determinant of the excretion of 8-oxoGua which is far larger than the excretion of 8-oxodG [20]. In theory, 8-oxoGua could also originate from metabolism of the RNA oxidation product 8-oxoguanosine which is excreted in urine in 3–4 times higher amounts than 8-oxodG [29]. However, in humans the excretion of 8-oxoGua and 8-oxodG are in the same range and both are increased by smoking [15, 18]. Accordingly, although the exact relative importance of the repair pathways remains to be determined, the urinary excretion of 8-oxodG reflects the general average risk of a promutagenic oxidative adduct in DNA of all tissues and organs. Possibly, determination of 8-oxoGua in the urine will be a valuable addition allowing a complete account of the repair of 8-oxodG in DNA.

The 24 h urinary excretion of 8-oxodG shows a seven-fold range within the studied populations and an intersubject coefficient of variation of 30–40% (Table 1). The intraindividual coefficient of variation was 22% in 8 subjects examined twice on a controlled diet for 10 weeks [30], and in 20 subjects studied twice after an interval of 130 days the measured 8-oxodG excretion rates were significantly correlated ($r = 0.73$) [20]. In urine stored at −20°C 8-oxodG was stable for at least 6 years ([30], and unpublished observations). For Tg and dTg excretion the interindividual coefficients of variation range from 28% to 92% (Table 1).

For convenience, the use of spot urine samples corrected for creatinine would be simpler than 24 h collection of urine. However, in 73 healthy subjects from whom we collected urine for 24 h and a spot urine sample from the subsequent voiding, the correlation between the 8-oxodG to creatinine ratio in the spot samples and the 24 h excretion of 8-oxodG was, rather poor ($r = 0.50$). In 20 soldiers studied before and after an extensive exercise program the intra-individual variation in the 8-oxodG to creatinine ratio was sufficiently low, i.e. a coefficient of variation of 50%, to detect a significant effect, despite the potential effects on both 8-oxodG and creatinine production. Accordingly, 8-oxodG to creatinine ratios can be used in cross-over studies with repeated sampling in the same individual whereas 24 h urine collection is preferable in cross sectional studies.

For the use of the urinary excretion of repair products as biomarkers, the extensive repair of oxidative lesions in DNA is assumed. Thus, after ionising radiation the increase in urinary excretion of Tg and 8-oxodG occurred within 24 h in humans whereas excess 8-oxodG was removed from mouse liver DNA after approximately 90 min [31–33]. In a study of 8-oxodG in human brain the accumulation rate in the nuclear DNA corresponded to 2 lesions per cell per day [34]. The reported values of the urinary excretion of the repair product, 8-oxodG, are in the range 200–600 pmol/kg per 24 h which corresponds to an average of 168–504 lesions per day for each of the assumed 5×10^{13} cells in the body [18, 30]. In addition, the alternative repair product, 8-oxoGua appears to be excreted in similar amounts [15]. Accordingly, the calculated repair efficiency under these assumptions ranges from 99.4% to 99.8%.

Due to the extensive and rapid repair the urinary excretion of the repair products will reflect the average rate of oxidative DNA damage in all the cells in the body. In contrast, the levels of oxidised bases in DNA lymphocytes or other accessible cells will reflect the steady state levels, i.e. the balance between damage and repair, albeit only in a surrogate for target tissues.Thus, the two groups of biomarkers are supplementary.

Table 1. Published values regarding urinary biomarkers of oxidative DNA damage in humans

Experimental protocol	Age (yr)	Lesion	Assay	Mean±SD or (range)	Reference
9 healthy subjects	unknown	dTg and Tg	HPLC	390±360 and 100±50 pmol/kg 24 h	[13]
10 healthy subjects *vs* 20 cancer patients	unknown	dTg	HPLC	435±120 *vs* 347±156 pmol/kg 24 h	[61]
10 healthy subjects *vs* 20 cancer patients	unknown	Tg	HPLC	174±54 *vs* 125±45 pmol/kg 24 h	[61]
2 cancer patients before *vs* after radiotherapy	57-59	dTg	GC/MS	8-10 *vs* 20-37 nmol/24 h	[31]
100% *vs* 60% energy in diet (n=1)	50	dTg	GC/MS	250 *vs* 106 pmol/kg 24 h	[16]
14 cancer patients before *vs* after adriamycin therapy	23-73	5-OHmU	GC/MS	74±9 *vs* 96±9 nmol/24 h*	[35]
5 healthy subjects	unknown	8-oxodG	HPLC	323±23 pmol/kg 24 h	[11]
63 healthy subjects	unknown	8-oxodG	HPLC	172±79 pmol/kg 24 h	[20]
23 healthy subjects	unknown	8-oxodG	GC/MS	300±100 pmol/kg 24 h	[16]
53 healthy non-smokers *vs* 30 healthy smokers	40-64	8-oxodG	HPLC	213±84 *vs* 320±99 pmol/kg 24 h*	[18]
21 healthy non-smokers *vs* 12 healthy smokers	33±10	8-oxodG	HPLC	318±130 *vs* 431±168 pmol/kg 24 h*	[36]
24 healthy non-smokers *vs* 10 healthy smokers	unknown	8-oxodG	HPLC	1.0±0.4 *vs* 1.3±0.4 nmol/mmol creatinine*	[19]
Smokers on placebo (n=57) *vs* 8-carotene 20 mg/day (n=65)	39±10	8-oxodG	HPLC	2.8±1.2 *vs* 3.0±1.1 nmol/mmol creatinine	[54]
20 smokers +/- change after vitamin E 100 mg/day	48±6	8-oxodG	HPLC	41±16 -3.8 (95% CI -9 to 1.8) nmol/24 h	[55]
38 smokers +/- change after vitamin C 500 mg/day	45±8	8-oxodG	HPLC	38±17 +2.3 (95% CI -3.8 to 7.8) nmol/ 24 h	[55]
39 smokers +/- change after coenzyme Q 90 mg/day	45±6	8-oxodG	HPLC	42±22 -2.9 (95% CI -5.1 to 10.0) nmol/ 24 h	[55]
19 smokers +/- change after placebo	45±7	8-oxodG	HPLC	53±23 -8.5 (95% CI -18.1 to 1.1) nmol/ 24 h	[55]
300 g vegetable diet *vs* 300 g Brussels sprouts diet (n=5)	young	8-oxodG	HPLC	300-630 *vs* 210-490 pmol/kg 24 h*	[57]
100% *vs* 80% energy in diet (n=16)	35-50	8-oxodG	HPLC	629±218 *vs* 78±189 pmol/kg 24 h*	[30]
100% *vs* 60% energy in diet (n=1)	50	8-oxodG	GC/MS	345 *vs* 110 pmol/kg 24 h	[16]
9 swimmers studied before *vs* immediately after swimming	20±1	8-oxodG	HPLC	2.3±0.5 *vs* 2.5±0.4 nmol/mmol creatinine	[47]
9 runners studied before *vs* immediately after running	21±2	8-oxodG	HPLC	1.4±0.5 *vs* 1.6±0.5 nmol/mmol creatinine	[47]
10 rowers studied before *vs* immediately after a rowing bout	23 (19-30)	8-oxodG	HPLC	(1.6-3.7) *vs* (1.4-14.7) nmol/mmol creatinine	[64]
20 soldiers before and after 30 days of 11 h exercise/day	22±2	8-oxodG	HPLC	1.03±0.59 *vs* 1.25±0.59 nmol/mmol creatinine	[65]

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20 soldiers before and after 30 days of 11 h exercise/day	22±2	8-oxodG	HPLC	1.03±0.59 *vs* 1.25±0.59 nmol/mmol creatinine	[65]

33 benzene exposed workers pre-shift and the following evening	32 (20-53)	8-oxodG	HPLC	0.72 and 0.99 nmol/mmol creatinine*	[50]
63 smoking glass workers *vs* 37 unexposed non-smokers	mean 42	8-oxodG	HPLC	14.2±7.0 *vs* 11.1±5.6 nM *	[66]
7 noncancer patients *vs* 10 cancer patients	unknown	8-oxodG	HPLC	1.1±0.6 *vs* 1.8 to 3.4 nmol/mmol creatinine*	[19]
27 healthy subjects *vs* 136 cancer patients	unknown	8-oxodG	HPLC	1.1±0.6 *vs* 2.1±1.4 nmol/mmol creatinine*	[22]
27 healthy subjects *vs* 136 cancer patients	unknown	8-oxodG	HPLC	15±8 *vs* 18±11 nmol/24 h	[22]
79 cancer patients before *vs* 357 cancer patients after chemotherapy	unknown	8-oxodG	HPLC	1.9±1.0 *vs* 2.6±2.5 nmol/mmol creatinine*	[22]
10 *vs* 11 healthy subjects	20-47 *vs* 62-82	8-oxodG	HPLC	(1.0-5.2) *vs* (0.8-3.9) nmol/mmol creatinine	[56]
10 RA[a] patients *vs.* 33 SLE[b] patients	57-80 *vs* 20-64	8-oxodG	HPLC	(25-32) *vs* (< 1) nmol/mmol creatinine*	[56]
10 healthy subjects *vs* 11 cystic fibrosis patients	mean 9 *vs* 5-16	8-oxodG	HPLC	1.5±0.4 *vs* 2.8±1.2 nmol/mmol creatinine*	[63]
2 cancer patients before and after radiotherapy	57-59	8-oxodG	GC/MS	8-14 *vs* 31-40 nmol/24 h	[31]
6 healthy non-smokers *vs* 7 healthy smokers	22-25 *vs* 23-60	8-oxoGua	HPLC	3.8±1.9 *vs* 7.41±1.5 ng/mg creatinine*	[15]
3 healthy subjects exposed to polluted air	22-25	8-oxoGua	HPLC	max. 12-18 ng/mg creatinine*	[15]
11 healthy subjects before *vs* after exercise	18-35	8-oxoG	HPLC	405±85 *vs* 310±85 pmol/kg 24 h	[29]
After vitamin C and E and 8-carotene supplementation	18-35	8-oxoG	HPLC	390±85 pmol/kg 24 h	[29]
53 healthy subjects	unknown	8-oxoG	HPLC	335±125 pmol/kg 24 h	[20]

* $P < 0.05$; [a]RA, rheumatoid arthritis; [b]SLE, systemic lupus erythemathosus.

DETERMINANTS OF URINARY BIOMAKERS OF OXIDATIVE DNA DAMAGE

The rate of oxidative DNA modifications in humans has been studied by means of the urinary biomarkers in relation to a large number of factors and conditions as summarised in Table 1. In the majority of studies 8-oxodG has been chosen for measurement. There is good agreement between different laboratories regarding the values of urinary excretion of the repair product, whereas the values obtained from DNA isolated from tissues or cells differ by several orders of magnitude, but some of the differences may be related to the choice of analytical method [10].

Cancer therapy

Ionising radiation, a classical and pure source of reactive oxygen species (ROS) particularly hydroxyl radicals, has in a few subjects been shown to increase the urinary excretion rate of 8-oxodG and dTg (Table 1) [22, 31]. Similarly, cancer chemotherapy with adriamycin which induces formation of ROS increased the 24 h urinary excretion of 5-

OHmU, a repair product of thymine oxidation in 14 cancer patients [35]. Many other forms of chemotherapy with or without radiotherapy appear to increase 8-oxodG excretion [22].

Age

So far, no published study has systematically addressed age as a determinant of the markers of oxidative modification of DNA in humans. From the studies involving different age groups it would appear that the urinary excretion rate of the repair products decreases with age (Table 1). In ageing rats urinary 8-oxodG excretion decreased along with accumulation in tissue DNA [3]. These data thus suggest that the rate of damage decreased with age, possibly along with the decreasing rate of metabolism, whereas the steady state levels increased due to failing repair.

Metabolism, exercise and diet

A close relationship between the urinary excretion rate of 8-oxodG and 24-h oxygen consumption or the change in resting metabolic rate after energy restriction has been demonstrated in humans [30, 36]. Similar correlations, including dTg excretion, have been shown across species [16, 37–39]. This apparent relationship between oxidative DNA damage and oxygen consumption is thought to be due to the 1–5% fraction undergoing single electron transfers to generate ROS during mitochondrial respiration [40].

Exercise would be expected to increase the metabolic rate and might thus increase the rate of oxidative DNA damage. However, this notion has not been supported by studies with short term exercise of medium intensity in athletes (Table 1). However, 10 h after a massive exercise effort in terms of a marathon run a 130% increase in the urinary 8-oxodG to creatinine ratio has been reported [41]. Moreover, after a period of 30 days with extensive exercise 8–11 h per day the 8-oxodG to creatinine ratio increased significantly in 20 soldiers [42].

In rodents energy restriction consistently increased the life span and reduced the incidence of spontaneous as well as carcinogen in-

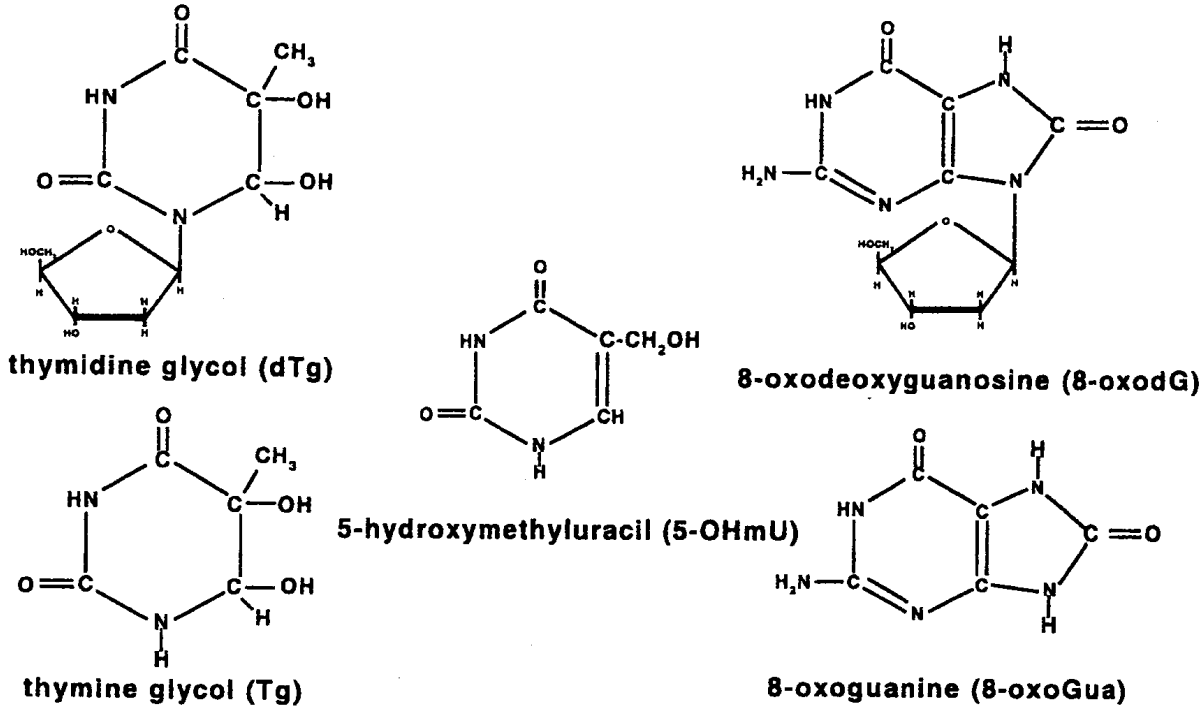

Figure 1. Products of repair of oxidative DNA damage identified in human urine.

duced tumours [43, 44]. This effect may be related to a decreased rate of oxidative DNA damage as shown in nuclear and mitochondrial DNA from liver and mammary gland in energy restricted rats [45, 46]. Similarly, in a single human subject after energy restriction by 40–50% for periods of 10 days, the urinary excretion rate of 8-oxodG and dTg was reduced by 50–80% [16]. However, in a controlled study of 16 subjects on a diet containing 80% of their weight maintaining energy for 10 weeks there was a slight increase in the rate of urinary 8-oxodG excretion (Table1) whereas there was no change in 8 subjects continuing on a diet with 100% of their weight maintaining energy [30].

Tobacco smoking and other environmental exposures

Tobacco smoking has consistently been shown to increase the urinary excretion rate of 8-oxodG by 30–50% (Table 1) [18, 19, 36, 47]. Moreover, 4 weeks after cessation of smoking the 8-oxodG excretion was reduced by 20% in 65 quitters whereas a much smaller decrease was seen in continuing smokers in a controlled study [48]. Similarly, tobacco smoking increased the creatinine corrected concentration of the alternative repair 8-oxoGua in the urine [15].

A number of toxic occupational and other environmental exposures could be expected to act partly by inducing oxidative damage to DNA. Indeed, the creatinine corrected urinary concentration of 8-oxodG correlated significantly ($r = 0.34$) with benzene exposure in 65 fuel filling attendants [49]. Similarly, there was a significant increase in the urinary 8-oxodG to creatinine ratio from pre-shift to the following evening and morning in 33 workers exposed to benzene in gasoline although there was no apparent dose-effect relationship and no corresponding samples available as controls [50]. Asbestos induced 8-oxodG in DNA *in vitro* [51] and rubber, azo dye and asbestos exposed workers from Poland have been reported to have increased ratios of 8-oxodG to creatinine in the urine [52]. In a study of industrial art glass workers increased urinary 8-oxodG concentrations were found in exposed smokers whereas exposed non-smokers, and unexposed smokers and non-smokers showed no significant differences (Table 1). Exposure to heavily polluted urban air for 3 h increased the urinary creatinine corrected concentration of 8-oxoGua, the alternative repair product of 8-oxodG in DNA [15].

Antioxidants and phytochemicals

Antioxidant supplementation could be expected to reduce the rate of oxidative DNA modification. Indeed, the 8-oxodG excretion decrease along with an increase in plasma vitamin C concentration in a controlled smoking cessation study [48, 53]. However, so far intervention studies have not provided support for the notion of a beneficial effect of antioxidants. In smokers daily administration of 8-carotene, vitamin C, vitamin E or coenzyme Q had no effect on the excretion rate of 8-oxodG (Table 1) [54, 55] or the RNA damage product, 8-oxoG [29]. Actually, a positive correlation between the creatinine corrected 8-oxodG levels in urine and the plasma levels of alpha-tocopherol was reported in patients with cystic fibrosis [56] although a biological mechanism for such an association is hard to conceive.

A potential DNA protective effect of specific vegetable components was suggested by a 28% reduction in the rate of urinary 8-oxodG excretion after a diet with 300 g of Brussels sprouts in comparison with 300 g of non-cruciferous vegetables [57]. This effect was reproduced in rats in our laboratory (unpublished data). In a repeat experiment involving humans, however, no sign of reduced 8-oxodG excretion was seen in women and a reduction did not reach statistical significance in men after a diet with 300 g of Brussels sprouts per day for a week [58]. Nevertheless, a new analytical procedure was used in that study and the val-

ues in some subjects were extremely high [58]. Indeed, cruciferous vegetables, such as Brussels sprouts and broccoli, contain certain phytochemicals that are anticarcinogenic possibly by inducing enzymes scavenging electrophiles and by mimicking the cellular protective response to oxidative stress [59, 60].

Diseases

In patients with malignant diseases an increased 8-oxodG to creatinine ratio has been found in two studies [19, 22] although the 24 h excretion was not significantly elevated, suggesting that the apparent effect could be related to decreased creatinine production (Table 1). In another study 24 h excretion rates of Tg and dTg were similar in cancer patients and healthy control subjects [61] (Table 1).

An important role of oxidative damage has been suggested in autoimmune diseases including rheumatoid arthritis and systemic lupus erythematosus. In the urine the 8-oxodG to creatinine ratio was increased in the former patients as compared to healthy controls whereas the latter patients excreted virtually no 8-oxodG and could have defective repair [56]. This could contribute to the pathogenesis and even to the increased risk of malignant diseases in these patients [56, 62].

In patients with cystic fibrosis the creatinine corrected concentration of urinary 8-oxodG in urine was elevated compared to healthy controls, offering a possible explanation of an increased cancer risk in those patients [63]. However, as creatinine production may be affected by this disease these data need confirmation from a study with quantitative urine collection.

CONCLUSION

The rate of oxidative DNA modifications in humans can be studied by means of measurement of the amount of the repair products as urinary biomarkers. Experience regarding the impact of a large number of factors and conditions has been gained, particularly with respect to urinary 8-oxodG excretion.

The data from studies on humans obtained so far indicate that the important determinants of the oxidative damage rate include tobacco smoking, oxygen consumption and some inflammatory diseases, whereas diet composition, energy restriction and antioxidant supplements have but a minimal influence, possibly with the exception of yet unidentified phytochemicals, e.g. from cruciferous vegetables. The data are consistent with the experimentally based notion that oxidative DNA damage is an important mutagenic and apparently carcinogenic factor. However, the search for a proof of a causal relationship in humans is still warranted. In the future the use of the biomarkers may provide this evidence and allow further investigation on the qualitative and quantitative importance of oxidative DNA modification and carcinogenesis in man, as well as elucidation of possible preventive measures.

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