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Direct inhibition of excision/synthesis DNA repair activities by cadmium: Analysis on dedicated biochips

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ABSTRACT

The well established toxicity of cadmium and cadmium compounds results from their additive effects on several key cellular processes, including DNA repair. Mammalian cells have evolved several biochemical pathways to repair DNA lesions and maintain genomic integrity. By interfering with the homeostasis of redox metals and antioxidant systems, cadmium promotes the development of an intracellular environment that results in oxidative DNA damage which can be mutagenic if unrepaired. Small base lesions are recognised by specialized glycosylases and excised from the DNA molecule. The resulting abasic sites are incised, and the correct sequences restored by DNA polymerases using the opposite strands as template. Bulky lesions are recognised by a different set of proteins and excised from DNA as part of an oligonucleotide. As in base repair, the resulting gaps are filled by DNA polymerases using the opposite strands as template. Thus, these two repair pathways consist in excision of the lesion followed by DNA synthesis. In this study, we analysed *in vitro* the direct effects of cadmium exposure on the functionality of base and nucleotide DNA repair pathways. To this end, we used recently described dedicated microarrays that allow the parallel monitoring in cell extracts of the repair activities directed against several model base and/or nucleotide lesions. Both base and nucleotide excision/repair pathways are inhibited by $CdCl_2$, with different sensitivities. The inhibitory effects of cadmium affect mainly the recognition and excision stages of these processes. Furthermore, our data indicate that the repair activities directed against different damaged bases also exhibit distinct sensitivities, and the direct comparison of cadmium effects on the excision of uracile in different sequences even allows us to propose a hierarchy of cadmium sensibility within the glycosylases removing U from DNA. These results indicate that, in our experimental conditions, cadmium is a very potent DNA repair poison.

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1. Introduction

Cadmium and cadmium compounds toxicity has long been established. Cadmium has been used in several industrial processes, resulting in professional exposure of workers, while air-borne cadmium results in environmental exposure of the general population through respiration and food contamination. Inhalation through cigarette smoke is also a major mode of contamination. Cadmium half life in humans is long (15–20 years), and Cd accumulates in several target organs. This accumulation over time represents a problem as Cd is a potent carcinogen and was classified in 1993 by IARC as Group I carcinogen to humans [1].

Cadmium toxicity has been investigated *in vivo* and *in vitro* in numerous experimental systems. Depending on the animal model or the cell type analyzed, the concentration, and the mode and duration of contamination, Cd exposure leads either to apoptosis or adaptation and cell survival. Cadmium interferes with several key cellular processes [2], which together control the transcription of a large number of target genes. Cd is intrinsically only weakly genotoxic. However, Cd interferes with intracellular homeostasis of redox metals like zinc, copper and iron [3]. Because of its strong affinity for sufhydril groups, it displaces Zn and Cu ions from Cys-rich proteins like metallothioneins or from the Zn-finger domains found in many enzymes, including some involved in DNA repair [4,5]. As Cd also binds Cys-rich glutathion, the cell faces at the same time an increase in intracellular level of redox metals and a depletion of anti-oxidant defences. These conditions create inside the cell

Abbreviations: BER, base excision repair; NER, nucleotide excision repair; AP, apurinic-apyrimidic site; APE1, AP endonuclease 1; 8 oxoG, 8-oxo-7, 8-dihydroguanine; OGG1, 8 oxoG glycosylase; ODN, oligonucleotide; NE, nuclear extract; THF, tetrahydrofuran; Tg, thymine glycol; CPD, cyclobutane pyrimidine dimers; 6-4 PP, 6-4 photoproducts; AAG, (6-4PP); AAG, alkyladenine DNA glycosylase; ds, double-stranded.

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a pro-oxidant environment [6,7] which can result in mutagenesis and transformation through excessive DNA damage [8].

Spontaneous and environmentally induced DNA lesions constantly appear on genomic DNA [9]. If left un-repaired, they can eventually result in sequence alterations and genetic rearrangements. To avoid these deleterious effects, several complementary, partially overlapping elaborate processes have evolved to detect and repair DNA damage [10]. Damaged bases and nucleotides are repaired mostly by excision/synthesis mechanisms: after detection, the lesion is removed together with a variable number of surrounding nucleotides, and the correct DNA sequence is faithfully restored using the opposite strand as template [11]. In the nucleotide excision repair (NER) pathway, bulky lesions that distort the DNA double helix structure are recognized either by proteins associated with the transcription machinery or by the XPC protein, and removed as part of an oligonucleotide [12]. In the base excision repair (BER) pathway, small base lesions are selectively removed by a specific glycosylase [13], leaving an abasic (AP) site which is then incised, either by the AP endonuclease 1 (APE1) or by the glycosylase itself, if it is endowed with AP lyase activity. The resulting DNA gap is subsequently filled with replacement of one or a few nucleotides depending on the damage excision/incision mechanism and the polymerase(s) recruited [14]. Thus, DNA repair through NER and BER depends on the sequential recruitment and activation at the damaged sites of numerous proteins in a timely manner [14,15]. These two pathways are fairly well biochemically defined and can be reconstituted *in vitro* with largely different sets of purified proteins. However, they are not fully compartmentalized as it appears that inactivation or mutation of typical NER proteins can impair the repair of typical BER substrates [16–19].

Numerous studies showed that the efficiency of DNA repair activities is affected in the context of a Cd-induced stress response [8,20]. Indeed, for example, the activity and expression of some of the glycosylases are regulated during the cell cycle [21,22], and Cd interferes with the regulation of cell cycle progression by several mechanisms [23,24]. In addition, Cd exposure can prevent DNA repair through the relocalization and sequestration in stress granules of DNA repair factors like OGG1 [25], the glycosylase responsible for removal of 8oxoG, the most frequent oxidative DNA lesion. Repair of 8oxoG can also be decreased through the modulation of OGG1 expression because of the Cd-dependent reduction of the Sp1 transcription factor activity [26–28]. In addition, Cd effects are not limited to base damage, as Cd exposure also affects the repair of DNA double-strand breaks by non-homologous end joining [29]. These few examples illustrate how Cd contamination indirectly decreases the cellular DNA repair activities. However, Cd can also directly affect DNA repair protein activity. *In vitro*, Cd exposure inhibits the DNA binding activity of purified XPA [30,31] and XPC [29], two key components of NER, the incision activity of purified APE1 [32] and the glycolsylase activity of OGG1 [25,33].

Thus, Cd exposure will at the same time generate an environment that is favourable to the generation of DNA lesions and interfere with DNA repair, and its carcinogenicity may be related to the deficient repair of an excessive load of lesions. However, because most of the previous studies were focused on the repair of 8oxoG by OGG1, we found important to address the direct effects of Cd on the repair of other types of DNA lesions repaired by BER and/or NER. In this paper, we used two recently described multiplexed assays to analyze at the same time the direct impact of Cd contamination on the DNA repair activities catalyzing excision/synthesis repair in an a-cellular system. Our results show that Cd directly inhibits the repair of several oxidative, spontaneous and UV-induced lesions, mainly by inhibiting the initial step of the repair process.

2. Materials and methods

The plasmid and oligonucleotide chips used in this study have recently been described [19,34]. According to their implementation, these two microsystems allow us to measure different parameters of the BER and NER pathways in Hela cell nuclear extracts (NE, Table 1). Hela cell NE, purchased in bulk quantities from CilBiotech (Mons, Belgium), was prepared according to Dignam et al. [35] and dialyzed in 20 mM HEPES (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM PMSF and 0.5 mM DTT. NE was aliquoted before storage at −80 °C. Each aliquot was used only once.

2.1. Plasmid chips

Briefly, damaged plasmids generated by chemical or physical treatment of supercoiled pBluescript are spotted onto an hydrogel-coated slide. Each plasmid bears a specific type of lesion(s) repaired by NER [cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PP), thereafter collectively referred to as CPD-64] or BER [8oxoG, glycols, alkylated bases (AlkB)]. Glycols can be recognized and excised by hNTH1 and NEIL1 [22,36], two bi-functional glycolsylases. 8oxoG is recognized and excised by OGG1, which is also a bi-functional glycosylase [37]. CPD and 6-4PP lesions are typical NER substrates. Alkylated bases can be recognized by AAG, a monofunctional glycosylase which therefore requires the subsequent action of APE1 [13], or can also be repaired by NER [38]. Undamaged plasmid is also spotted alone to control for non-specific activities. The plasmid microarrays are incubated at 30 °C with nuclear extracts in the presence of reaction buffer, ATP and dNTPs, including Cy5-labelled dCTP which is incorporated into the plasmid following excision/synthesis repair of the lesion by the nuclear extract. Alternatively, the incubation with NE is performed in the absence of ATP and dNTPs and in the presence of aphidicollin. In these conditions, the activity of nuclear extract DNA polymerases is inhibited and only excision of the lesions takes place. These excised sites are then revealed in a second incubation in the presence of Klenow DNA polymerase and dNTPs, including Cy5-labelled dCTP [19]. The fluorescence incorporated in each plasmid spot, proportional to the repair activity is quantified at 635 nm using a Genepix 4200A scanner (Axon Instrument) and the Genepix Pro 5.1 software (Axon Instrument).

2.2. Oligonucleotide biochip

The oligonucleotide chip is composed of double stranded (ds) oligonucleotides (ODN) anchored on streptavidin coated slides. They consist in duplexes, formed by the specific hybridization of one short, Cy3-labelled, lesion-containing ODN and one long ODN that are addressed onto biotinylated anchoring ODN complementary to the long ODN of the duplex. The lesions included in the duplexes are: thymine glycol paired with A, tetrahydrofuran (THF, an AP site equivalent) paired with A, U paired with A and U paired with G. A control duplex is also prepared by using an undamaged Cy3-labelled short ODN. Each duplex, lesion and control, is printed in duplicate in each well. Recognition and excision of the lesion by a glycosylase, followed by incision of the resulting AP site by APE1, or excision and incision by a bi-functional glycosylase, upon incubation with NE results in disassembly of the duplex and loss of the fluorochrome. Therefore, the decrease of fluorescence at lesion-containing ODN spots measures the cleavage efficiency of the lesion. Non specific activities are measured from control ODN without lesions. Excision rate for a given DNA lesion is defined as the percentage of residual fluorescence after incubation with the NE corrected for the eventual degradation of the control oligonucleotide with no lesion [34].

Table 1
Overview of the activities measured on the different biochips.

Assay	Repair pathways	Activities measured	Activities performed by	
			Excision	Synthesis
Plasmid Biochip	NER and BER	Excision and synthesis	Cell extract	Cell extract
	NER and BER	Excision	Cell extract	Klenow DNA polymerase
ODN Biochip	BER	Excision	Cell extract	N/A[a]

[a] Not applicable.

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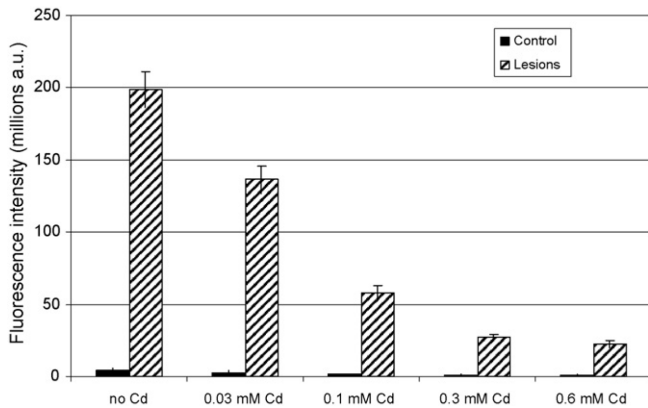

Fig. 1. Global inhibition of excision/synthesis DNA repair activities in the presence of Cd. Excision/synthesis repair of the indicated lesions by Hela NE at a final concentration of 0.5 mg/mL was allowed to proceed for 3 h at 30 °C without Cd or in the presence of 0.03, 0.1, 0.3 and 0.6 mM Cd in the reaction mix. The histograms represent the total fluorescence incorporation in undamaged plasmids (black bars) vs plasmids bearing DNA lesions (stripped bars) following incubation with Hela cell NE in the absence and the presence of $CdCl_2$ at increasing concentrations.

3. Results

3.1. Measure of BER and NER excision/synthesis activities on plasmid biochips

We addressed the effects of Cd on excision/synthesis repair of representative lesions substrates for BER (8oxoG, Glycols, AlkB) and/or NER (CPD-64, AlkB) on supercoiled ds plasmids arrayed on plasmid chips as described in Section 2. This plasmid chip assay allows the simultaneous measurement of the repair activities directed against each of the individual lesions. As a first measure of Cd effects on DNA repair activities, we compared the total amount of fluorescence incorporated in all damaged plasmids spots in each condition, i.e. we compared the sum of the repair activities directed against each of the individual lesions, in the absence and in the presence of increasing concentrations of Cd. As shown in Fig. 1, Cd induces a strong reduction of the fluorescence incorporated into the damaged substrates at each of the concentrations tested. This dose-dependent reduction is of about 30% for 0.03 mM, 70% at 0.1 mM and 90% at 0.3 and 0.6 mM $CdCl_2$. Thus, it appears that the inhibitory effects of Cd on DNA repair reach a plateau at the higher concentrations. It must be noted that the incorporation of labelled dCTP in undamaged control plasmids does not increase in the presence of Cd, showing that Cd does not activate unspecific nucleases in the Hela NE and that the specificity of the repair reaction is maintained.

The effects of Cd on the repair of each of the 4 lesions were then analyzed individually. Fig. 2A shows the level of labelled dCTP incorporation in one experiment where all the different $CdCl_2$ concentrations were tested in parallel, and Fig. 2B shows the average percentage of inhibition obtained in different experiments for the repair of each lesion in each of the conditions. It is clear from these data that Cd differentially affects the repair activities directed against each of the lesion present on the biochip. The repair of 8oxoG is not affected at 0.03 mM $CdCl_2$, whereas repair of CPD-64, alkylated bases and glycols is reduced, and in this group the reduction is stronger for the repair of glycols than for the repair of alkylated bases. In the presence of 0.1 mM $CdCl_2$, the repair of 8oxoG and alkylated bases is decreased by about 50% and 65%, respectively, and it is further inhibited (to about 80%) when Cd concentration in the assay reaches 0.3 mM. In contrast, the maximum level of inhibition is already reached at 0.1 mM $CdCl_2$ for the repair of CPD-64 and glycols and does not further evolve when Cd concentration is increased. It appears that a higher level of inhibition

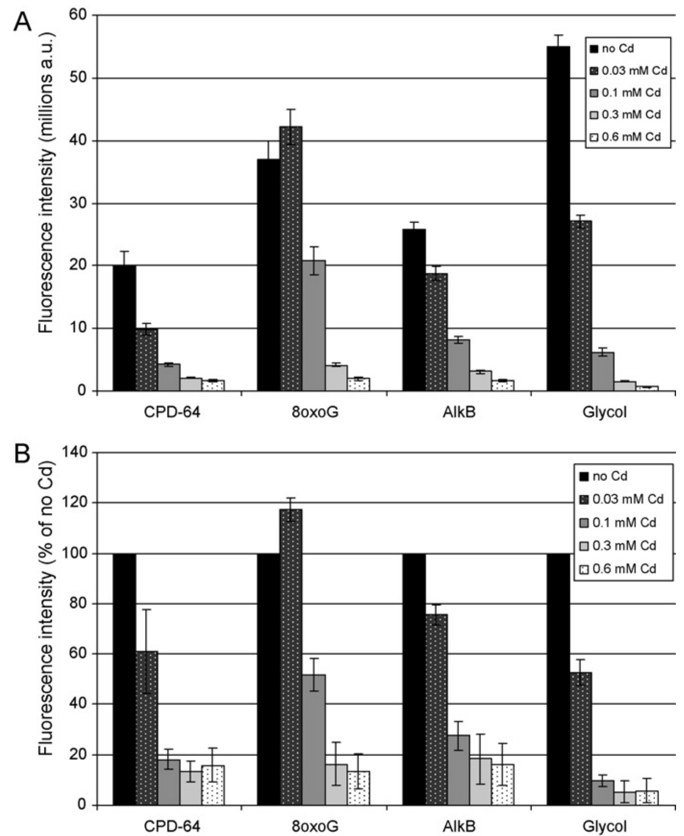

Fig. 2. Cd inhibition of excision/synthesis repair of the different lesions. (A) Cd dose-dependant decrease of dCTP-Cy5 incorporation in damaged plasmids. Data represent the total fluorescence incorporated following repair of each lesion in the presence of increasing $CdCl_2$ concentrations. Histogram bars show the average value (±SD) from 2 microarrays for each condition in the same experiment. (B) Percentage of remaining fluorescence intensity following incubation with Hela cell NE without and with $CdCl_2$ at the indicated concentration, expressed as a percentage of the fluorescence intensity obtained in absence of $CdCl_2$. These data represent the average (±SD) value obtained from 4 to 8 microarrays per condition

is attained for the repair of glycols (around 90%) than for the other three lesions (around 80%).

Thus, we observe a strong and steep inhibition of the repair of glycols at the lower Cd concentration tested, whereas the repair of 8oxoG is affected only after a higher concentration is reached, is more progressive and not so complete. The repair of CPD-64 and alkB represent intermediate situations in the steepness and/or the extent of the inhibitory effects of Cd. These repair processes are complex but can be roughly divided in two main stages: the damage recognition and excision, followed by DNA synthesis to restore DNA integrity. The fact that the threshold required for – and the extent of – Cd effects varies for each of the lesion indicates that the repair stage, performed essentially by the same replicative polymerases in our assay [19], is probably not the main target of Cd inactivation. Therefore, we decided to adapt our assay to focus on the recognition/excision step of the repair processes.

3.2. Measure of BER and NER excision activities on plasmid biochips

The same plasmid biochips were then used with an alternative protocol. Deoxynucleotides (including Cy5-dCTP) were omitted and aphidicollin was added to the reaction mix to block the activity of the DNA pol ε/δ contained in the NE. Consequently, DNA lesion recognition/excision and incision of the resulting AP site are still performed but subsequent DNA polymerisation is prevented. The

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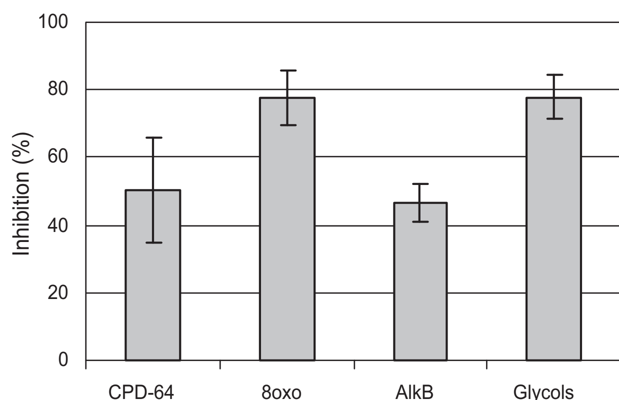

Fig. 3. Cd inhibition of excision of the different lesions on plasmid biochips. The percentage of inhibition of the excision activities following pre-incubation of Hela cell nuclear extract (0.25 mg/mL) with 1 mM $CdCl_2$ for 10 min at 4 °C. Bars represent the average value (±SD) of 6 microarrays

reaction was allowed to proceed for 20 min at 30 °C, and then was stopped by washing in PBS/Tween buffer. All the sites that were incised following excision are revealed during a second incubation in the presence of Klenow DNA polymerase and dNTPs, including labelled dCTP. In this scheme, the incorporation of labelled dCTP is proportional to the number of sites where DNA lesion repair was initiated during the first incubation, and therefore reflects the recognition/excision/incision activities present in the NE tested. Because the reaction time here (20 min) is much shorter than in the previous excision/synthesis assay (3 h), we used a higher $CdCl_2$ concentration (1 mM). NE were preincubated with $CdCl_2$ for 10 min at 4 °C before performing the reaction. The effects of Cd on the excision activities were tested in these conditions at two different NE concentrations, 0.05 mg/mL and 0.25 mg/mL. In these conditions, the global repair activity is decreased by about 55% for both protein concentrations (data not shown).

As previously observed for the analysis of excision/synthesis repair reactions, the excision activities directed against each of the different lesions are differentially affected. Fig. 3 shows the results obtained with 0.25 mg/mL of NE. Here again, the effects of Cd exposure appear more pronounced on the excision of glycols than on the excision of CPD-64 and AlkB. Excision of 8oxoG is affected to the same extend as excision of thymine glycols. Overall similar results are observed with 0.05 mg/mL of Hela NE (data not shown).

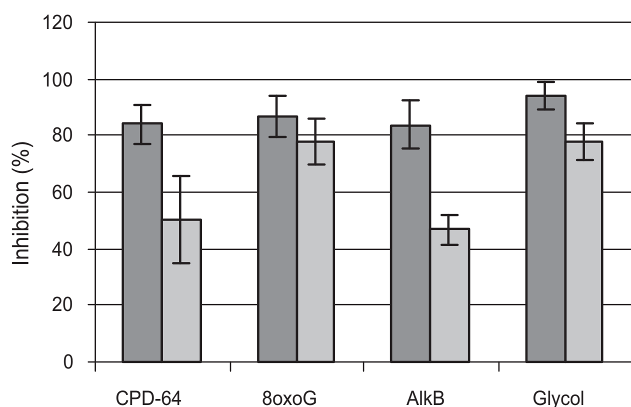

Fig. 4. Comparison of the extent of inhibition imposed by cadmium on excision/synthesis and excision of the lesions. This figure shows a side by side comparison of the average values of inhibition obtained with the plasmid biochips in excision/synthesis (dark grey bars) and excision/klenow (light grey bars) protocols in the presence of 0.6 mM and 1 mM $CdCl_2$, respectively

Interestingly, Fig. 4 shows that for glycols and 8oxoG, the inhibitory effect of 1 mM Cd on excision represents more than 80% of the inhibition of repair observed in the excision/synthesis assay in the presence of 0.6 mM Cd. In contrast, in the same conditions, the effects on excision of CPD-64 and alkylated bases represent only between 55% and 60% of the level reached in the excision/synthesis assay. Thus, depending on the lesion considered, repair inhibition results mainly from the toxic effects of Cd on the initial recognition and excision steps of the process (glycols, 8oxoG), or from the cumulative effects on both the recognition/excision and subsequent synthesis steps.

3.3. Measure of BER excision activities on ODN biochips

We used ODN biochips to obtain complementary data on the cleavage step of BER by glycosylases and/or APE1. In this assay, representative DNA lesions are borne onto Cy3-labelled double stranded ODNs. Cleavage of the lesion following recognition and excision of the damaged site and incision of the resulting AP site results in the loss of the ODN fragment bearing the fluorescent label. Therefore, the efficiency of the initial step of BER activities is measured by a loss of fluorescence. We focused our analysis on a set of 4 lesions that are reproducibly well cleaved by Hela NE in this assay: thymine glycols (Tg), cleaved by NTH1 and/or NEIL1, to allow a comparison with the previous experiments, THF, representing AP sites cleaved by APE1 and U, incorporated either in front of A or in front of G; U:A pairs represent mis-incorporation of dUMP during DNA synthesis, whereas U:G pairs mimic cytosine deamination, either spontaneous or enzymatically induced. Our assay also includes, on each biochip, several spots in which the substrate is an undamaged ds ODN to control for nonspecific activities.

As shown in Fig. 4A, in the absence of Cd, THF and U:G lesions are more efficiently recognized and cleaved than Tg and U:A. With a cleavage rate around 90% in the absence of Cd, THF and U:G lesions are very efficiently cleaved by Hela NE at 0.02 mg/mL, whereas the excision rate is around 40% and 30% for Tg and U:G in these conditions. To determine the extent of Cd effects on this cleavage, Hela NE were pre-incubated 10 min at 4 °C in the presence of increasing Cd concentration ranging from 0.1 mM to 0.5 mM. The DNA repair activities directed against the four lesions clearly exhibit different dose-response patterns (Figs. 5 and 6). Excision of Tg is the most sensitive. It is already diminished by about 28% in the presence of 0.1 mM Cd, and the inhibition is essentially complete at 0.3 mM and 0.5 mM. The activities directed against THF, U:A and U:G are unchanged at 0.1 mM. The cleavage of U:A and U:G is first inhibited at 0.3 mM Cd, but to widely different levels: whereas excision of U paired with G is reduced only by 20%, there is an 75% inhibition for U paired with A. Both of these activities are completely inhibited at 0.5 mM. Finally, cleavage of THF sites by APE1 is the less sensitive to the inhibitory effects of Cd in this assay. Its excision rate is diminished only at 0.5 mM Cd, and even at this relatively high concentration, the Hela NE still retains 13% of residual activity. Interestingly, cleavage of U:A and U:G are affected before any effect is visible on the cleavage of AP sites. Therefore, the effects of Cd on the recognition/excision of U:A and U:G pairs clearly reflect an inhibition of the glycosylases involved in this processes.

Thus, these results show again that the repair activities directed against each of the lesions are differentially affected by Cd at the recognition/excision/incision level.

4. Discussion

In this paper, we used two different parallelized assays to monitor simultaneously the effects of Cd on the BER and NER activities directed against several model DNA lesions representing oxidative

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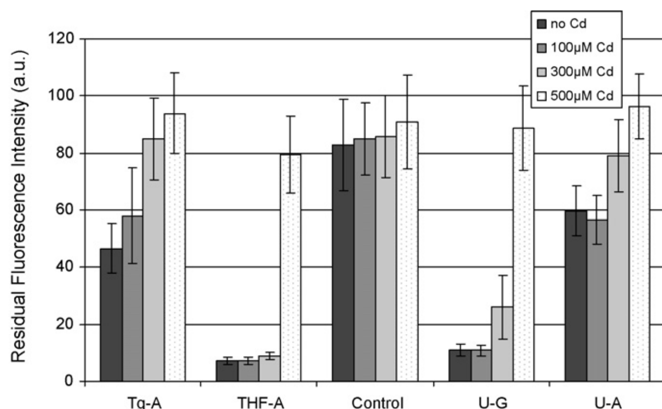

Fig. 5. Cd inhibition of the excision activities on ODN biochip. Shown is the residual fluorescence intensity (a.u. ± SD) measured on ODN microarrays following incubation in the presence of Hela cell NE without or with $CdCl_2$ at the indicated concentration, normalized for the fluorescence intensity obtained following incubation in repair buffer only. The identity of the lesion borne onto the labelled ODN duplex and its opposite base is indicated beneath the corresponding histogram bars. The "control" duplex ODN bears no lesion and loss of fluorescence on this spot reveals non-specific activities, which remain constant irrespective of $CdCl_2$ concentration

(8oxoG, glycols), UV-induced (CPD-64) or spontaneous (U, AP sites) damage repaired by BER and/or NER.

Our results show that Cd exerts a direct inhibitory effect on the repair of all the lesions considered here, irrespectively of whether they are BER or NER substrates. This inhibition of excision/synthesis repair could be due to the inactivation of only one (or a few) crucial factor(s) common to all the pathways, like for example XRCC1 or the gap filling activities. XRCC1 seems to participate in different steps of the BER and NER processes [39–41], whereas different DNA polymerases participate in gap filling in BER and NER. If one (or all) of these molecules was a direct target of Cd, one would expect a similar level of inhibition for the repair of each of the lesions at a given Cd concentration, and it is clearly not the case. Furthermore, even if this explanation could be evoked to explain the results obtained in our first set of experiments on excision/synthesis repair, we later show that, at least in the case of glycols and 8oxoG, the inhibitory effects of Cd are already imposed onto the activities required for recognition/excision/incision of the DNA lesions. Indeed, the comparison of the levels of inhibition attained during excision/synthesis and excision/klenow experiments conducted on the same substrates clearly indicates that for these two lesions, inhibition of excision/incision activities accounts for most of the observed effects. In addition, the side by side comparison of activities in the same experimental conditions shows that the response of glycosylases to Cd is not homogeneous: excision of glycols appears sensitive to lower Cd doses and is strongly inhibited, whereas activities directed against 8oxoG or U are less sensitive and require higher Cd concentration in the same assay; cleavage of THF sites, representing AP sites, is less sensitive to Cd inhibition than cleavage of U, either in front of G or in front of A. Thus, although Cd interferes with all the steps of excision/synthesis DNA repair, our multiplexed assays allow us to define a hierarchy of sensitivities to the direct toxic effects of Cd on DNA repair mechanisms within the glycosylases, and from glycosylases to APE1 to post cleavage steps.

In both assays, the excision/cleavage of glycols is the activity the most sensitive to Cd inhibition. Thus, even at relatively low Cd concentration, when APE1 activity is not yet impacted by Cd, repair of glycols is prevented because of an effect on the initiation of the BER process. Glycols can be recognized and excised by NTH1 or NEIL1 [22,36]. These two proteins are bi-functional enzymes endowed with both glycosylase and AP lyase activities. However, they do not share structural homology, and they use distinct mechanisms for the removal of the damaged bases and for the APE1-independent incision of the resulting AP site [37,42]. Despite these differences, the sharp and deep decrease in glycols excision/cleavage by Cd indicates that all repair activities, irrespective of their nature, are affected. How this inhibition is achieved can only be hypothesized at that stage. A common mechanism could be that these proteins are directly and irreversibly inactivated, as was shown for purified OGG1 *in vitro* [33]. However, other non-mutually exclusive possibilities exist to explain Cd inhibition of these two proteins. For example, NTH1 possesses a [4Fe–4S] cluster that is required for proper positioning of the molecule onto its target [43]. Cd might displace Fe ions from this cluster and inactivate the protein, as was shown for another transition metal (Co) in a bacterial system [44]. To investigate this possibility, we tried to mimic this potential Cd effect by pre-incubating our NE in the presence of dimethyl bipyridyl, an iron chelator, before measuring DNA lesion excision using our oligonucleotide biochips. This treatment did not change the efficiency of the reactions (data not shown). Thus, if Cd-induced Fe ions displacement results in NTH1 inactivation, NEIL1 activity compensates this loss and maintains a normal level of glycol excision in our assay. NEIL1 does not display any obvious feature like a Zn-finger or a Fe–S cluster that could explain its high sensitivity to Cd. It is, however, predicted to be a Zn binding protein (see the NEIL1 Gene Card at http://www.genecards.org/) [45]. Therefore, NEIL1 inactivation might result from changes induced upon Zn/Cd exchange at a site close to structures like the "Zn-finger like" or the H2TH domains [46] that would alter their conformation and/or function in DNA recognition.

Interestingly, our cleavage assay revealed a clear difference in the Cd sensitivity of the repair activities responsible for U cleavage in U:G and U:A base pairs. In the presence of 0.3 mM $CdCl_2$, the cleavage rate of U in U:A pairs is inhibited by 75%, whereas it is inhibited by only 20% in U:G pairs. The activities responsible for the recognition/excision/incision of U in these two contexts are therefore different. In our assay, three uracil-DNA glycosylase can potentially perform U cleavage: UNG2, SMUG1 and TDG [47]. In human cells, U opposite A seems to be excised only by UNG2, whereas U in U:G pairs is excised mainly by UNG2 with a minor contribution by SMUG1 and TDG, which can increase in conditions where UNG2 is limiting [48,49]. Our data would then indicate that UNG2 is more Cd-sensitive than SMUG1 and/or TDG: in the presence of 0.3 mM $CdCl_2$, cleavage of U:A by UNG2 is strongly inhibited while U:G cleavage is still performed at a high level, probably by SMUG1 and/or TDG. In U:G pairs, U results from cytosine deamination and is mutagenic, leading to C → T transitions if unrepaired,

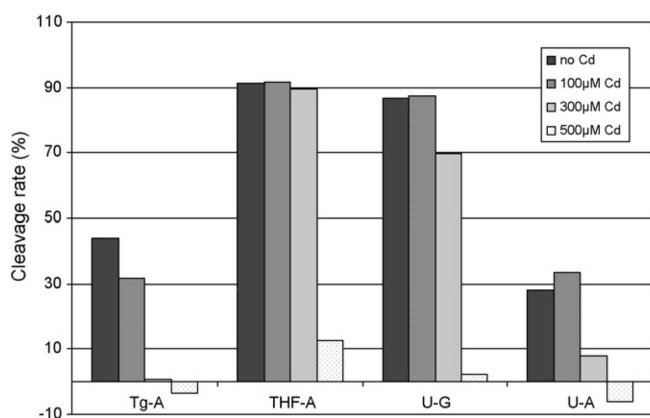

Fig. 6. Effects of Cd on the cleavage rate of modified bases and AP site. The cleavage rate represents the percentage of residual fluorescence at each lesion spot after incubation with the NE without or with $CdCl_2$ at the indicated concentration, corrected for the non-specific activities measured on the control duplex ODN shown in Fig. 5.

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whereas U:A mismatches resulting from faulty dUMP incorporation in DNA are not mis-coding [9]. Thus, the differential sensitivity of U:G repair activities in the presence of Cd have important functional consequences as they allow to maintain a good level of protection in the cell in a context of stress. However, it should also be noted that although not mis-coding, U incorporation in front of A is not totally neutral; it can for example alter the binding of transactivating factors onto their cognate target sequences [50]. The pattern of expressed genes in a contaminated cell will therefore be modified not only through the effects of Cd on multiple signalling pathways [2], but also because of its inhibition of DNA repair.

5. Conclusions

In conclusion, we show in this study that cadmium directly inhibits the activity of the proteins responsible for the excision/synthesis repair of a variety of lesions representing spontaneous and environmentally induced DNA damage. We used nuclear extracts prepared from un-manipulated Hela cells. Therefore, the repair events observed in our assays result from the activity of nuclear proteins expressed stoichiometrically at their normal respective levels. The simultaneous analysis of the repair of several lesions allowed us to define a hierarchy in the sensitivity of the different repair activities to the detrimental effects of Cd. We could show that the effects of Cd are already targeted at the initial recognition/excision steps of the BER and NER processes, which are both severely affected. In our cleavage assay, inhibition of APE1 activity required a higher Cd concentration than inhibition of the glycosylases, independently of whether they are mono- or bi-functional. Consequently, the repair of each type of lesion will already be individually inhibited at the lower Cd concentration, making Cd a very effective DNA repair poison, irrespective of the requirement for APE1. NER has been shown to participate in the repair of BER substrates [16–19] and could possibly substitute for Cd-induced BER inactivation in contaminated cells. However, our results clearly show that the repair of NER substrates is inhibited as efficiently as the repair of BER substrates. This inhibition can be related to the reported effects of Cd on XPA [30,31] and XPD [29] activities. Therefore, the cross talk between BER and NER activities may eventually provide more flexibility to a cell confronted with DNA damage, but it does not confer any beneficial advantage in case of Cd contamination and the cells are left defenceless and unable to handle mutagenic oxidative base damage.

Finally, our work shows for the first time, to the best of our knowledge, that Cd inhibits the repair of U in DNA, resulting both from mis-incorporation and from C deamination. These lesions, as AP sites, are common in any cell [9], and must constantly be repaired to avoid mutagenic events. The necessity to continuously repair these lesions is underscored by the high levels of expression of UNG2 and APE1 [51]. The inhibition of these enzymes by Cd may result in an excessive burden of unrepaired U and Ap sites in the nucleus of contaminated cells. This genotoxic consequence of Cd exposure might participate in the deregulation of physiological cellular processes by altering the pattern of gene expression on the one hand (U), and increasing the mutation rate on the other hand (AP site), thereby interfering with the normal control of cell growth and division. Thus, in addition to its effects on signal transduction and intra-cellular redox status, Cd activity toward DNA repair pathways, including UNG2 and APE1, most certainly contributes to its carcinogenic properties.

Conflict of interest statement

None declared.

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