Free Radical Biology & Medicine, Vol. 36, No. 11, pp. 1378–1382, 2004

0891-5849/$-see front matter

ELSEVIER

doi:10.1016/j.freeradbiomed.2004.02.070

Original Contribution

OLIGONUCLEOTIDES IN HUMAN URINE DO NOT CONTAIN 8-OXO-7,8-DIHYDRODEOXYGUANOSINE

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(*Received* 18 *August* 2003; *Revised* 17 *November* 2003; *Accepted* 19 *February* 2004)

Available online 18 March 2004

Abstract—The promutagenic DNA modification 8-oxo-7,8-dihydrodeoxyguanosine is the most frequently used marker for oxidative stress to DNA. The unmodified base and nucleoside and the 8-hydroxylated guanine base and nucleoside are found in urine, the latter used as a global measure of oxidative stress to DNA. Nucleotide excision repair (NER) excises a 27- to 29-mer oligonucleotide with oxidative lesions, and if found in urine, it could be used as a measure of DNA repair in vivo. Enzymatic hydrolysis of human urines followed by HPLC–tandem mass spectrometry was not able to reveal oligonucleotides and/or mononucleotides with the 8-oxo-7,8-dihydrodeoxyguanosine modification. The recovery of a synthetic oligonucleotide with the modification was complete (95% confidence limits: 98–124%). These experiments show that oligonucleotides are excreted into urine, but that 8-oxo-7,8-dihydrodeoxyguanosine is found only as the mononucleoside and is not present in any significant amounts in oligonucleotides. We conclude that oligonucleotides are excreted into urine, and they do not contain oxidized lesions. Either NER products are degraded after excision or NER functions differently in vivo in humans compared with cellular systems.

INTRODUCTION

Urinary excretion of the oxidized guanine moiety 8-oxo-7,8-dihydrodeoxyguanosine (8-oxodG) has been advocated as a measure of oxidative stress to DNA [1–3] and it has been used as such in both humans and experimental animals. With this methodology it has been demonstrated that environmental and lifestyle factors like tobacco smoking [4], extensive exhaustive exercise [5], and urban air pollution [6], to DNA, in addition to physiological factors such as sex [4], and energy expenditure [7], and pharmacological factors such as estrogen supplementation [4], are important in oxidative stress to DNA. It has been suggested that urinary excretion of oligonucleotides with oxidative modifications may represent an unrecognized source of 8-oxodG [8,9] and that this could lead to underestimation of the urinary excretion of 8-oxodG.

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The origin of 8-oxodG excreted in urine has not been established. In experimental animals the excess amount of 8-oxodG induced in various organs corresponds to excess excretion into urine within 24 h when repair has normalized the organ levels of 8-oxodG [10]. This finding speaks in favor of the origin of 8-oxodG being products from DNA repair. Several other origins are possible but have so far not been evaluated quantitatively and experimentally: cell turnover, cell death, oxidation of the nucleotide pool. Dietary origin has also been suggested but does not appear to be a relevant source in humans [11].

Several DNA repair enzyme systems specific for 8-oxodG have been identified. The present concept is that the different repair systems are guardians of longevity and protection against mutagenesis leading to cancer, which can explain why different inborn errors or mouse knockout models are prone to develop aging syndromes or cancer [2]. Base excision repair (BER) of 8-oxodG is a multiple enzyme pathway initiated by the glycosylase OGG1 that cleaves the glycosidic bond and liberates the oxidized base (8-oxoGua) from DNA [13]. Other glycosylases include

Table 1

	Free nucleosides[a]				Bound in oligonucleotides[b]				Ratio of bound/free nucleosides[c]			
	dA	dC	dG	dT	dA	dC	dG	dT	dA	dC	dG	dT
Urine A	9.2	12.4	5.2	14.5	116.4	60.0	95.0	63.5	13	5	18	4
Urine B	16.8	12.7	5.7	13.6	36.9	23.3	39.6	27.4	2	2	7	2
Urine C	15.6	16.3	7.6	25.3	637.2	436.3	518.2	604.0	41	27	68	24
Urine D	25.0	13.3	8.3	11.3	20.1	12.1	18.2	17.3	1	1	2	2
Urine E	41.5	22.4	15.8	21.8	92.1	52.6	70.4	84.9	2	2	4	4
Urine F	34.9	15.4	22.7	25.3	69.1	45.3	56.5	64.5	2	3	2	3

[a] Urinary free nucleoside concentrations (nM) are given for the four nucleosides in DNA.
[b] Concentrations (nM) of the four DNA nucleosides liberated from urine after enzymatic hydrolysis (Bound in oligonucleotides); the contribution from the free nucleosides is not included.

hMYH (human mutY homolog), uracil DNA glycosylase, *N*-methylpurine glycosylase, human NTH (hNTH1), and ribosomal S3 protein [12,14]. The nucleotide excision repair pathway (NER) gives products consisting of 27- to 29-mer oligonuclotides containing the lesion [15], which, on later cleavage, could be excreted into the urine. Also 8-oxodGTP in the nucleotide pool, which is sanitized by hMTH1, the human homolog of mutT, could result in 8-oxodG. If oligonucleotides containing 8-oxodG were excreted into urine they might provide a way to estimate NER activity in vivo in humans.

In the present paper we examined if urines contain oligonucleotides and if they contain 8-oxodGs, based on a recently developed LC-MS/MS assay that makes it possible to quantify the 8-oxo-modified and unmodified guanine base, nucleoside, and ribonucleoside [16].

MATERIAL AND METHODS

Urine stored at −20°C originated from a published intervention trial [17] where our database contained information about 24 h urinary 8-oxodG excretion on all subjects. Six urines from the control group were selected so that three samples contained a small amount of 8-oxodG and three samples contained a very large amount of 8-oxodG. This strategy was chosen to cover a broad range of urinary excretion patterns based on the idea that oligonucleotides in the urine could relate to or could relate inversely to the excretion of 8-oxodG.

Two oligonucleotides (22-mers) were synthesized as previously described [18]. One oligonucleotide contained one 8-oxodG and no dGs, while the other contained 5 dGs and no 8-oxodG. By spiking urine samples with these oligonucleotides (final concn. of 8-oxodG was 16.1 nM), artificial oxidation could be controlled and liberation of nucleosides from the oligonucleotides could be estimated. In addition to the spiking with the defined oligonucleotides, urine was subjected to enzymatic hydrolysis following the same protocol as enzymatic hydrolysis of tissue DNA for the measurement of 8-oxodG in nuclear DNA [19].

Table 2. 8-oxodG Content of Six Urine Samples Subjected to Five Different Pretreatments before Analysis[a]

	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Hydrolysis contribution	% Change	Increase from added oligonucleotide
Urine A	4.2	4.3	4.1	21.7	4.6	0.0	0.9	17.4
Urine B	4.5	4.6	4.5	22.4	4.9	0.0	0.8	17.8
Urine C	4.2	4.8	4.4	21.8	4.5	0.6	15.1	16.9
Urine D	15.6	15.3	16.5	34.4	15.7	-0.3	-2.1	19.1
Urine E	18.5	17.0	17.7	35.9	17.6	-1.5	-8.1	18.9
Urine F	10.4	11.4	10.5	28.3	10.5	1.0	9.5	16.9
Mean						0.0	2.7	17.9
SD						0.86	8.3	0.96

The Hydrolysis contribution is calculated by subtracting the value under Treatment 1 from the value under Treatment 2. The increase from added oligonucleotide is calculated by subtracting the value under Treatment 2 from the value under Treatment 4. The added amount of 8-oxodG from the synthetic oligonucleotide corresponds to 16.1 nM. All values in nanomolar unless otherwise indicated.

[a] Treatments: (1) raw urine; (2) urine treated with nuclease P1 and alkaline phosphatase; (3) raw urine spiked with a synthetic oligonucleotide containing 8-oxodG, but no dG; (4) as in (3) but treated with nuclease P1 and alkaline phosphatase; (5) raw urine spiked with a synthetic oligonucleotide containing dG, but no 8-oxodG, and treated with nuclease P1 and alkaline phosphatase.

We also tested that synthetic di- and trimers, as well as monophosphate and triphosphate monomers, were hydrolyzed by the enzymes to ensure that total hydrolysis of small oligonucleotides occurred (data not given).

The free nucleosides were measured with a previously described LC-MS/MS method slightly modified to include dA, dC, and dT [20].

RESULTS

The six urine samples contained oligonucleotides and/or mononucleotides as evidenced by the increased concentrations of dA, dC, dG, and dT after hydrolysis of the urine (see Table 1). The hydrolysis liberated 2- to 69-fold greater amounts of the four deoxynucleosides in the urines, though in four of the persons the liberation was only 2- to 8-fold (Table 1). However, no increase in the level of 8-oxodG could be found after hydrolysis of the endogenous oligonucleotides in the urine (Table 2, treatment 1 vs. treatment 2). The hydrolysis of oligonucleosides in the urine was effective as indicated by the experiments with addition of synthetic oligonucleotides and measurements with and without hydrolysis (Table 2). By comparison of treatments 1 and 3 it is seen that no 8-oxod-G is liberated from an 8-oxodG-containing oligonucleotide without enzymatic hydrolysis. By comparison of treatments 1 and 4, it is seen that 8-oxodG is liberated on enzymatic hydrolysis of the synthetic 8-oxodG-containing oligonucleotide. By comparison of treatments 1 and 5, it is seen that no artificial 8-oxodG is formed on enzymatic hydrolysis of dG-containing oligonucleotides. To ensure that the nucleosides from very small oligonucleotides were also liberated, hydrolysis of monomers and di- and trimer oligonucleotides was tested and they too were found to be fully hydrolyzed (data not shown). Table 2 also gives the results of the experiment as means and SD of the analysis of the six urine samples. The values are given as percentage change in 8-oxodG from the analysis of untreated urine, i.e., no oligonucleotide addition and no enzymatic hydrolysis, as the levels of the absolute amount of 8-oxodG varied in the chosen urine samples.

The 95% confidence limits of the 8-oxodG released by enzymatic hydrolysis were −1.2 to 6.2%, indicating that oligonucleotides containing 8-oxodG are not present in human urine. As can be seen from Table 2, spiking the urine with an oligonucleotide containing 8-oxodG followed by hydrolysis increased the concentration of 8-oxodG by an average of 17.9 ± 0.96 nM (95% confidence limits, 98–124%). Because the added 8-oxodG from the synthetic oligonucleotide was 16.1 nM we can conclude that all the 8-oxodG was released.

DISCUSSION

During metabolic processes in the cell, free oxygen radicals are formed that modify important cellular macromolecules, including DNA. The most abundant identified base modification is 8-hydroxylation of the guanine moiety in DNA, a lesion that is mutagenic, leading to GC–TA transversion mutations such as those found in important genes like p53 and various oncogenes [21]. On this basis the 8-oxodG lesion has been suggested as an important mechanism for endogenously induced mutations and a cause of cancer and aging in humans [12].

The importance of endogenous induced DNA lesions is also indicated by the multiple specific DNA repair systems that are highly efficient in the repair of oxidative lesions [14]. It has been estimated that if these DNA repair systems did not operate, several percent of DNA would be oxidized within a few years [22]. Two main DNA repair pathways have been described: base excision repair (BER) and nucleotide excision repair (NER). Although it has been suggested by several authors that the urinary excreted nucleoside 8-oxodG results from NER and that the urinary excretion of the corresponding base 8-oxoGua results from BER, this is supported only by cellular studies, and there are no quantitative data in vivo supporting this hypothesis, except the finding that the modified base is excreted in about 10-fold larger amounts than the nucleoside [16]. Oxidative stress, e.g., from tobacco smoking, increases the urinary excretion of 8-oxodG [4,23]; however, only few data describe the excretion of the modified base and so far there are no published data on its relationship to oxidative stress in humans.

Commercial immunologically based methods have been proposed for the analysis of 8-oxodG in urine; however, they appear to be unspecific and to clearly overestimate the urinary concentration [24]. It has been argued that oligonucleotides from the NER pathway containing the 8-oxodG lesion were present in urine and only detected by the immunological methods. We now present direct measurements that demonstrate (1) human urine contains only limited amounts of oligonucleotides and/or mononucleotides and (2) these oligonucleotides and/or mononucleotides do not contain oxidative modifications in the form of 8-oxodG. We verified the methodology of hydrolyzing the urinary oligonucleotides by adding synthetic 22-mer oligonucleotides with and without the 8-oxodG modification and can conclude that urine from humans does not contain oligonucleotides and/or mononucleotides with the 8-oxodG modification in any significant amounts. In one of the subjects, a rather high concentration of oligonucleotides was measured (subject C, Table 1), whereas another (subject A) excreted an intermediate concentration of oligonucleotides, measured as nucleosides. We do not

know the size distribution of the nucleotides; however, these two persons could have excreted a few very large oligonucleotides, e.g., as a result of urinary tract cell breakdown.

The overall limited excretion of oligonucleotides speaks against a contribution in urine of oligonucleotides or nucleosides originating from cell death in the urinary tract, and certainly oxidatively modified oligonucleotides do not seem to be present.

The origin(s) of the oligonucleotides and/or mononucleotides found in urine is not known. They can originate from the various types of DNA repair of the multiple different lesions found in DNA. As argued above, an origin from cell breakdown/turnover in the body and excretion via the kidneys to urine, or locally in the kidneys, the urinary tract, and the bladder epithelium is not particular likely. Oligonucleotides developed as drugs or originating from infectious agents are metabolized in the body and are also excreted into urine [25–27], but detailed pharmacokinetic analysis of labeled oligonucleotides is not available and thus cannot help to identify the origin of the oligonucleotides found in this study. Presently, it is not possible to determine the origin of the nucleotides excreted into urine.

We conclude that human urine contains oligonucleotides but without oxidative lesions in the form of 8-oxodG. Either NER products, i.e., 27- to 29-mer oligonucleotides with oxidative lesions, must be degraded after excision or NER functions differently in vivo in humans compared with cellular systems. The organ or tissue origin of the nucleotides remains obscure, and it has not been determined if 8-oxodG excreted into urines originates from NER or another source.

Acknowledgments—We thank Mads Sabroe Jensen and Lis K. Hansen for expert technical assistance. Work was supported by the EU Framework 5 program DNAge and the Danish Medical Research Council.

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ABBREVIATIONS

BER — base excision repair
LC-ECD — HPLC with electrochemical detection
LC-MS/MS — HPLC coupled with tandem mass spectrometry
NER — nucleotide excision repair
8-oxodG — 8-oxo-7,8-dihydrodeoxyguanosine
8-oxoGua — 8-oxo-7,8-dihydroguanine