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A methodology for estimating localization of apurinic/apyrimidinic sites in DNA using fluorescence resonance energy transfer

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ABSTRACT

We have developed a methodology for estimating localization of lesions on double-stranded DNA using fluorescence resonance energy transfer (FRET). We focused on apurinic/apyrimidinic (AP) sites, which are typical DNA lesions induced by radiation and chemicals and produced spontaneously under physiological conditions. Donor–acceptor fluorescent probes with *O*-amino groups (Alexa Fluor 350–Alexa Fluor 488 dye pair) were used for selectively labeling AP sites. pUC19 plasmid subjected to heat treatment (pH 5.2, 70 °C) was used as a model double-stranded DNA containing AP sites. The results of both FRET analysis and theoretical study enabled us to prove that AP sites induced by the heat treatment are distributed almost randomly along the DNA molecule. This methodology will be useful for estimating the risk of ionizing radiation and chemicals (e.g., pollutants and anticancer agents) based on the probability of producing "clustered DNA damage sites," which are considered to be less easily repairable and, therefore, more harmful to living systems.

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DNA damage induced by radiation, chemicals, and physiological causes is responsible for mutation and carcinogenesis [1,2]. Most isolated lesions are corrected by repair enzymes and recombination systems. By contrast, clustered damage sites, in which two or more lesions occur within a few helical turns, are not expected to be repaired completely [3,4]. Studies using plasmid vectors carrying an artificial clustered damage site have clearly demonstrated that such sites are highly mutagenic [5,6]. On the other hand, the existence of clustered damage sites induced by ionizing radiation has been confirmed only indirectly by experiments quantifying single- and double-strand breaks using base excision enzymes as probes [7–11] or predicted by Monte Carlo track simulations [12]. Three questions arise from these approaches to investigating the quantity and quality of clustered damage sites. First, are "artificial" clustered damage sites similar to those actually produced by DNA-damaging agents? Second, to what extent do previously used quantitative methods that depend on functions of base excision enzymes reflect the real amount of "unrepairable" clustered damage sites? Third, what are the differences among types of ionizing radiation (or chemical agents) in terms of the probability of producing a clustered damage site? Regarding the second question in particular, there are uncertainties about the behaviors of base excision repair (BER)[1] enzymes at clustered damaged sites. After all, little is known about the "real" structure of these sites. To answer these questions, a novel methodology that can directly evaluate the spatial distribution of DNA lesions needs to be developed.

In this study, focusing on the manner in which damage is distributed on DNA, we have developed a methodology for estimating the proximity of lesions on DNA. Our method is an application of a photochemical phenomenon, fluorescence resonance energy transfer (FRET), and does not rely on the use of BER enzymes. The FRET technique, a "nanometer ruler," has been widely used for estimating distances between biomolecules and studying their molecular dynamics [13]. Fortunately, the range of FRET is generally shorter than 10 nm, comparable to the standard definition of a clustered damage site (two or more lesions within a few helical turns, i.e., <7 nm). For this study, we selected the apurinic/apyrimidinic (AP) site as the target lesion because it has an aldehyde moiety that can selectively react with a probe containing an *O*-amino group via Schiff's base-type covalent bonding [14]. In fact, a lot of applications using *O*-amino group for AP site detection have already been

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[1] *Abbreviations used:* BER, base excision repair; FRET, fluorescence resonance energy transfer; AP, apurinic/apyrimidinic; ARP, aldehyde reactive probe; MS, mass spectrometry; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; AF, Alexa Fluor; DMSO, dimethyl sulfoxide; UDG, uracil DNA *N*-glycosylase; PDI, phosphodiesterase I; ex, excitation; em, emission; dsb, double-strand break; ssb, single-strand break; oxPu, oxidized purine; oxPy, oxidized pyrimidines.

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reported. In particular, the aldehyde reactive probe (ARP) with biotin moiety for enzyme-linked immunosorbent assay (ELISA)-like assay is useful for high-sensitive AP site detection in vitro [15,16] and in living cells [17]. Moreover, Roberts and coworkers reported an accurate AP site detection method without overestimation based on mass spectrometry (MS), high-performance liquid chromatography (HPLC)–electrospray ionization (ESI)–tandem MS (MS/MS) [18]. However, most of the previous reports were not concerned with AP site localization or distribution in damaged DNA.

To develop a method to measure the distance between AP sites, it was necessary to discover the relationship between FRET efficiency and the separation of AP sites. We first developed a model to calculate the distance between fluorophores as a function of base separation and experimentally determined the distance between the labeled fluorophores at AP sites. This enabled us to semiempirically determine the relationship between the AP site separation (i) in base pairs and the corresponding FRET efficiency (e_i). To demonstrate that the FRET method could actually measure the spatial distribution of the AP sites, we constructed a de novo theoretical model that estimates the observed FRET efficiency (E) for randomly distributed AP sites. Using this model combined with the (e_i) versus (i) relationship, theoretical E values were calculated. The theoretical curves of E exhibited a good fit to experimental E values for heat-induced AP sites, providing support for the idea that our new FRET methodology could evaluate the spatial distribution of DNA lesions. The methodology developed here enables us to determine how differences among types of radiation influence the AP site (or aldehyde/ketone moiety) distribution in DNA. This method will also be applicable for lesions caused by DNA-damaging chemicals such as pollutants and anticancer agents.

Materials and methods

Chemicals

pUC19 plasmid vector (2686 bp, 1 mg/ml, in 10 mM TE [Tris–ethylenediaminetetraacetic acid] buffer at pH 8.0) was purchased from Bayou Biolabs (Haralan, LA, USA). The "donor" (***D***) and "acceptor" (***A***) fluorescent probes with *O*-amino functional groups (Alexa Fluor 350 [AF350] and Alexa Fluor 488 [AF488] dyes, respectively) were purchased from Life Technologies Japan (Tokyo, Japan). Dimethyl sulfoxide (DMSO) and phenol were obtained from Nacalai Tesque (Kyoto, Japan). In advance, each of the fluorescent dyes (net: 1–1.05 mg/tube) was dissolved in DMSO to a concentration of exactly 10 mM. Synthetic single-stranded DNA 31-mers with a 2′-deoxyuridine in the center of each strand (Strand1: 5′-ctgcgcgtggtttct**u**agatcataataggag-3′; Strand2: 5′-ctcctattatgatc t**u**agaaaccacgcgcag-3′), purified by polyacrylamide gel electrophoresis (PAGE), were obtained from Tsukuba Oligo Service (Ibaraki, Japan).

Enzymes

Uracil DNA *N*-glycosylase (UDG, from *Escherichia coli ung* gene, 1 U/μl) was obtained from Eurogentec (Liege, Belgium). DNase I (2 U/mg, from bovine pancreas) was obtained from Wako Pure Chemicals Industries (Osaka, Japan). *Sma*I (from *Serratia macescens* Sb, 12 U/μl) was obtained from Toyobo (Osaka, Japan). Phosphodiesterase I (PDI, from *Crotalus adamanteus* venom, 36.2 U/mg) was obtained from USB (Cleveland, OH, USA). The unit definitions of these nucleases are as follows. For UDG, 1 U is defined as the amount of enzyme that catalyzes the release of 60 pmol of uracil per minute from double-stranded uracil-containing DNA in the reaction buffer (20 mM Tris–HCl [pH 8.0], 1 mM EDTA, and 1 mM dithiothreitol [DTT]) at 37 °C. For DNase I, 1 U is defined as the amount of enzyme that increases absorbance of 0.001 at 260 nm of the reaction mixture (pH 5.0) containing DNA substrate at 25 °C. For *Sma*I, 1 U is defined as the amount of enzyme required to completely digest 1 μg of λ DNA in 1 h at pH 7.5 and 30 °C in 50 μl of assay buffer. For PDI, 1 U is defined as the amount of enzyme required to hydrolyze 1 μmol of *p*-nitrophenyl thymidine 5′-phosphate per minute at pH 8.9 and 25 °C.

Fluorimetry

Fluorimetry was performed using a spectrofluorometer equipped with a temperature-controlled cell holder and FL-1044 dual polarizers (SPEX FluoroMAX-3, Horiba, Kyoto, Japan). In general, a quartz microcuvette (type 23-3.45, 3 × 3 mm [internal length and width], Starna Scientific, Essex, UK) containing a sample solution was set into the cell holder, kept at 10 °C, and held for more than 90 s before measurement to stabilize fluorescence intensity. The excitation (ex)/emission (em) polarizers were set at the magic angle (ex: 0°, em: 55°). The fluorescence spectra were obtained from 360 to 600 nm; excitation was at 347 nm.

Syntheses and purification of fluorescence-labeled DNA oligomers

To estimate the average lengths between ***D*** and ***A*** (see Supplementary Fig. 1 in supplementary material) perpendicular to the DNA axis in aqueous solution, we synthesized two complementary DNA oligomers (31-mer) with an AP site at the center conjugated to ***D*** or ***A***. The ***D***–***A*** distance after hybridization of these includes unknown lengths of the fluorescent dyes (d) and unknown distance between DNA core axis and an AP site (a) (Fig. 1).

In a microtube, 10 mM dye in DMSO, 0.1 mM DNA oligomer, 100 mM Tris–HCl (pH 7.0), and 1 U/μl UDG were mixed in the ratio of 2:2:5:1 (v/v/v/v). Mineral oil was added to the tube to avoid concentration by evaporation. Reactions were incubated for 24 h at 37 °C. The reaction mixture was purified twice using ProbeQuant G-50 (GE Healthcare Japan, Tokyo, Japan), followed by HPLC purification using a CLASS-VP system (Shimadzu, Kyoto, Japan) equipped with a reverse-phase C30 column (Develosil RPAQUEOUS, 4.6 i.d. × 150 mm, Nomura Chemical, Aichi, Japan). The DNA–Alexa dye conjugates obtained were detected using a fluorescence detector (350 dye: ex = 347 nm, em = 449 nm; 488 dye: ex = 480 nm, em = 520 nm). The concentrations of DNA–Alexa dye conjugates were determined using absorbance at 260 nm by comparison with each of the parent oligomers. The derivatives of the DNA oligomers used in this study are listed in Table 1. The concentrations of these complexes were adjusted to be 1.4 μM in 10 mM Tris–HCl/1 mM EDTA buffer (pH 8.0).

Hybridization experiment to determine the distance between the Alexa dyes on DNA

The values of quenching ($q_{D,A}$), ($q_{D,dna}$), mentioned later, that determine the distance between ***D*** and ***A*** perpendicular to the DNA core axis, $2(a + d)$ (Fig. 1), were obtained by the following hybridization experiments [19]. In brief, 5 μl of ***D***-16AP–Strand1 (Table 1) was mixed with 150 μl of TE buffer (pH 8.0) at 0 °C in the quartz microcuvette. The fluorescence spectra before and after adding 7 μl of ***A***-16AP–Strand2 (Table 1) were measured as a function of time (ex = 347 nm). An additional 2 μl of ***A***-16AP–Strand2 was needed to allow all ***D***-16AP–Strand1 molecules to be annealed with ***A***-16AP–Strand2. In addition, the fluorescence spectra before and after adding Strand2 were also measured to observe background quenching by the complementary strand without ***A***. At least 3 μl of 16AP–Strand2 was needed for complete hybridization

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