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An oligonucleotide microarray for the monitoring of repair enzyme activity toward different DNA base damage

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Abstract

Characterization of DNA-*N*-glycosylase activities in cell extract is a challenging problem and could represent a major concern for medical applications. Synthetic oligonucleotides which contain base lesions located on specific sites constitute suitable substrates for their study. An in vitro miniaturized assay was developed that allows the measurement of cleavage activities of DNA repair enzymes on a set of oligonucleotides (ODNs) that contained different lesions. The modified ODNs were indirectly hybridized onto probes chemically fixed at defined sites on a circlar format within each well of a 96-well microtiter plate (Oligo Sorbent Array, OLISA). The lesions were selected among oxidative damage (8-oxo-7,8-dihydroguanine, formylamine), deaminated bases (uracil, hypoxanthine) and alkylated base N^6-etheno-adenine). Cleavage specificity was checked using different enzymes: Fapy-DNA-*N*-glycosylase, 3-methyladenine DNA glycosylase II, uracil-*N*-glycosylase, endonuclease V and endonuclease VIII. The extent of excision could be monitored simultaneously for the selected base damage. For this purpose, we used automated fluorescence imaging analysis of the residual ODNs that contained lesions and remained on the support after release of the cleaved ODNs recognized by the repair enzymes. The results indicated that this assay could advantageously replace the analysis of glycosylase activites by PAGE techniques. Finally we show that this in vitro repair assay represents an interesting tool for the determination of cellular repair activites.

Oligonucleotide microarrays have been developed for the parallel monitoring of gene expression levels [1]. Methods using anchored primers may also be used to determine nucleotide polymorphisms using a single base extension approach [2] or discrimination by target hybridization [3]. Oligonucleotides addressed on microarray may also be used to specifically guide the hybridization of DNA fragments tagged with unique sequences (''zip-code'') [4].

Synthetic oligonucleotides which contain base lesions located on specific sites constitute substrates suitable for the study of damage recognition and excision by DNA-*N*-glycosylases [5–7]. Typically these enzymes, which belong to the base excision repair (BER)[1] pathway, remove oxidated, alkylated, or deaminated bases [8]. They recognize the lesion and catalyze the cleavage of the *N*-glycosylic bond, leaving an abasic site. This action is followed by the incision of the sugar–phosphate backbone by an apurinic/apyrimidinic (AP) endonuclease.

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[1] *Abbreviations used:* OLISA, oligo sorbent array; BER, base excision repair; PAGE, polyacrylamide gel electrophoresis; 8-oxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; dF, *N*-(deoxy-β-D-*erythro*-pentofuranosyl)-formylamine; εdA, N^6-etheno-deoxyadenosine; Fpg, Fapy-DNA-*N*-glycosylase; UNG, uracil-*N*-glycosylase; AlkA, 3-methyladenine-DNA glycosylase; Endo VIII, endonuclease VIII; Endo V, endonuclease V; AP, apurinic/apyrimidinic; PBS, phosphate-buffered saline; HB, hybridization buffer; WB, washing buffer; DB, digestion buffer; SA-PE, streptavidin-R-phycoerythrin.

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Bifunctional DNA glycosylases possess a concomitant AP lyase activity that cleaves the phosphate in the 3′ position of the abasic site.

The emergence of original pathways for the synthesis of modified oligonucleotides and improvement in the chemistry of protective groups have allowed the site-specific insertion of a broad range of DNA lesions [6]. The oligonucleotides used for the repair studies are usually radioactively labeled. The cleavage products are then analyzed after electrophoresis on polyacrylamide gel (PAGE).

Determination of glycosylase activities and lesion properties is challenging and complex. Time-consuming experiments are necessary to fully characterize the repair enzymatic activities.

To facilitate the study of DNA repair enzyme properties, we propose a miniaturized method based on the follow-up of the digestion by DNA-*N*-glycosylases of labeled oligonucleotides that have incorporated different DNA lesions and are immobilized on the same microarray.

A set of enzymes and lesions incorporated within different sequence contexts was chosen to evaluate the method. The aims of this approach were to (i) test the feasibility of the assay on a variety of substrates, (ii) detect the activity of enzymes possessing either glycosylase or lyase activities or both, and (iii) check the specificity and the efficiency of the digestion on support for already characterized lesions and enzymes and hence, validate the simultaneous sandwich hybridization of oligonucleotides of different sequences.

The DNA repair biochip was addressed with six different lesions representative of specific families. Two lesions arise from deamination of normal DNA nucleosides. Inosine is provided by adenosine and uridine by cytosine residue. 8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), which is miscoding and mutagenic [9], is considered one of the most important DNA markers of the oxidation processes within cells [7]. Furthermore formylamine (*N*-(deoxy-β-D-*erythro*-pentofuranosyl)-formylamine; dF), a major oxidative DNA lesion formed upon ionizing irradiation [10], was added. The latter lesion is considered an abasic site by certain authors [11]. Finally we incorporated N^6-etheno-deoxyadenosine (εdA). εdA is formed by reactive aldehyde [12] and by reaction with several chemical carcinogens [13]. Nicking efficiency was monitored using enzymes from *Escherichia coli*. Among them, Fapy-DNA-*N*-glycosylase (Fpg) possesses glycosylase/AP lyase activity [14] whereas uracil-*N*-glycosylase (UNG) and 3-methyladenine-DNA glycosylase (AlkA) are monofunctional enzymes and therefore generate AP sites (for review see [15]). AlkA possesses broad specificity toward unrelated base structures whereas UNG has rather narrow substrate specificity. Endo VIII is a class I AP endonuclease with an associated *N*-glycosylase activity for a number of specific substrates [16], among which is the hypoxanthine base from inosine. Endo V recognizes deaminated bases such as xanthine and hypoxanthine [17]. Moreover, it was shown to possess an activity toward urea residue and AP sites [18], deoxyuridine, and, with a much lower activity, base mismatch [19].

DNA repair is a biological mechanism important to monitor in humans to evaluate protective mechanisms against cancer risk and sensitivity to radiotherapy for cancer patients. The results obtained show that this miniaturized and parallelized assay is specific and convenient to process. It may be used for the screening of repair activities contained in cell extracts.

Materials and methods

Oligonucleotides

The sequences and names of the support oligonucleotides, lesion oligonucleotides, and junction oligonucleotides used in this study are listed in Table 1. The support oligonucleotides, extended by a $(dT)_5$ at their 5′ end, were chemically fixed onto the support by their 5′ aminated end. The correspondence between the name of the lesion oligonucleotides and the lesion that they contained was the following: U for dU, O1 and O2 for 8-oxodGuo, Ino for inosine, dF for dF, and Et for εdA. The MutY oligonucleotide was named according to the *E. coli* enzyme that excised the adenine opposite a 8-oxoGua [9]. It thus contained a dA positioned opposite an 8-oxodGuo located within the corresponding junction oligonucleotide. Within the different sequences, the lesions were designated by letters as described in Table 1. The lesion oligonucleotides were either purchased from Proligo (Paris, France) or synthesized in the laboratory (see [20] for the dF oligonucleotide). All were purified by PAGE. The chemical structures of the base lesions and their corresponding localizations within each well are depicted in Fig. 1. The 3′ terminal position of each lesion oligonucleotide was linked to a molecule of biotin to permit a fluorescent detection by binding to streptavidin-R-phycoerythrin (SA-PE).

Enzymes

Fapy-DNA-*N*-glycosylase and 3-methyladenine-DNA glycosylase were generously provided by S. Boiteux (CEA Fontenay aux Roses, France). Other enzymes (*E. coli* uracil-*N*-glycosylase, *E. coli* endonuclease VIII, *E. coli* endonuclease V) were purchased from Trevigen (Interchim, Montluçon, France). Reaction buffers (1×) for each enzyme are described in Table 2.

Table 1
Sequences of the oligonucleotides used in this study with their corresponding names

		Name	Lesion ODNs—3′ biotin (5′ → 3′)
Support ODNs (5′ → 3′)			
K	TTTTT CAC GTG GCT ACC ATG CCA TT	U	CCT GCC CTG TG**U** AGC TGT GGG-bio
H	TTTTT CCA CAC GGT AGG TAT CAG TC	O1	GAA CTA GTG **O**AT CCC CCG GGC TGC-bio
		O2	GAA CTA GTG GAT CCA TC**O** GTC TGC-bio
		C	GAA CTA GTG **G**AT CCC CCG GGC TGC-bio
J	TTTTT ACC ATC GCT TCC AGA GAC TC	Ino	GGA CTA ACG T**I**C CTT CCA AG-bio
I	TTTTT ATC CGT TCT ACA GCC GTA CA	MutY	TTC CGC GAT TG**A** CCA AGT CC-bio
M	TTTTT CAA GCC TTG GCA GAT GCT GT	dF	CAC TTC GGA TCG **F**GA CTG ATC T-bio
N	TTTTT CGA GTA GTG CTA TCG ACG TT	Et	CCT GCC CTG TGC **E**GC TGT GGG-bio
	Junction ODNs (5′ → 3′) (cLesion-*cSupport*)		
cKcU	CCC ACA GCT **G**CA CAG GGC AGG *AAT GGC ATG GTA GCC ACG TG*		
cHcO1	GCA GCC CGG GGG AT**C** CAC TAG TTC *GAC TGA TAC CTA CCG TGT GG*		
cHcO2	GCA GAC **C**GA TGG ATC CAC TAG TTC *GAC TGA TAC CTA CCG TGT GG*		
cJcIno	CTT GGA AGG **T**AC GTT AGT CC*G AGT CTC TGG AAG CGA TGG T*		
cIcMutY	GGA CTT GG**O** CAA TCG CGG AA*T GTA CGG CTG TAG AAC GGA T*		
cMcdF	AGA TCA GTC ACG **A**TC CGA AGT G*AC AGC ATC TGC CAA GGC TTG*		
cNcEt	CCC ACA GC**T** GCA CAG GGC AGG *AAC GTC GAT AGC ACT ACT CG*		

The modified nucleosides in the lesion oligonucleotides are represented by letters in boldface (see Materials and methods). Each junction oligonucleotide consisted of two parts: one complementary of a given support oligonucleotide (part in italic letters) and one complementary of a given lesion oligonucleotide. The bases located opposite the lesions are in boldface letters; **O** in cIcMutY represents 8-oxodGuo.

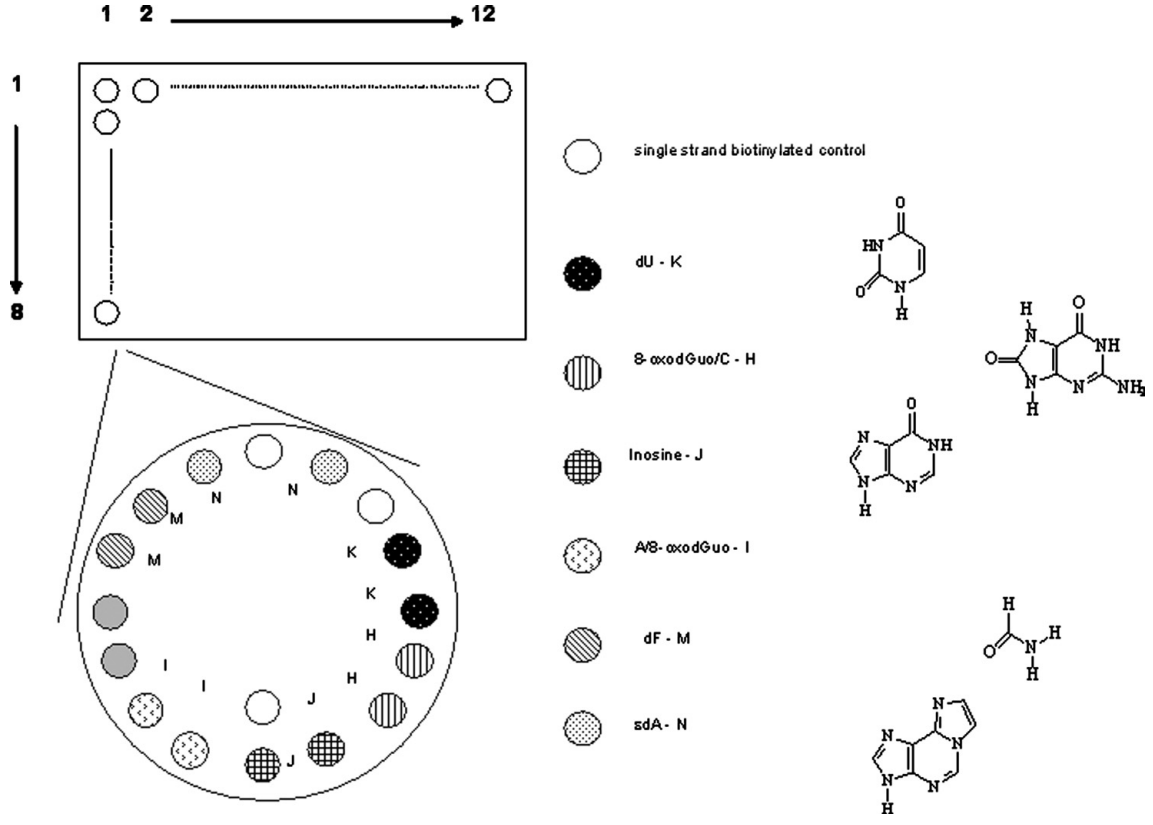

Fig. 1. Design of the DNA repair biochip. Each well of the microplate was functionalized by indirect hybridization with six different oligonucleotides that contained DNA damage (dU in position K, 8-oxodGuo opposite a dC in position H, inosine in position J, dA opposite a 8-oxodGuo in position I, dF in position M, and εdA in position N). Each position was duplicated. Three biotinylated single-stranded controls (open circles) were chemically fixed onto the support. The chemical structures of the modified bases are shown.

Table 2
Composition of the enzyme buffers

	Hepes/KOH (mM)	Tris/HCl (mM)	KCl (mM)	EDTA (mM)	NaCl (mM)	$MnCl_2$ (mM)	2-Mercaptoethanol (mM)	Dithiothreitol (mM)
Endo V	10				50	0.5		
Endo VIII	10		100	1				
AlkA	70			1			5	
Fpg		40	100	0.5				
UNG		20		1				1

Preparation of the whole-cell extracts

The cell extracts were prepared from exponentially growing cells following the protocol of Redaelli et al. [21] with minor modifications. Approximatively 10^7 cells (either HeLa or human normal primary fibroblasts) were suspended in 100 μL of ice-cold buffer A (45 mM Hepes–KOH, pH 7.8, 0.4 M KCl, 1 mM EDTA, containing 10% glycerol and 1 mM dithiothreitol). The membranes were disrupted by three cycles of freezing/thawing at −80 and 4 °C, respectively. A 1% solution of Triton X-100 in buffer A was then added (12 μL for 50 μL of cell mixture). After centrifugation of the lysate at 14,000*g* for 5 min at 4 °C, 3 volumes of buffer B (45 mM Hepes–KOH, pH 7.8, 0.25 mM EDTA) containing 0.3 mg/mL of bovine serum albumin, 2% glycerol, antiproteases (Complete-mini; Roche Applied Science, Meylan, France), and phenylmethylsulfonyl fluoride were added to the supernatant. The cell extracts were kept at −80 °C until use.

The protein content was determined using the micro BCA kit (Interchim). The protein concentration was approximately 16 mg/mL for all extracts.

Support

The supports consisted of black polystyrene microplates separated into eight-well modules (strips). Each well was addressed with a series of oligonucleotides of different sequences chemically tethered by oriented coupling on the activated plastic surface, according to Fig. 1 configuration (oligo sorbent array (OLISA) technology; Apibio, France). Fifteen spots were printed per well. Three of them corresponded to a single-stranded biotinylated oligonucleotide that was used as control (vide infra). Six other oligonucleotides 20 nucleotides long were printed in duplicate within each network on a circular format (Fig. 1). They were called the support oligonucleotides.

Typical diameter of each spot was 150 μm, corresponding to a deposit of 2–3 nL of each probe solution at 10 μM concentration in appropriate buffer. At best, 30 fmol of oligonucleotide could be immobilized at each fixation site.

Hybridization of the lesion oligonucleotides

For the first hybridization step, the junction oligonucleotides were mixed together in ice-cold PBS/NaCl 0.2 M (hybridization buffer; HB) at a concentration of 0.5 pmol/35 μL. Typically, a volume of 35 μL was distributed in each well kept on ice. The strips were then incubated for 2 h at 37 °C in a humidified atmosphere. After removal of this solution, the wells were rapidly rinsed with 200 μL of HB, and a mixture of the lesion oligonucleotides was added under the same conditions as those for the first hybridization. The second hybridization step lasted 2 h at 37 °C. The strips were then rinsed (3 × 5 min) at room temperature with 200 μL of HB.

Digestion with the E. coli enzymes

The strips were placed on ice and incubated 3 × 5 min with the prechilled buffer corresponding to the enzyme to be tested (Table 2). Enzymes were diluted as specified under Results. At indicated times, the strips were removed, rinsed 3 × 5 min with 200 μL of HB containing 0.1% Tween 20 (washing buffer; WB) and kept in the same buffer at 4 °C until all the digestions were completed. The last wash lasted 30 min at 37 °C in WB.

Digestion with the cell extracts

The strips were first incubated for 60 min at 4 °C with 200 μL of the digestion buffer (DB; 1 volume of buffer A and 3 volumes of buffer B); 50 μL of the diluted extract was added (150 μg/mL of proteins in the same buffer). The digestion proceeded at 30 °C. At indicated time, the strips were removed and rapidly rinsed with 200 μL of WB to remove the active extracts. They were then kept in the DB alone at 30 °C until all the digestions were completed. Washes (3 × 20 min) were finally performed at 37 °C with 200 μL of the WB. Experiments were replicated eight times.

Detection

The strips were incubated in the dark with 50 μL of HB containing 5 μL of SA-PE solution (Sigma Chemicals, St. Louis, MO, USA) for 1 h at 37 °C. The strips were washed 3 × 5 min at 37 °C in WB in the dark. The fluorescent signals were recorded immediately.

Signal recording and image analysis

The images were automatically recorded using an Apibio high-throughput biochip imaging system (Apimager) in which acquisition is charge-coupled device camera based. The signals were integrated upon 1500 ms excitation time. Image processing parameters such as brightness and contrast were standardized. The images were subsequently analyzed using the automated Apimager software developed by Apibio. The position of the control oligonucleotides was pointed manually and from the latter data every other spot was automatically identified. The fluorescence intensity was calculated as the average gray level of each spot multiplied by its surface. The signal obtained at each oligonucleotide site upon incubation with the enzyme buffer alone for 1 h at 37 °C was set to 100. For each site, all the results were calculated relative to this value. A mean was calculated for the duplicated points within each well.

Statistical analyses

Statistical analyses were conducted using StatView software. Data were expressed as means ± standard error. Data were compared using ANOVA and Bonferroni/Dunn posttest. Comparison between HeLa and fibroblast repair profiles was assessed using three variables (type of extract, digestion time, and type of lesion). For one given extract type, the lesion effect (significance between the cleavage of the different lesions) was determined using two variables (digestion time and type of lesions).

Results

Solid support assay

To site-specifically immobilize the oligonucleotides that contained the lesions (called the lesion oligonucleotides), a sandwich construction was elaborated. As a first step, long junction oligonucleotides (approximatively 40 nucleotides long) were mixed together and hybridized to their complementary support oligonucleotides. In a second step, the lesion oligonucleotides were mixed together and hybridized to their complementary single strand part of the different junction oligonucleotides already hybridized onto the support.

Each lesion oligonucleotide bore a biotin at its 3′ end. After the second hybridization step, the microwells were incubated in the presence of the enzyme of interest. The incision of the damage by the enzyme yielded oligonucleotide fragments with sizes of between 6 and 14 nucleotides. Extensive washes at 37 °C led to the dehybridization of the cleaved lesion oligonucleotides whereas the full-length oligonucleotides remained hybridized onto the support. The last step was the detection of the biotin located at the 3′ end of the lesion oligonucleotides by a streptavidin-phycoerythrin conjugate. The fluorescent signal located at each site was quantified using automated customized fluorescent image analysis (Fig. 2). Examples of the images obtained in the course of a digestion experiment are shown in Fig. 3.

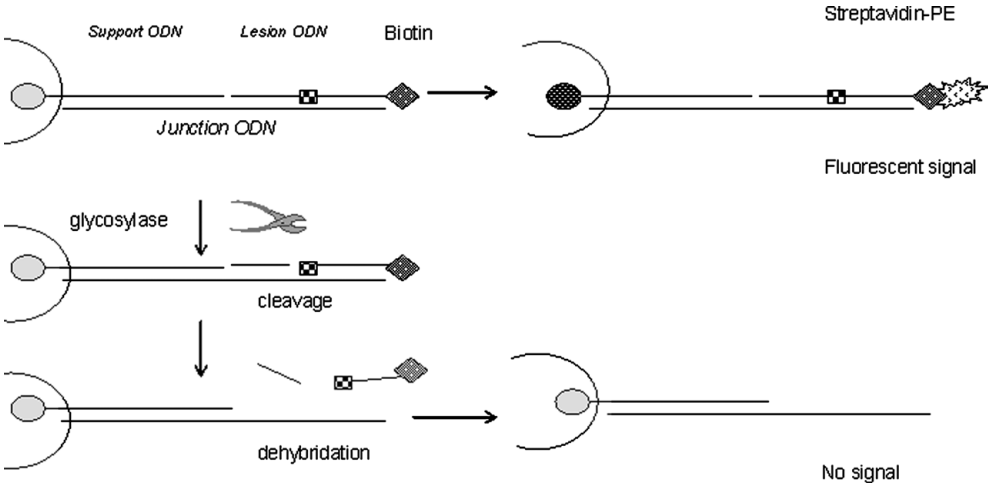

Fig. 2. Principle of the detection of cleavage activity of DNA-*N*-glycosylases on the biochip. Each oligonucleotide that contained a base lesion (lesion oligonucleotide) was biotinylated at its 3′ end. Its fixation within the support was directed by a longer junction oligonucleotide hybridized onto a probe (support oligonucleotide) chemically fixed at a specific site of a microplate well. The sandwich hybrids could be detected using a streptavidin-phycoerythrin conjugate and quantified by fluorescence imaging. After incubation with a DNA-*N*-glycosylase and in case of cleavage of the lesion by the enzyme, the nicked product labeled by the biotin was eliminated by washes. We thus observed signal loss at the site of the recognized substrate.

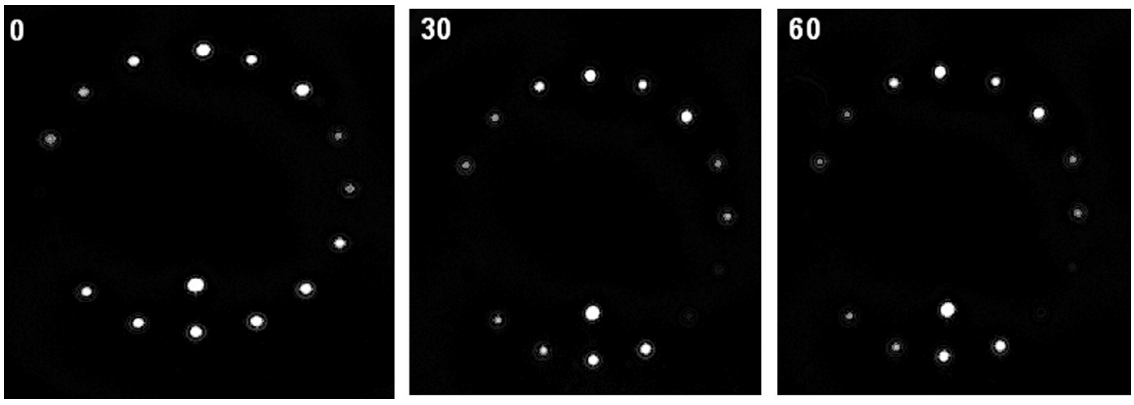

Fig. 3. Examples of images recorded under the fluorescent microscope by the charge-coupled device camera after 0, 30, and 60 min of Fpg digestion of the wells functionalized by U, O2, MutY, Ino, dF, and Et. The images were subsequently analyzed using the Apimager software as described under Materials and methods.

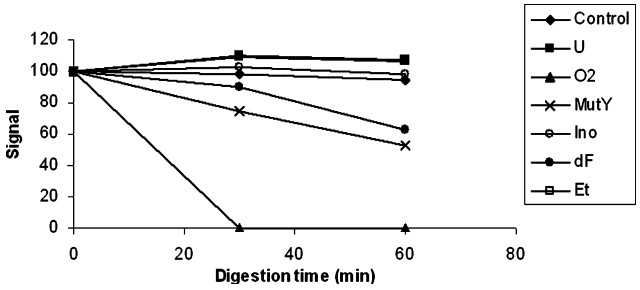

Fig. 4. Signal evolution as a function of the digestion time. The mean for each duplicate is reported. The total excision of the 8-oxodGuo contained in O2 was observed after 30 min of digestion. Diminution of the signal at the MutY position was due to the cleavage of the 8-oxodGuo contained in the junction oligonucleotide that led to the release of the biotinylated MutY oligonucleotide. dF was also excized by Fpg, nevertheless less efficiently than by 8-oxodGuo.

Control of hybridization specificity and efficiency

We checked for the specificity of hybridization of the different support oligonucleotides with their complementary strands and for the lack of cross-hybridization with the other strands. For this purpose, independently for each location, oligonucleotides that were fully complementary to the support oligonucleotides and labeled with a biotin at their 3′ end were incubated within the addressed wells. The signal localization was then controlled within the wells. The hybridizations were site specific (data not shown).

The same individual experiments designed to control the site-specific hybridization were performed using the indirect hybridization system for each pair of oligonucleotides (junction oligonucleotide + lesion oligonucleotide; Table 1). In the latter case the hybridization was sequential: hybridization of one junction oligonucleotide for 2 h at 37 °C followed by the hybridization of the corresponding lesion oligonucleotide under the same conditions. Sandwich hybridization was specific for each site.

Stability of the hybrids and of the cleaved oligonucleotides

Series of washing experiments were performed to determine the stability of the constructions hybridized onto the support. Different temperatures (between 25 and 45 °C) and two buffers differing by their ionic strength (PBS or PBS + 0.2 M NaCl) were tested: all the hybrids remained stable between 25 and 37 °C in PBS containing 0.2 M NaCl and 0.1% Tween 20 (data not shown).

Washing experiments were also performed under the same conditions to determine the stability of the cleaved oligonucleotide. After enzymatic digestion, washing for 30 min at 37 °C in PBS containing 0.2 M NaCl and 0.1% Tween 20 led to the elimination of all cleaved oligonucleotides from the support.

Digestion experiments with the E. coli enzymes

Representative results of series of experiments that were performed are presented. Various combinations of the lesion oligonucleotides were tested in the different experiments.

Fpg digestion of U, O2, MutY, Ino, dF, and Et. At the concentration used (4.4 μg/mL; 35 μL/well), Fpg totally cleaved O2 very fast and as expected dF with a lesser efficiency (Fig. 4). We could also observe that the duplex MutY-cIcMutY was partially cleaved by Fpg. Complementary experiments were conducted by PAGE analysis to obtain further insights into this last result. We confirmed (data not shown) using the same oligonucleotides that, in solution and above an enzyme concentration of 0.1 ng/fmol of duplex, a 30% cleavage of the 8-oxodGuo located in the long junction oligonucleotide opposite the dA was observed after 1 h of digestion. As expected, dA was not cleaved in the MutY oligonucleotide.

Endo V digestion (0.75 U) of U, O1, MutY, dF, and C and Endo V digestion (0.075 U) of U, O1, MutY, dF, Ino,

and Et. At low concentration (0.075 U), (Fig. 5B), Endo V recognized and excised inosine (ino oligonucleotide) which is its known favorite substrate. It also cleaved dF with an almost equal efficiency. At higher enzyme concentration (0.75 U) (Fig. 5A) as expected, all the lesions tested were substrates with various cleavage efficiency.

AlkA and Endo V digestion of U, O1, Ino, and dF. AlkA creates AP sites; thus to induce a strand break it was necessary to complement its activity by adding an enzyme that possessed a lyase activity. At low concentration (0.015 U/well) (Fig. 6B), Endo V induced the cleavage only of abasic sites. The signal decrease observed after the complementary action of AlkA and Endo V could thus be related to AlkA activity. Under the conditions that were used (AlkA 5 μg/well and Endo V 0.015 U/well) (Fig. 6A), the favorite substrate of AlkA was Ino whereas Et and O1 were recognized with lower efficiency. As expected U was not a substrate at all.

UNG and Endo VIII digestion of U, MutY, and C. We tested the AP endonuclease activity of Endo VIII (0.1 U/well) associated with the glycosylase activity of UNG (1 U/well) on MutY and U (Fig. 7). Only U was cleaved with high efficiency. UNG alone (1 U/well) and Endo VIII alone (0.1 U/well) did not show nonspecific activity either on the control or on the lesion oligonucleotides (data not shown).

Fpg digestion: comparison of O1 and O2 digestion. The O1 oligonucleotide was only partially digested by Fpg on the biochip as revealed by the residual signal measured on the addressed spot (Fig. 8). The O1 oligonucleotide was also never completely cleaved by Fpg in solution, whatever the concentration of the enzyme tested (data not shown), and the rate of cleavage was in the same range as that determined on support.

The shift of the 8-oxodGuo 6 bases from the 3′ end of the oligonucleotide (O2, Table 1) instead of 14 bases (O1, Table 1) modified the digestion kinetic and rate. A very fast and efficient signal disappearance was observed on the support (Fig. 8) with 11 μg/mL of Fpg. The same feature was observed in solution (PAGE analysis; data not shown).

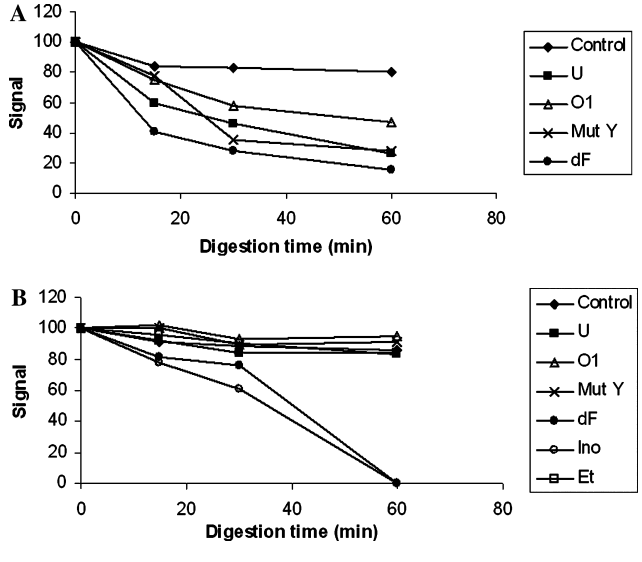

Fig. 5. Damage excision by Endo V. The mean for each duplicate is reported. Whereas at high concentration of enzyme (A) (Endo V 0.75 U), all the oligonucleotides were more or less substrates for Endo V, the use of 0.075 U per well (B) resulted in the digestion only of Ino and dF.

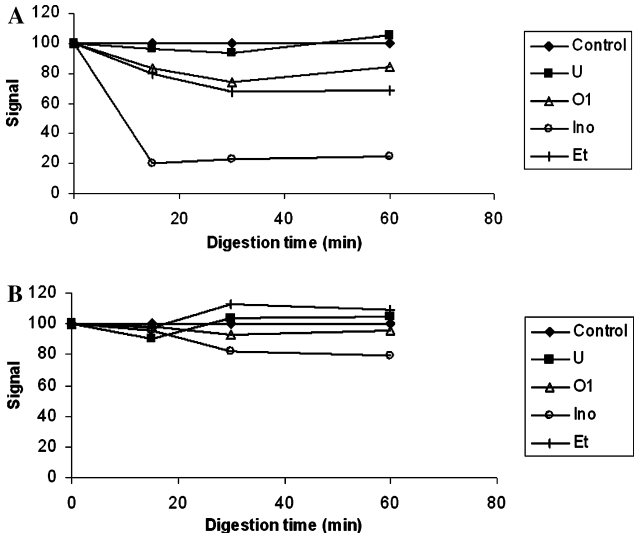

Fig. 6. Damage excision by AlkA complemented by the activity of Endo V. The mean for each duplicate is reported. (A) AlkA (5 μg/well) + Endo V (0.015 U/well) digestion. The oligonucleotides were incubated for the indicated time with AlkA and subsequently for 1 h at 37 °C with Endo V. Ino was very efficiently excised by AlkA + Endo V while Et and O1 were recognized with lower efficiency. At the concentration used, Endo V alone (B) (0.015 U/well) failed to cleave the lesion oligonucleotides.

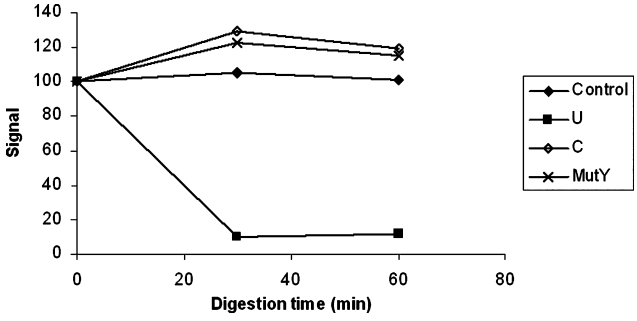

Fig. 7. Damage excision by UNG (1 U/well) complemented by the activity of Endo VIII (0.1 U/well). The mean for each duplicate is reported. UNG and Endo VIII were active in the UNG buffer (Table 2); as a consequence they could be used simultaneously. U was totally excised by this combination of enzymes whereas the other lesions were not recognized.

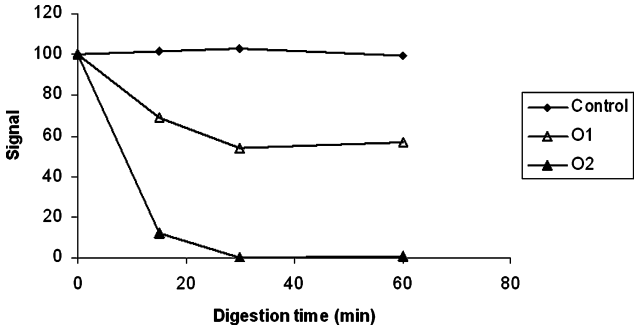

Fig. 8. Influence of the position of 8-oxoGua within the oligonucleotide on the efficiency of excision by Fpg. The sequences of the O1 and O2 oligonucleotides were identical, except for the position of the lesion. In O1, 8-oxoGua was located 14 bases from the 3′ end of the oligonucleotide whereas in O2 it was located 6 bases from the 3′ end.

Digestion experiments with the cell extracts

The microarrays were addressed with the six lesion oligonucleotides for these experiments (Fig. 9). Approximately 8 μg of proteins was used per well (150 μg/mL). The use of higher protein content resulted in enhanced single-strand control oligonucleotide digestion while the extent of cleavage of lesion oligonucleotides did not improve (data not shown). Using the HeLa extracts, after 15 min incubation, the rate of cleavage of the different lesions reached a plateau. We observed cleavages of approximately 20% for O2, Ino, and MutY, 40% for dF, and 60% for U. Data obtained with the primary human fibroblast extracts were different. Incision at the sites of the lesions was time dependent over a 60-min period. As with the HeLa extract, cleavage activity was the highest for U (40%) and dF (30%). O2 was inefficiently cleaved, while incision of Ino, MutY, and Et reached 20 to 25%. ANOVA and Bonferroni/Dunn posttest revealed that the two profiles were significantly different ($p < 0.0001$). We looked at the lesion effect for each type of extract. For HeLa extract, there was no statistical difference between cleavage of the control and O2. Relative comparison of Ino/MutY, MutY/Et, and Ino/Et pairs revealed no statistical difference whereas cleavage was statistically different for all the other pairs ($p < 0.0001$). For fibroblast extract, all the comparisons gave statistically significant differences ($p < 0.0001$) except for the pairs O2/Ino and O2/MutY.

Discussion

Design of the DNA repair biochip

Versatility of the system was assured by the indirect hybridization of the lesion oligonucleotides via junction oligonucleotides: (i) By simply changing the junction oligonucleotides and more precisely the part complementary to the lesion oligonucleotides, we may use the same microarray support with a large variety of distinct substrates or sequences. (ii) This indirect hybridization system possibly allows the fixation of oligonucleotides that contain different lesions within the same sequence context.

In the format that we used, the hybridization was directed by the sequence of the different support oligonucleotides. We first checked by performing independent experiments at each site that cross-hybridization did not occur on the support under the conditions used. Nevertheless, it was not possible to directly demonstrate that hybridization of all the oligonucleotides mixed to-

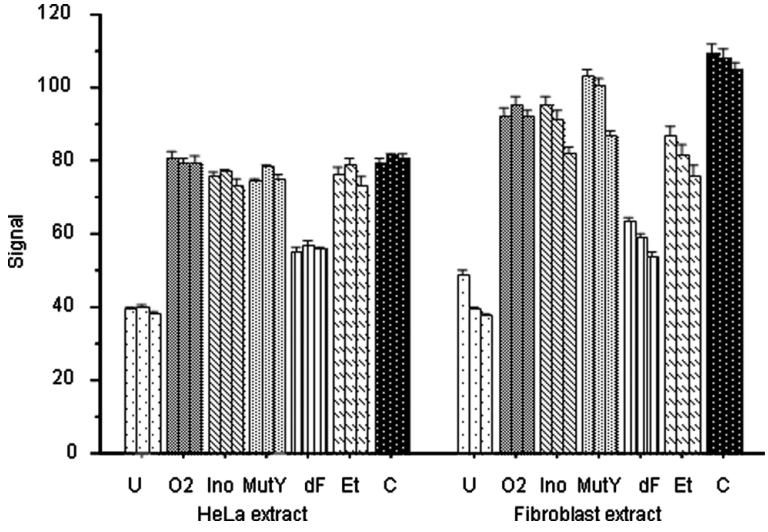

Fig. 9. Histogram representation of the cleavage of the lesion oligonucleotides by cell extracts on the microarray. The support was addressed with the six lesion oligonucleotides. The extracts (150 μg/mL) were prepared from HeLa cells (left) and human normal primary fibroblasts (right). The means of eight experiments (±standard error) are reported.

gether were site specific. Hence different known enzymes had to be tested on known substrates to accumulate results aimed at validating this approach.

Function of the control oligonucleotide

Each well contained a biotinylated single-strand control oligonucleotide (Fig. 1) fixed at three specific and predetermined sites in each well by the supplier of the OLISA support. This oligonucleotide (20 nucleotides long) was chemically attached by its 5′ end on the support and carried a biotin moiety at its 3′ end. It remained single-stranded during the whole process.

Thanks to the biotin attached at its 3′ end, the oligonucleotide was detected after incubation with the SA-PE conjugate and thus served as positive control for the imaging step. The function of the control was essentially to direct the automatic localization of the different spots theoretically present in each well during the course of the image analysis with the Apimager software.

Digestion by E. coli enzymes

The use of Endo V or Endo VIII in complement to exclusive glycosylase activities allowed the revelation of the AP sites. However, Endo VIII presented two advantages over the other enzyme: it had a narrower specificity and was active in the glycosylase buffer as it is not inhibited by the EDTA.

New experimental data on the recognition and very efficient cleavage of dF by Endo V were obtained. Urea residues and abasic sites are known substrates for Endo V [18], so the fact that this endonuclease also cleaved dF was not surprising. As expected, the latter lesion was also cleaved by Fpg [20].

Apart from the last result which is new, all the digestion curves that were obtained correlated well with relative known substrate specificity for the enzymes tested. To obtain more precise insights into some of the results, complementary PAGE analyses were conducted. In particular, analysis of the Fpg digestion experiment (Fig. 4) showed a digestion at the position I of the microwell. It concerned the MutY-biotinylated oligonucleotide that presented a dA opposite 8-oxodGuo. The PAGE analysis revealed that this signal diminution was due to a partial cleavage of the 8-oxodGuo located in the junction oligonucleotide and not to a cleavage in the biotinylated strand, as already demonstrated by Tchou et al. [22] for this last lesion. Indeed the elimination of a fragment of the junction oligonucleotide induced the dehybridization of the corresponding biotinylated oligonucleotide and thus to the loss of the label at the corresponding position.

The results obtained on dF, O1, and O2 oligonucleotides were also confirmed by PAGE analysis (data not shown).

Digestion by cell extracts

BER activities and absence of nuclease activity in the prepared extracts were checked using PAGE analysis of the ^{32}P-labeled substrates (data not shown). The in vitro assay was very sensitive and less than 8 μg of proteins per well was required to complete the excision reaction. Higher amounts of protein did not improve the rates of digestion of the different substrates. Statistical analysis revealed an overall significant difference in the profile of cleavage of the substrates for the two types of extracts (Fig. 9) and a significant effect of the lesions on the cleavage efficiency for fibroblast extracts. Two groups of damage categories could be defined: the first one formed by the most easily excised lesions dU and dF (30 to 60% cleavage) and the other one that included 8-oxodGuo, dA/8-oxodGuo pairs, εdA, and inosine (0 to 20% cleavage). The poor repair of 8-oxodGuo compared to dU is a known feature of human cell extracts [23]. The purpose of our work was not to elucidate the nature of the activity present in the extracts but rather to present the microarray assay. Hence we did not investigate which activity, remained undescribed in cell extracts, excised dF. However, as it is relatively high, the last lesion could be an interesting substrate to incorporate into a selection of lesions aimed at fully characterizing an extract activity. Efforts will focus in further study to elucidate the nature of this activity.

Choice of the oligonucleotide sequences

For this first approach, we chose to study lesions within different sequence contexts. It is established that for BER, the repair rate is dependent on the sequence surrounding the lesion [24]. Thus, the cleavage differences that we saw with the repair enzymes were due not only to the nature of the lesion but also to the sequence context and as a consequence to the position of the lesion within a given sequence (Fig. 7: comparison of O1 and O2 cleavage by Fpg). The distance of the lesion from the 3′ end of the oligonucleotide could also influence the enzyme turnover. However, this latter point has to be further explored. From the data that we have, it would be advisable to position the lesion near the 3′ end of the oligonucleotide to promote the fast elimination of the cleaved product. Experimental data have to be taken into account for the choices of optimal sequences and the position of the lesion within the DNA fragment.

Conclusion

This functional assay allows the monitoring of the cleavage activity of repair enzymes simultaneously toward a broad spectrum of DNA base lesions using a

new approach on a microarray. We adapted an existing generic format which is a low-density biochip. It has the advantage of being simple and very convenient to process. The multiparametric analysis approach is very powerful and generates abundant results and information in a very short time. A very important parameter to explore is the choice of the oligonucleotide sequences. It raises the question of standardization of the repair assays, which also remains a problem for experiments performed in solution. We have selected lesions issued from deamination, oxidation, and alkylation of normal bases. Indeed, as the tool is generic, it may be extended to the study of other substrates provided that they may be incorporated into synthetic oligonucleotides. The assay described is compatible with a small-scale preparation of extracts. Moreover it requires sufficiently small quantities of proteins to be performed with primary cells. It opens new perspectives in the development of tests to study the influence of genotoxic agents and chemicals on repair induction and modulation. The possibility offered by our test to be used to evaluate DNA repair in a small biological sample such as tumor extract opens potential clinical applications. Anticancer treatment such as irradiation by X- or γ-rays [25] and administration of drugs such as bleomycin, adriamycin, *cis*-platinum, or nitrosourea kill tumor cells by damaging DNA. They create single- or double-stranded breaks, abasic sites, or oxidative base and sugar damage. Hence, the resistance of tumors to various chemotherapeutic agents depends partly on their DNA repair capacities. DNA repair biochips can also be useful tools to screen for agents able to improve efficiency of ionizing radiation or anticancer drugs. Inversely we can test DNA repair capacities to detect abnormally low activity that can lead to a dramatic hypersensitivity to radiation. Hypersensitivity to ionizing radiation could be related in humans to an increased risk of breast cancer [26]. Such hyperreactivity can eventually lead to the death of patients when treated with usually safe radiation doses.

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