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Repair of oxidative damage of thymine by HeLa whole-cell extracts: simultaneous analysis using a microsupport and comparison with traditional PAGE analysis

Valérie Guerniou [a], Delphine Rapin [a], Jean-François Millau [a], Emmanuel Bufflier [b], Alain Favier [a], Jean Cadet [a], Sylvie Sauvaigo [a,*]

[a] *Laboratoire Lésions des Acides Nucléiques, Service de Chimie Inorganique et Biologique, CEA/DSM/Département de Recherche Fondamentale sur la Matière Condensée, CEA-Grenoble, 17, rue des Martyrs, 38054 Grenoble cedex 9, France*
[b] *APIBIO, 15, rue des Martyrs, 38054 Grenoble cedex 9, France*

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Abstract

In mammalian cells, the base excision repair (BER) pathway allows the remove of small DNA base lesions such as oxidized bases. It is initiated by glycosylases that removed the modified base leaving an abasic site that is subsequently processed by AP endonuclease activities. Measurement of BER activities in cell extracts is time consuming and hazardous when radioactive material is used. We report in this study, the parallelized fluorescent analysis of excision of several oxidation products of thymine by cell extracts. To conduct the study, 5-(hydroxymethyl)uracil, 5-formyluracil, 5-carboxyuracil and formylamine together with uracil and the control thymine, were incorporated into oligonucleotides of identical sequences and paired either with adenine or with guanine containing DNA fragments. The oligonucleotides were fixed by sandwich hybridization in wells of a microplate (OLISA technology). Excision by HeLa extracts of the six different DNA base lesions could be followed simultaneously in the same well. Our results showed that the extent of excision of the lesions was the same on support and in solution using classical PAGE analysis approach with modified ^{32}P-labeled oligonucleotides. We demonstrated that the simultaneous analysis on support is a successful approach to facilitate high-throughput screening of BER activities present in cell extracts. Moreover, extended study of 5-carboxyuracil revealed that this lesion displays similar biological properties as 5-formyluracil.

Keywords: 5-(Hydroxymethyl)uracil; 5-Formyluracil; 5-Carboxyuracil; *N*-(2-deoxy-*β*-D-*erythro*-pentofuranosyl)-formylamine; Base excision repair; Biochip

1. Introduction

Considerable and growing interest is devoted to the assessment of the consequences of oxidative DNA damage in term of cancer induction and aging. Reactive oxygen species produced by normal aerobic metabolism, inflammatory response, exogenous sources such as ionizing radiations or chemical oxidants, are considered to be mostly responsible for the occurrence of DNA-damaged bases and strand breaks. Base excision repair (BER) pathway is the major mechanism devoted to correction of these latter lesions. The main features of BER deal with recognition and removal of aberrant base from the DNA by an *N*-glycosylase followed by incision of the resulting AP site by an AP-endonuclease or an AP-lyase [1]. Repair is completed in subsequent steps by processing of the resulting termini, filling of the gap by polymerases and intervention of a ligase to reseal the repaired strand. The BER mechanism can proceed through two different sub-pathways for gap-filling: short patch BER and long patch BER [2].

Abbreviations: A, adenine; BER, base excision repair; CaU, 5-Carboxyuracil; DTT, dithiothreitol; F, formylamine; FoU, 5-Formyluracil; G, guanine; HmU, 5-(Hydroxymethyl)uracil; HPLC-MS/MS, high performance liquid chromatography coupled to tandem mass spectrometry; IdU, 5-iodo-2′-deoxyuridine; ODN, oligonucleotide; OHC, 5-hydroxycytosine; OLISA, oligo sorbent array; PAGE, polyacrylamide gel electrophoresis; Pol I Kf (exo-), Klenow exonuclease-free fragment of polymerase I; ROS, reactive oxygen species; SMUG1, single-stranded monofunctional uracil DNA *N*-glycosylase; T, thymine; U, uracil; UV, ultra-violet.

* Corresponding author. CEA Grenoble, DRFMC-SCIB-LAN, 17, rue des martyrs, 38054 Grenoble cedex 9, France. Tél. :+33-4-38-78-37-52; Fax : +33-4-38-78-50-90.
E-mail address: ssauvaigo@cea.fr (S. Sauvaigo).

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Unrepaired bases and sugar damage are likely to have a high mutagenic potential, to block DNA replication and to lead to cell death. Indeed, biological consequences of DNA lesions are linked to efficiency of their repair. Despite the fact that basic steps of BER could be reconstituted in vitro with only four enzymes, it appears that many non-repair proteins are involved in the coordination and assembly of the repair complex in mammalian cells [3,4]. Moreover BER proteins could interact with factors that are associated with transcription and replication [5]. Another feature of BER is the redundancy of the enzymes involved. For example multiple enzymes have evolved for the repair of U, one of the most abundant base lesions found in mammalian cells [5]. It must be noted also that many *N*-glycosylases possess overlapping substrate specificity. These data imply a complex and versatile regulation and activity of BER.

In mammalian cells, no evidence clearly establishes the importance of *N*-glycosylase activities in maintenance of genome integrity [1]. However, several studies emphasize correlations between some polymorphisms in BER genes and cancers ([6,7] and for review see Ref. [8]). Determination of variation in repair capability among individuals thus represents a challenging problem. Such a determination must take into account the complexity of the repair systems. Several in vitro assays have been developed for BER using whole-cell extracts [9]. It is of interest to determine repair ability for individual base lesions. As a consequence, characterization of enzymatic activities as well as cell extract repair capacities must include studies on a wide range of substrates as illustrated by Shen et al. [10].

Here we report an approach based on the simultaneous determination of excision capacity of cell extracts toward a panel of DNA lesions, recognized by the BER system. For this purpose, lesions were included within oligonucleotides attached to a support in a customized miniaturized assay [11]. Briefly, 12 oligonucleotides containing different lesions in an otherwise identical sequence were immobilized by sandwich hybridization into a microplate well. Cleavage of the lesion by repair enzymes present in the extract led to elimination of the nicked fragments and to modification of the fluorescent signal emitted at the site of the recognized oligonucleotides. Automated simultaneous quantification of residual fluorescent signal emitted at each site of the well of the microplate allowed comparative determination of cleavage efficiency of each lesion by enzymes present in the cell extract.

We have focused our research on the cleavage of several methyl oxidation products of thymine that include 5-(hydroxymethyl)uracil, 5-formyluracil and 5-carboxyuracil. In parallel, we have also investigated the repair of formylamine, a major oxidation product of thymine formed by ionizing radiation [12]. The normal bases thymine and uracil were also added to this panel.

The 5-formyluracil is a major oxidative product of DNA formed upon exposure to ionizing radiation in presence of oxygen or when treated with Fenton-type ROS-generating systems [13,14]. It can also be formed in yield comparable to that of 8-oxo-7,8-dihydroguanine together with the minor product 5-carboxyuracil, upon menadione- or quinone-mediated sensitization of thymidine to UVA radiation [15]. The 5-(hydroxymethyl)uracil is also an oxidation product of thymine formed upon exposure to OH radical or a type I photosensitizer [16].

Cleavage efficiency of the oligonucleotides by HeLa cell extracts was examined for two different duplex constructions: the bases of interest were paired either with an adenine or with a guanine.

The ability of the fluorescent support assay to deal with the assessment of repair efficiency of whole-cell extracts was compared with that of the usual solution digestion approach that involves PAGE analysis.

Finally, to bring more data on 5-carboxyuracil whose biological properties have never been explored, primer extension experiments with exo- fragment of Klenow polymerase were conducted. Insights into the mutagenic properties of the last lesion were gained.

2. Materials and methods

2.1. Reagents

The NAP25 column, microspin G-25 column were from Amersham Biosciences (Orsay, France). 2′-Deoxyuridine and 5-trifluoromethyl-2′-deoxyuridine were from Pharma Waldhof (Düsseldorf, Germany). The 5-Iodo-2′-deoxyuridine, Triton X-100, HEPES were from Sigma Aldrich (St-Louis, MO). Phosphoramidite monomers used for oligonucleotide synthesis were from Glenn Research (Sterling, VA). Non-modified oligonucleotides were purchased from Proligo (Paris, France).

2.2. Synthesis of the oligonucleotides

The modified oligonucleotides were synthesized using the phosphoramidite chemistry on solid support. The 5-(hydroxymethyl)-2′-deoxyuridine phosphoramidite derivative was synthesized in four steps from 2′-deoxyuridine following the method reported by Sowers and Bearsley [17]. The 5-formyl-2′-deoxyuridine containing oligonucleotide was obtained by mild oxidation of an oligonucleotide in which 5-(1,2-dihydroxyethyl)-2′-deoxyuridine was initially site-specifically inserted. The phosphoramidite building block of 5-(1,2-dihydroxyethyl)-2′-deoxyuridine was prepared from 5-iodo-2′-deoxyuridine (IdU) in seven steps as already described [18]. Before use, 1 nmole of 5-(1,2-dihydroxyethyl)-2′-deoxyuridine containing oligonucleotide was oxidized in water at room temperature by 50 equivalents of $NaIO_4$ during 1 min (98% yield). This led to 5-formyl-2′-deoxyuridine-containing oligonucleotide. The DNA fragment was subsequently desalted on a microspin G-25 column.

The 5-carboxy-2′-deoxyuridine phosphoramidite building block was synthesized from 5-trifluoromethyl-2′-deoxyuridine in four steps [19]. The *N*-(2-deoxy-*β*-D-*erythro*-

pentofuranosyl)-formylamine phosphoramidite building block was synthesized in three steps from thymidine [20]. The building blocks for each DNA base lesion were incorporated at defined location into a 22-mer oligonucleotide using an Applied Biosystem Inc. 392 DNA synthesizer. The oligonucleotides were deprotected using appropriated conditions prior to being purified by PAGE. The analysis of the content of the oligonucleotides was performed by HPLC-MS/MS after enzymatic hydrolysis [21]. All the oligonucleotides had the expected composition (data not shown).

2.3. Principle of the assay

The principle of the assay has been described previously [11] and is presented in Fig. 1. Selected lesions were incorporated into oligonucleotides (called lesion oligonucleotides) that were hybridized into a well of a microplate through a sandwich construction. Each well was functionalized with six different oligonucleotides fixed in duplicate in a circular format. The ODNs attached to the support were called the support ODNs. Each lesion containing oligonucleotide bore a biotin at its 3′-end that subsequently allows its affinity labeling by streptavidin linked to the fluorescent molecule phycoerythrin. Each well contained also a control biotinylated oligonucleotide fixed in triplicate at specific sites of the well. The role of the control oligonucleotide was to direct automatic and simultaneous detection of fluorescent signals within each well using an automated fluorescent microscope (Apimager, Apibio, France). The wells functionalized by the lesion oligonucleotides were incubated with extract of interest for indicated times. Washings led to elimination from the support of the lesion oligonucleotides that had been cleaved by the enzymatic activities present in the extract. The last step was the incubation with streptavidin-R-phycoerythrin for the affinity labeling of the lesion oligonucleotides remaining on the support, through their 3′ biotinylated end. Controls consisted of wells incubated with boiled extracts. Quantification of the fluorescent signals related to residual damage allowed the calculation of the percentage of lesion oligonucleotides remaining on the support, relative to the controls. Then the percentage of excision was calculated for each lesion.

2.4. Oligonucleotides

The sequences of the ODNs used in this study are listed in Table 1. The junction ODNs that formed the sandwich on the support were combinations of the complementary sequence of one lesion ODN for the 5′-end part and one support ODN for the 3′-end part.

Six lesion ODNs were synthesized (Table 1): the control sequence containing a thymidine at position 10 from the 3′ terminus and five other modified oligonucleotides containing either a 2′-deoxyuridine (U base), a 5-(hydroxymethyl)-2′-deoxyuridine (HmU base), a 5-formyl-2′-deoxyuridine (FoU base), a 5-carboxy-2′-deoxyuridine (CaU base) or a *N*-(2-deoxy-*β*-D-*erythro*-pentofuranosyl)-formylamine (F base) at the same position. The structures of the lesions are shown in Fig. 2.

2.5. Preparation of HeLa whole-cell extracts

HeLa whole-cell extracts were prepared from semi-confluent cells according to a protocol initially developed by Redaelli et al. [22] and slightly modified by Collins et al. [23]. Typically, 10^7 pelleted cells were suspended in 50 µl of buffer A (90 mM Hepes-KOH (pH 7.8), 800 mM KCl, 2 mM EDTA, 1 mM DTT, 20% glycerol). Cells were then submitted to two freezing (–80 °C)–thawing (4 °C) cycles. Subsequently, 12 µl of buffer B (1% of Triton X-100 in buffer A) was then added and a new freezing–thawing cycle was performed. Finally, 38 µl of buffer C (45 mM Hepes–KOH (pH 7.8), 0.25 mM EDTA, 2% glycerol, 17.9 µg/µl PMSF, antiprotease (Complete-mini, Roche Applied Science, Meylan, France)) was added. The cells were then homogenized vigorously at 4 °C for 30 s and centrifuged at 4 °C for 5 min at 14,000 *g*. The supernatant was collected and the protein con-

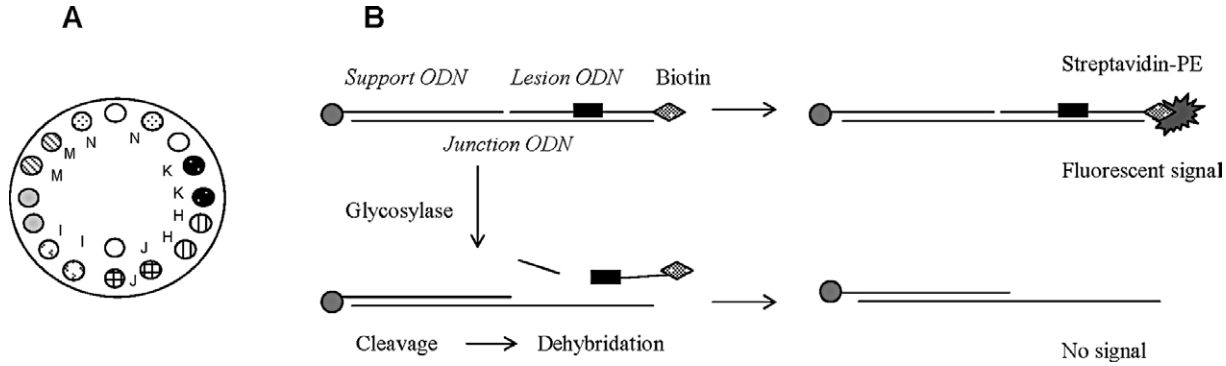

Fig. 1. Principle of the measurement of DNA-*N*-glycosylases activities on the biochip. Six support ODNs were fixed in duplicate in a circular format within the wells of a microplate (Positions K, H, I, J, M, N; Fig. 1A). Three single-strand ODNs, biotinylated at their 3′, end were also fixed at the bottom of each plate and served as positive control for the automatic detection of the different fixation sites (white circles). Each lesion ODN was biotinylated at its 3′ end (Fig. 1B). A duplex was initially formed in solution between each lesion ODN and a longer junction ODN. The single strand part of the junction ODN subsequently directed the hybridization of the duplex onto a support ODN of complementary sequence fixed at a specific site of a microplate well. The sandwich hybrids were detected using a streptavidin–phycoerythrin conjugate and quantified by fluorescence imaging. After incubation with HeLa extract and in case of cleavage of the lesion by a DNA-*N*-glycosylase, the nicked products labeled by the biotin were eliminated by washes. We thus observed signal loss at the site of the cleaved substrate.

Table 1
Sequences of the ODNs used in this study

Name	Support ODNs (5′ NH_2 →3′)	Name	Support ODNs (5′ NH_2 →3′)
K	TTTTT CAC GTG GCT ACC ATG CCA TT	J	TTTTT ACC ATC GCT TCC AGA GAC TC
H	TTTTT CCA CAC GGT AGG TAT CAG TC	M	TTTTT CAA GCC TTG GCA GAT GCT GT
I	TTTTT ATC CGT TCT ACA GCC GTA CA	N	TTTTT CGA GTA GTG CTA TCG ACG TT
Lesion ODNs (5′-3′)			
CAC TTC GGA TCG (X)GA CTG ATC T			
Junction ODNs (5′→3′) (cLesion-*cSupport*)			
CKcX AGA TCA GTC (A/G) CG ATC CGA AGT G*AA TGG CAT GGT AGC CAC GTG*			
CHcX AGA TCA GTC (A/G) CG ATC CGA AGT G*GA CTG ATA CCT ACC GTG TGG*			
CJcX AGA TCA GTC (A/G) CG ATC CGA AGT G*GA GTC TCT GGA AGC GAT GGT*			
CicX AGA TCA GTC (A/G) CG ATC CGA AGT G*TG TAC GGC TGT AGA ACG GAT*			
CMcX AGA TCA GTC (A/G) CG ATC CGA AGT G*AC AGC ATC TGC CAA GGC TTG*			
CNcX AGA TCA GTC (A/G) CG ATC CGA AGT G*AA CGT CGA TAG CAC TAC TCG*			

Support ODNs (K, H, I, J, M, N) were aminated at their 5′-end and chemically fixed onto the support. All the lesion ODNs had the same sequence surrounding the lesion (X). The X represents one of the following nucleoside: HmdU, FodU, CadU, FadU, T and dU. The junction ODNs were complementary to the lesion ODNs for their 5′ part, with either a A or a G opposite the lesion, and complementary to the support ODNs for their 3′part (in italic letters).

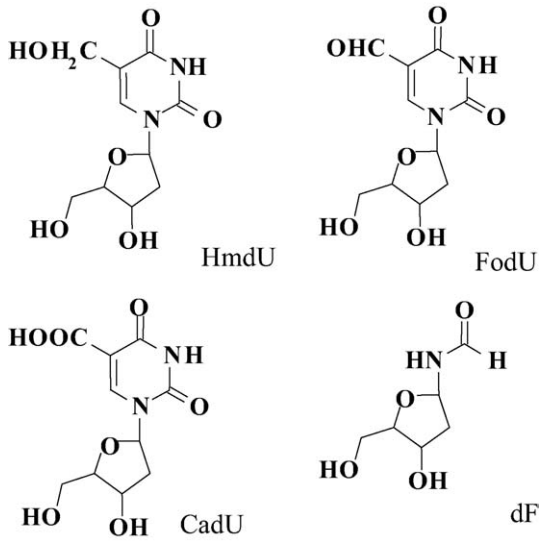

Fig. 2. Chemical structure of the four oxidation products of thymidine that were studied: HmdU (5-(hydroxymethyl)-2′-deoxyuridine), FodU (5-formyl-2′-deoxyuridine), CadU (5-carboxy-2′-deoxyuridine) and FadU (*N*-(2-deoxy-*β*-D-*erythro*-pentofuranosyl)-formylamine).

tent was determined using the Micro BCA protein assay (Interchim, Montluçon, France). The extracts were divided into 10 µl aliquots and then stored at –80 °C.

2.6. Support

The microarrays (Oligo Sorbent Array, OLISA™) were purchased from Apibio (Grenoble, France). They consisted of black polystyrene 8-well module microplates. Each well was functionalized with six support ODNs chemically tethered in duplicate by their 5′-aminated end to the support (Table 1). Three identical control ODNs were also covalently bound. The control ODNs were single stranded sequences that were biotinylated at their 3′-end [11].

2.7. Excision assay in solution

Solution excision assays were conducted on double stranded DNA fragments that contained a thymine, uracil, 5-(hydroxymethyl)uracil, 5-formyluracil or 5-carboxyuracil residue. Typically, 10 pmol of radiolabeled modified oligonucleotides were annealed with 15 pmol of appropriate complementary strand to obtain either A or G opposite the base of interest, in 10 µl of hybridization buffer (HB: PBS/0.2 M NaCl). Enzymatic reactions were performed at 30 °C using 0.1 pmol of duplex in 10 µl of repair buffer (RB: 10 mM Hepes–KOH (pH 7.8), 80 mM KCl, 2 mM EGTA, 1 mM DTT) that contained the HeLa extracts. Reactions were stopped by adding an equal volume of gel loading buffer. The samples were heated to 85 °C for 5 min before being loaded onto a 20% denaturing polyacrylamide gel. After electrophoresis, the gels were exposed to phosphorimager screens. The bands corresponding to the intact oligonucleotide and the cleavage products respectively were quantified using the Biorad Quantity One v4.2.2 software.

2.8. Excision assay on support

2.8.1. Hybridization of oligonucleotides onto the support

Each modified ODN (75 pmoles) and 50 pmoles of the chosen corresponding junction ODN were hybridized in 100 µl of HB by heating at 85 °C for 1 min, followed by slow cooling down to room temperature. Duplexes were kept at 4 °C until use. Just prior to the experiment and in order to functionalize the support, the duplexes were mixed together in the wells of the microplate at the final concentration of 0.75 pmol/50 µl of HB and subsequently incubated for 90 min at 37 °C in a water-saturated atmosphere. The complementary sequences of the support oligonucleotides directed the hybridization of the pre-formed duplexes onto the support. Wells were then rinsed three times at room temperature with 250 µl of washing buffer (WB: PBS/0.2 M NaCl /0.1% Tween 20).

2.8.2. Digestion with the cell extracts

Serial dilutions of the cell extracts were prepared at 4 °C. Wells were placed on ice and quickly rinsed with 250 µl of pre-chilled RB. Wells were incubated with 50 µl of the diluted

cell extract at 30 °C in a water-saturated atmosphere. At indicated time, the wells were removed and rinsed 3 × 5 min with WB at room temperature. Wells were kept at 4 °C until digestions were completed. The WB was then changed and the wells were finally washed for 3 × 20 min at 37 °C.

2.8.3. Detection

Wells were incubated in the dark with 50 μl of WB containing 5 μl of streptavidin-R-phycoerythrin solution (Sigma, St Louis, MO) for 1 h at 37 °C. Wells were then washed three times for 5 min at 37 °C in the WB in the dark. Fluorescent signals were recorded immediately.

2.8.4. Signal recording and image analysis

Images were automatically recorded using the biochip-imaging system (fluorescence microscope) developed by Apibio (Apimager). Signals were integrated upon 1500 ms excitation time. The images were subsequently analyzed using Bio-Rad Quantity One software. Fluorescence intensity was calculated as the average grey level of each spot multiplied by its surface.

2.8.5. Data analysis

For each incubation time, the control consisted of a well incubated with denatured cell extract in the RB. The value of the signal emitted at each ODN site in the control well was set up to 100. The results obtained from the digestion experiments were calculated relative to this value. For each lesion a mean for the duplicates within each well was calculated. Standard deviations were also calculated using values from different experiments performed in the same conditions.

2.9. Primer extension assay

A 9-mer oligonucleotide complementary of the 3′ part of the lesion oligonucleotide (5′ AGA TCA GTC 3′) was used for the extension analysis. 3 pmol of ^{32}P 5′-end labeled 9-mer primer was annealed with 15 pmol of the 22-mer oligonucleotide in 60 μl of HB. The mixture was heated to 85 °C for 2 min and then slowly cooled down to 4 °C.

For replication experiments, 0.05 pmol of each duplex was incubated for 5 min with 0.02 U of Klenow exonuclease-free fragment of polymerase I (Pol I, Kf exo-; Roche Applied Science, Meylan, France) diluted in 10 μl of 50 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 0.05 mg/ml bovine serum albumin, 1 mM DTT and 100 μM of the appropriate single dNTP. Control reactions contained the 4 dNTPs. Six microliters of gel loading buffer was added to stop the reaction. Samples were heated at 85 °C for 3 min. Then 4 μl of the mixture was loaded onto a 20% denaturing polyacrylamide gel. Gels were analyzed using Bio-Rad Molecular Imager and Bio-Rad Quantity One v4.2.2 software.

3. Results

3.1. Solution incision assay

In a first approach, the solution incision assay was conducted to check that HeLa extracts did not induce non-specific cleavage of the oligonucleotides. Highly concentrated extracts could have nuclease activities on the ODNs. Hence a range of concentration was selected that gave incisions at the site of the lesions and no cleavage activities on the non-modified ODNs. It must also be underlined that extracts prepared from confluent cells had much more non-specific cleavage activities than extracts prepared from cells in exponential growing phase, in which nuclease activities were negligible.

In solution, cleavage of the lesion ODNs was time-dependent and a maximum was observed after 2 h incubation with HeLa extracts (protein content 0.2 μg/μl and 0.5 μg/μl) (Fig. 3). Percentages of incision of the lesion ODNs at this time point are listed in Table 2.

We found that HeLa cell extracts cleaved HmU and FoU at the respective rates of 22% and 24%, when they were paired with a guanine base. Incision was much more efficient in the case of the lesion:G substrate than of the lesion:A substrate. Less than 10% of these two lesions were found to be excised when paired with an adenine base. The CaU residue, repair of which has never been investigated, was also incised when

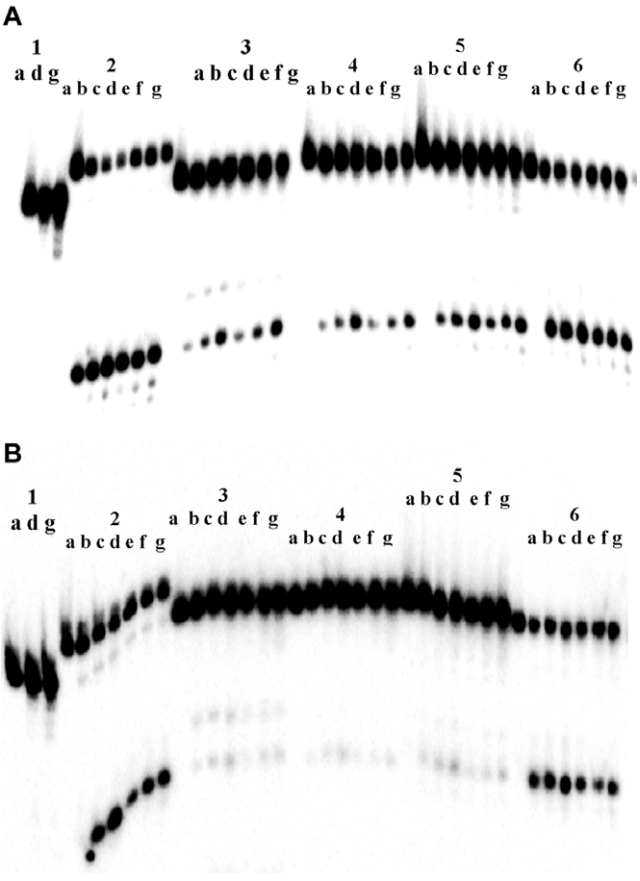

Fig. 3. Activity of the HeLa extracts in solution on 0.1 pmol of ^{32}P-labeled oligonucleotides: the ODNs that contained a thymine lesion were paired with a guanine (Fig. 3A) and with an adenine (Fig. 3B). The oligonucleotides that contained thymine (1), uracil (2), 5-(hydroxymethyl)uracil (3), formyluracil (4), carboxyuracil (5), and formylamine (6) were incubated for 30 (b and e), 60 (c and f) and 120 (d and g) min with cell extracts ((b–d) 0.2 μg/μl of proteins and (e–g) 0.5 μg/μl of proteins). Lanes a show control ODNs incubated with denatured cell extracts.

Table 2
Extent of excision of the base lesions in solution and on the support by the HeLa extracts

Base	Solution assay		Support assay	
	G (%)	A (%)	G (%)	A (%)
T	0	0	31 ± 7	6 ± 5
U	89	67	84 ± 3	73 ± 9
F	68	58	53 ± 1	31 ± 5
HmU	22	≈ 9	29 ± 6	12 ± 8
FoU	24	≈ 4	22 ± 6	0 ± 10
CaU	29	≈ 6	30 ± 14	12 ± 8

For solution assay, analysis was conducted using 0.1 pmol of duplexes. Quantification was performed after 2 h incubation with 0.5 μg/μl of proteins (Fig. 2, lanes g). The OLISA assay was performed with 0.4 μg/μl of protein and quantification was made after 1 h incubation. The bases of interest faced either a guanine (row G) or an adenine (row A).

incubated with the HeLa cell extracts. As for other thymine oxidation products, the incision was about 5-fold more efficient when it was paired with a guanine (29% incision) than with an adenine (6% incision).

High rates of cleavage were measured for the U residue when it was paired with an adenine (67%) or with a guanine (89%). This also applies to the removal of F since DNA cleavage at the site of the lesion was efficient in front of either adenine (58%) or guanine (68%).

3.2. Support incision assay

Parallel studies were conducted on the same oligonucleotides immobilized on the OLISA biochip. Excision of the lesions was monitored by fluorescent measurement of residual oligonucleotides that remained on the support after treatment by the extracts. For each experiment, the value of the excision rate was the mean of the two duplicated spots in the same well. Curves presented in Fig. 4 have been obtained from four independent experiments performed with the same cell extract used at the optimized concentration of 0.4 μg/μl of protein. Table 2 listed the excision rates obtained after 1 h incubation with the whole-cell extracts.

Interestingly, except for the mispaired thymine, we could observe similar excision rates for each of the lesions whether they were in solution or immobilized on the biochip (Table 2). Cleavage was fast and almost reached completion after 15 min incubation when the lesions faced a guanine (Fig. 4A). Calculated standard deviation of quantification of excision on support was at most 14% (Table 2) with a mean of 8%. Excision of the 3-methyl oxidation products of thymine was not significantly different from the control, on support, when paired with adenine (Fig. 4B).

3.3. Primer extension assay

Under the conditions used, Pol I Kf (exo-) directed incorporation of dAMP and to a lesser extent dGTP opposite FoU in the presence of a single dNTP (Fig. 5). A faint band corresponding to incorporation of dCTP was also observed. Using the CaU containing oligonucleotide, dAMP, and to a lesser

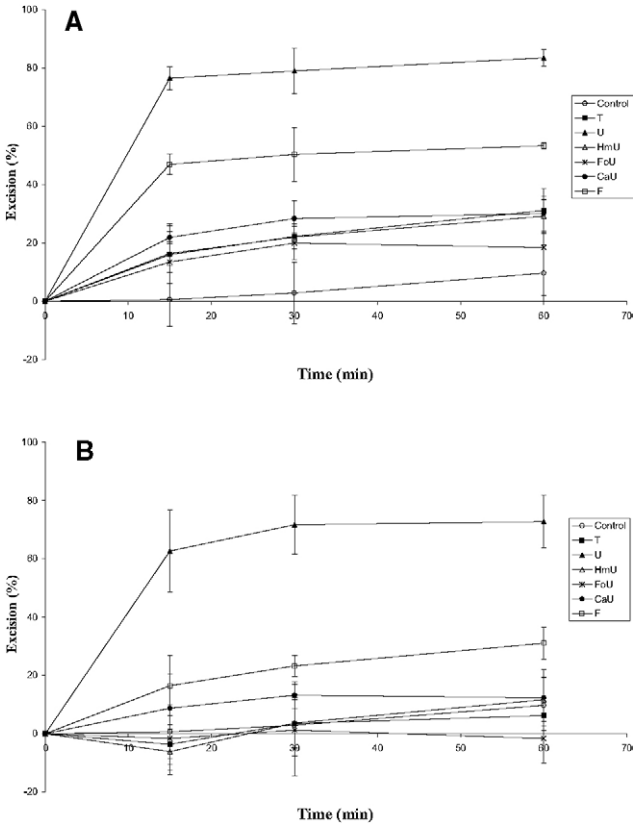

Fig. 4. Excision of the base lesions incorporated into ODNs fixed onto the biochip, by 0.4 μg/μl HeLa cell extracts as a function of the digestion time. The mean of four independent experiments performed with the same extract is represented. Panel 4A shows the extent of excision of the lesions when paired with G. Panel 4B shows the extent of excision of the lesions when paired with A.

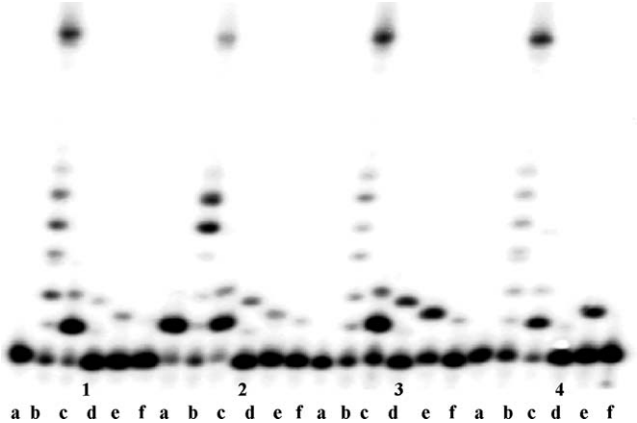

Fig. 5. Primer extension assay conducted with Klenow exonuclease-free fragment of polymerase I using the oligonucleotides that contained the methyl oxidation products of thymine as template. A 9-mer primer was annealed with the 22-mer modified oligonucleotides that contained thymidine (lanes 1), 5-(hydroxymethyl)-2′-deoxyuridine (lanes 2), 5-formyl-2′-deoxyuridine (lanes 3) and 5-carboxy-2′-deoxyuridine (lanes 4). Extension was performed in the presence of the 4 dNTPs (b), dATP (c), dCTP (d), dGTP (e), dTTP (f). Lanes a show the non-extended oligonucleotides.

extent dGTP, were incorporated opposite the latter lesion. According to published data [24], only dAMP was inserted in front of HmU. It may be noted that none of the lesions blocked extension by the polymerase. Control experiments performed with the normal oligonucleotide showed the sole incorporation of dATP in front of T.

4. Discussion and Conclusion

This paper describes an assay that is suitable for the assessment of DNA repair ability to cleave modified oligonucleotides at sites of a range of dedicated lesions using whole-cell extracts. The specificity of this methodology has been previously validated using several enzymes from *E. coli* on a panel of DNA base lesions (8-oxo-7,8-dihydroguanine, hypoxanthine, 1,N6-ethenoadenine, formylamine, uracil) [11]. This assay has been shown to allow determination of cleavage profile of specific lesions for two different types of cell extracts (HeLa cells and human primary fibroblasts). Consistent with our previous report, we now show that low amount of protein (20 μg/well) was sufficient to cleave the panel of oxidized bases at a comparable rate to that seen in solution using a unique lesion with a given extract. Approximately 30 fmol of each duplex was immobilized on the support [11]. For comparison, experiments in solution were performed on 100 fmol of each oligonucleotide construction using 5 μg of HeLa extract. To compare the relative rates of excision of different substrates, all the lesions were incorporated within the same sequence context. Hence, the junction ODNs were composed of an identical sequence containing the lesion at their 5′ part and a specific "zip-code" sequence at their 3′ part. For biochip experiments, constructions that were made of the hybrid between lesion ODN and junction ODN were elaborated individually for each lesion ODN before being pooled within the well of the microplate. Then, the "zip code" part of junction ODN that remained single-stranded, conducted hybridization of the duplex on support ODN.

We chose as a model a family of lesions that all arise from radical oxidation of the thymine, namely: FoU, HmU, CaU and F. It must be underlined than the outcome of CaU in cells has never been investigated until now. Control T oligonucleotide was added to this panel of lesions as well as an oligonucleotide that contained a U base at the same site.

During replication, FoU forms a mispair with guanine in addition to the correct base pair with adenine [25]. Oxidation of thymine can lead to formation of a base pair between HmU and adenine, whereas a base pair between HmU and guanine can be generated by deamination of 5-methylcytosine, in DNA [26]. Cells have evolved the ability to counteract these base instabilities at the origin of mutagenic mispairs. Several publications have reported the reparability of the different mispairs using either purified bacterial glycosylases or cell extracts [26–31].

It was thus interesting to undertake the study of incision of oxidative lesions of thymine using the two bases (either G or A) within the complementary ODN. Both studies were conducted in solution and on the microsupport. Our results are in accordance with previously published data on efficiency of incision of methyl oxidation thymidine damage by cell extracts [26].

An interesting result was that CaU was excised approximately at the same rate as the other oxidation products of the methyl group of thymine. The current study shows that a base lesion formed at a very low yield compared to HmU and FoU in isolated DNA and at a non-detectable level in cellular DNA after gamma radiations (unpublished data), i.e., CaU is recognized and cleaved with high efficiency by activities present in the cell extracts. To further document the features of this lesion, we performed preliminary complementary experiments on its mutagenic properties. Primer extension analysis conducted with the Klenow exonuclease-free fragment revealed that CaU, as FoU, directed the incorporation of A and to a lesser extent G as an opposite base. As a major oxidation product, biological implications of FoU have been well documented. Our results are in accordance with published data [32–34]. CaU, like the other methyl oxidation products of thymine, did not block replication by the polymerase tested. Our data suggested that CaU displays similar biological properties to FoU.

SMUG1 excises uracil and uracil derivatives bearing an oxidized group at C5 [26,28,34,35]. It would be of interest to evaluate in vitro the ability of this glycosylase to excise CaU. Further, the information of the comparative ability with which the different methyl oxidation products of the thymine are removed from DNA should be of interest to understand global mechanisms of recognition for the cellular pool of enzymes involved in the base excision repair (BER) system.

As already demonstrated [11], F was removed with high efficiency by HeLa extracts. However, as for CaU, the enzyme responsible for recognition and cleavage of the F lesion remained to be investigated.

Different authors have described a poor repair of the T residue in T:G base pair by enzymatic activities in cell extracts, even in the presence of ATP [26,36,37]. In agreement with these reports, the rate of cleavage of T in T:G pair was undetectable in our ATP-free conditions. Whereas the results we obtained either in solution or using the biochip assay were almost similar, surprisingly we observed a significant rate of incision on the microsupport (about 30%) using the same HeLa extract. G:T mismatches are produced by errors during DNA replication. Spontaneous deamination of 5-methylcytosine also leads to this mispair [38]. In humans, CpG dinucleotides are exclusive sites of 2′-deoxycytidine demethylation and the correction of this error is initiated by the thymine DNA glycosylase which excises the mismatched thymine [39]. More recently, it was shown that the glycosylase MBD4 is also able to remove the thymine in such a mispair [40]. The CpG sequence context that is the preferred site of cleavage is not absolutely required for the activity of the latter enzyme. However it is a necessity for the activity of the former TDG enzyme. There is no evident explanation for the

discrepancy we have obtained using the support assay since the sequence context of the lesion was the same. The only difference we can note is that the duplex oligonucleotides used in solution experiments were shorter (22 bases long) than the oligonucleotides used for the biochip assays (42 bases long). We can hypothesize that enzymatic activities that recognize and excise mispaired thymine may be more efficient on longer oligonucleotides; in addition we cannot exclude an effect of the support that could block access to other cellular factors thus impeding cleavage of the mismatch in solution. The latter point remains to be clarified.

Except for the mispaired thymine, results obtained in solution and on support were comparable. In humans, four different enzymes have been identified for their ability to allow removal of U and the methyl oxidation products of thymine in DNA [27]. These glycosylases generate abasic sites that must then be processed by an AP-endonuclease activity. The observation that excision of the lesions in solution and using the microsupport is equivalent suggests that elimination of AP sites is not rate-limiting when lesions are processed together within the same support and using the same extract.

Simultaneous determination of excision of a variety of substrate is interesting for identification and characterization of repair enzymatic activities in cell extract [41]. Moreover, major information on mechanisms of repair can be inferred from experiments performed by adding rate-limiting BER components to repair reactions involving a panel of lesions [42]. The biochip assay offers the advantage of measuring simultaneously, very conveniently without requiring the use of radiolabeled probes, a global DNA repair ability on a variety of selected lesions. We show here that it can facilitate monitoring of cleavage activity of enzymes that are presented in whole cell extract. Applications of this assay include screening for patients at higher risk to develop cancer. As an illustration, Paz-Elizur et al. [43] have shown using an oligonucleotide cleavage assay that low OGG1 activity in blood peripheral mononuclear cells was associated with an increased risk of lung cancer. It will thus be of interest to more fully characterize the functionality of enzymes that remove oxidized bases while taking into account the complexity of repair systems to design strategies of preventing cancer.

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