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Generation of Guanine -Thymine Crosslinks in Human Cells by One-Electron Oxidation Mechanisms

Guru S. Madugundu[†], Richard Wagner[†], Jean Cadet^{†,‡}, Konstantin Kropachev[§], Byeong Hwa Yun^{§,‡}, Nicholas E. Geacintov[§], and Vladimir Shafirovich^{§,*}

[†]Département de Médecine nucléaire et Radiobiologie, Faculté de Médecine, 3001 12e Avenue Nord, Université de Sherbrooke, Québec J1H 5N4, Canada

[#]Institut Nanosciences & Cryogénie, CEA/Grenoble, 17 Avenue des Martyrs, 38054 Grenoble, France

[§]Department of Chemistry, New York University, 31 Washington Place, New York, NY 10003-5180, United States

Abstract

The one-electron oxidation of cellular DNA in cultured human HeLa cells initiated by intense nanosecond 266 nm laser pulse irradiation produces cross-links between guanine and thymine bases (G*-T*), characterized by a covalent bond between C8 guanine (G*) and N3 thymine (T*) atoms. The DNA lesions were quantified by isotope dilution LC-MS/MS methods in the multiple reaction-monitoring mode using isotopically labeled [¹⁵N, ¹³C]-nucleotides as internal standards. Among several known pyrimidine and 8-oxo-7,8-dihydroguanine lesions, the G*-T* cross-linked lesions were detected at levels of ~0.21 and 1.19 d(G*-T*) lesions per 10⁶ DNA bases at laser intensities of 50 and 280 mJ/cm²/pulse, respectively.

The initiation of many human cancers is tightly associated with chronic inflammation.¹ At sites of chronic inflammation cellular DNA is under continuous attack from free radicals, electrophiles and other reactive species that generate a wide spectrum of DNA base modifications.² Failure of removal of these lesions by the DNA repair machinery, which maintains the integrity of the human genome, increases the risk of malignant cell transformation and further cancer development.³ The major target of oxidizing species overproduced during persistent oxidative stress associated with chronic inflammation is guanine, the most easily oxidizable natural DNA base.⁴

The mechanisms of initiation of the oxidation of guanine in cellular DNA are of significant current interest.^{5–7} The best known and most ubiquitous stable oxidation product is 8-oxo-7,8-dihydroguanine, 8-oxoGua (Chart 1), derived from free radicals or singlet oxygen attack on DNA.⁸ Guanine radical formation in vitro can occur by two principle pathways: (i) reaction with hydroxyl radical (·OH),⁹ and (ii) the one-electron oxidation of guanine.^{10, 11} The 8-hydroxy-7,8-dihydroguanyl radical, 8-HO-G·, arising from the addition of ·OH to C8

*Corresponding Author: vs5@nyu.edu. Telephone: (212) 998 8456.

[‡]Present Address

Division of Environmental Health Sciences, Wadsworth Center, NYS Department of Health, Empire State Plaza, P.O. Box 509, Albany, New York 12201-0509, United States

ASSOCIATED CONTENT

Supporting Information. Protocols of DNA extraction from HeLa cells, enzymatic digestion of DNA, synthesis of isotope labeled standards and LC-MS/MS methods for quantitation of DNA lesions. This material is available free of charge via the Internet at <http://pubs.acs.org>.

The authors declare no competing financial interest.

of guanine is reducing and is rapidly oxidized by oxygen, or other weak oxidants, to yield the 8-oxoGua lesion.¹² The guanine radical cation, $G^{\cdot+}$, derived from the one-electron oxidation of guanine is a strong electrophile, that can react with nucleophiles such as water¹⁰ and amino acids,¹³ and deprotonates to yield the neutral guanine radical intermediate, $G(-H)^{\cdot}$.¹⁴ In double-stranded DNA, reaction of $G^{\cdot+}$ with water is dominant, and the 8-HOG $^{\cdot}$ radical intermediate is then oxidized to yield 8-oxoGua.¹² The $G(-H)^{\cdot}$ radical is a long-lived,¹⁵ relatively strong electrophile. We have found¹⁶ that in single-stranded DNA, the $G(-H)^{\cdot}$ radicals readily react with nearby thymine bases to form the intrastrand guanine-thymine cross-links, G^*-T^* with a covalent bond between the guanine C8 (G^*) and thymine N3 (T^*) (Chart 1). These cross-links are also formed in double-stranded native DNA when exposed to oxidizing carbonate radical anions that are derived from the spontaneous decomposition of nitrosoperoxycarbonate, an important chemical mediator of inflammation, that arises from the combination of peroxynitrite and CO_2 in aqueous solutions.¹⁷ Enzymatic digestion of ... G^*pT^* ... cross-links yields the $d(G^*pT^*)$ dimer, while digestion of ... G^*NT^* forms the full digestion product $d(G^*-T^*)$.^{16, 17}

The formation of the G^*-T^* lesions is base sequence context-dependent and their levels should be significantly lower than the formation of 8-oxoGua which has an abundance of the order of one 8-oxoGua lesion per ~ million guanines.¹⁸ However, there is as yet no evidence that G^*-T^* cross-links are formed in human cells.

In this work we demonstrate that, in principle, $G^{\cdot+}$ radical cations and/or their $G(-H)^{\cdot}$ deprotonation products¹⁵ can react with nearby thymine nucleophilic centers other than with water in a cellular environment. Specifically, we demonstrate that G^*-T^* cross-linked products are produced in cultured human HeLa cells when $G^{\cdot+}$ radicals are generated. The one-electron oxidation of guanine in DNA in HeLa cells was initiated by intense nanosecond 266 nm Nd: Yag laser pulses. It is well established that this mode of irradiation induces two-photon ionization of nucleobases in isolated native double-stranded DNA; as a result, the oxidatively generated DNA damage is predominantly localized at guanine sites.¹⁹ The HeLa S3 cells (ATCC, Manassas, VA) were grown in culture using standard methods. The cells were harvested by trypsinization followed by centrifugation at 1,000 rpm for 5 min at 4 °C. The cell pellets were washed with cold 20 mM phosphate buffer, pH 7.8. The samples of HeLa cells (~10⁷ cells) were resuspended in this buffer solution (1.5 mL), placed in a 1×1 cm square quartz cell equipped with a magnetic stirrer, and exposed to 266 nm Nd: Yag laser pulse (~6 ns duration) train (10 Hz) to deliver a total energy of 120 J. To explore the effect of laser pulse intensity, the samples were irradiated at two different laser intensities of 50 and 280 mJ/cm²/pulse. The irradiated samples were placed in liquid nitrogen to terminate any ongoing DNA repair processes. The DNA was extracted using the NaI method^{20, 21} and subsequently digested by the protocols described in Supporting Information. The lesions produced by the laser excitation were quantified by isotope dilution LC-MS/MS methods²² using isotopically labeled [¹⁵N, ¹³C]-nucleotides as internal standards (for additional details, see the Supporting Information). These results are summarized in Table 1.

We found, as expected, that the major product is 8-oxoGua, detected at a level of 263±85 8-oxoGua/10⁶ DNA bases at a laser intensity of 50 mJ/cm²/pulse that is significantly higher than 4.8±3.4 8-oxoGua/10⁶ DNA bases in control non-irradiated samples (Table 1). The level of 8-oxoGua lesions increases up to 936±28 8-oxoGua/10⁶ DNA bases when the laser intensity is increased to 280 mJ/cm²/pulse at the same total energy dose of 120 J. This is a clear indication that the precursor of 8-oxoGua lesion is a guanine radical cation produced by two-photon ionization of guanine bases.¹⁹ The hydration of guanine radical cations leads to the formation of the 8-hydroxy-7,8-dihydroguanyl radical, which is rapidly oxidized by O₂, or other weak oxidants, to the 8-oxoGua lesion.⁸

The G*-T* cross-linked lesions were detected at a level of 0.21 ± 0.11 d(G*-T*) per 10^6 DNA bases at the weaker laser intensity of $50 \text{ mJ/cm}^2/\text{pulse}$. This level increased to 1.19 ± 0.12 d(G*-T*) per 10^6 DNA bases when the laser intensity was increased to $280 \text{ mJ/cm}^2/\text{pulse}$ (Table 1). This enhancement in d(G*-T*) levels is correlated with the increase in 8-oxoGua levels; this is consistent with the assumption that guanine radicals, derived from the two-photon ionization of guanine, are common intermediates in the formation 8-oxoGua and the G*-T* cross-links. In contrast, we were unable to detect d(G*pT*) lesions that might have arisen from neighboring ...GT... bases in the irradiated and the controlled samples. This d(G*pT*) lesion is resistant to enzymatic digestion under our conditions and cannot be a precursor of the d(G*-T*) lesion formed in ...GpT... sequence context.^{16, 17}

We also measured the levels of some other representative pyrimidine lesions, such as 5-hydroxycytosine (4), 5-(hydroxymethyl)uracil, (5), 5-formyluracil (6), and 5,6-dihydroxy-5,6-dihydrothymine (7), all shown in Chart 1. The levels of cytosine lesions increase from ~21 to ~51 5-OHCyt per 10^6 DNA bases when the laser intensity is increased from 50 to $280 \text{ mJ/cm}^2/\text{pulse}$ and is significantly greater than the background level of ~0.1 5-OHCyt per 10^6 DNA bases in the unirradiated control samples. In turn, variations in the levels of thymine lesions are in the range of 28 – 70 5-HmUra and 28 – 71 5-ForUra per 10^6 DNA bases. These levels are also significantly higher than the background levels of ~1.9 HmUra and ~1 5-ForUra per 10^6 DNA bases, respectively, in the unirradiated control samples. The levels of thymine glycols were in the range of 11 – 22 Tg per 10^6 DNA bases that are lower by a factor of ~3 than the levels of 5-HmUra and 5-ForUra lesions. A similar product distribution was reported in the DNA of human monocytes upon exposure to high-intensity 266 nm pulses.²³ The higher than expected amount of 8-oxoGua in non-irradiated cells may be attributed to auto-oxidation of Gua during sample preparation likely because we did not prepurify the nuclear DNA before extraction. Nevertheless, the increases of 8-oxoGua were overwhelming in the DNA of irradiated cells. The much higher yield of 8-oxoGua relative to pyrimidine oxidation products may be at least partly rationalized in terms of preferential hole transfer to guanine bases.

The formation of oxidatively generated pyrimidine damage may, potentially, arise from two different pathways: (i) the direct photoionization of pyrimidine bases, or (ii) by hydroxyl radical-mediated mechanisms. Guanine has the lowest ionization potential among the four natural DNA bases, but pyrimidines can be also ionized by intense 266 nm laser pulse irradiation, although with lower efficiencies than guanines.¹⁹ Highly reactive $\cdot\text{OH}$ radicals, which rapidly and unselectively react with all DNA bases,⁹ may also have contributed to the observed oxidatively generated pyrimidine damage in cellular DNA.^{5, 24} In our experiments, the precursor of $\cdot\text{OH}$ radicals may be the unreactive superoxide radical anion ($\text{O}_2^{\cdot-}$) that arises from the reduction of molecular oxygen by hydrated electrons derived from the two-photon ionization of DNA bases, and/or by photoexcitation of diverse endogenous cellular chromophores that are capable of absorbing light in the UV spectral range.⁵⁻⁸

Box and co-workers^{25, 26} discovered that $\cdot\text{OH}$ radicals produced by ionizing radiation generate intrastrand cross-links between guanine and adjacent pyrimidine (C or T) bases. In these so-called tandem lesions, C8 of guanine is covalently linked either to C5 of cytosine or to the methyl group carbon atom of thymine. Tandem lesions produced by $\cdot\text{OH}$ radicals have been investigated in detail *in vitro*²⁷ and detected in HeLa S3 cells.^{28, 29} Since the existence of these d(G[8-5m]T) lesions have already been demonstrated in HeLa cells by the Y. Wang group,^{28, 29} we focused here on the detection of the recently discovered G*-T* (G[8-3]T) lesions. Furthermore, our previous work suggests that the d(G[8-5m]T) cross-links are not observed when one-electron oxidants¹⁶ are used to oxidize guanine in DNA, while the d(G[8-5m]) cross-links are generated by hydroxyl radical mechanism.^{25, 26}

In cells exposed to ionizing radiation, the levels of guanine (1) and pyrimidine (4–7) DNA lesions (Chart 1) formed are in the range of 5 – 11 lesions/10⁹ DNA bases/Gy;²² these values are well correlated with the levels and distributions of these lesions produced by ionizing radiation in isolated native DNA.^{23, 24} The unselective generation of oxidatively damaged DNA bases is characteristic of the levels found when native DNA is exposed to ·OH radicals produced in aerated aqueous solutions by the well-known Fenton-like reactions (Fe²⁺ or Cu⁺ with H₂O₂ and ascorbate).²² In HeLa cells exposed to intense 266 nm laser pulse irradiation, the levels of 8-oxoGua lesions are higher by factors of 10 – 40 than the levels of pyrimidine lesions because guanine is selectively oxidized by the one-electron oxidation of guanine bases by the two-photon ionization mechanism (also by hole transfer migration to guanine residues).

In summary, we show here for the first time, that the nucleophilic addition of thymine bases to guanine radicals can lead to the formation of detectable levels of G*-T* cross-links in cultured human cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

LC-MS/MS	liquid chromatography coupled to tandem mass spectrometry
MRM	multiple reaction monitoring
G*-T*	guanine-thymine cross-link with a covalent bond between the C8 (G*) and thymine N3 (T*) atoms
8-oxoGua	8-oxo-7,8-dihydroguanine
5-OHCyt	5-hydroxycytosine
5-HmUra	5-(hydroxymethyl)uracil
5-ForUra	5-formyluracil
Tg	5,6-dihydroxy-5,6-dihydrothymine

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Chart 1.
Structures of DNA lesions.

Table 1

DNA Damage (in units of damage per 10^6 bases) Detected in HeLa Cells Exposed to Intense Nanosecond 266 nm Laser Pulse Excitation (120 J)^a

DNA damage	Laser Intensity, mJ/cm ² /pulse		Unirradiated control
	280	50	
G*-T*	1.19±0.03	0.21±0.11	0.02±0.05
8-oxoGua	936±28	263±85	4.8±3.4
5-OHCyt	51.0±6.7	21.0±7.2	0.08±0.19
5-HmUra	70.2±4.8	28.4±3.7	1.9±0.4
5-ForUra	71.3±5.1	28.4±3.8	1.0±2.0
Tg	22.3±2.8	11.9±2.4	1.9±1.2

^aThe results are averages derived from five samples.