DNA repair activity in protein extracts from rat tissues

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Abstract Among DNA repair pathways, nucleotide excision repair (NER) is able to recognize and process a wide variety of DNA lesions. The NER mechanism can be summarized in two stages: incision/excision of the lesion and DNA repair synthesis. Here, we have assessed the repair synthesis activity of protein extracts from different rat tissues by an in vitro biochemical assay that reproduces the entire NER reaction. The protein extraction procedure was adapted to rat tissues and the biochemical parameters of the assay (high salt concentration, addition of EGTA) in order to minimize non-specific nuclease activity which allows the measurement of repair activity. Using this repair assay we detected a small increase in the extent of repair synthesis in liver compared to brain and lung tissue protein extracts. Similar results were obtained using a derivative assay that allows the measurement of the incision activity of tissue protein extracts with lower incision activity in lung tissue extract.

Key words: DNA repair; Tissue

1. Introduction

Accumulation of DNA damage in tissues leads potentially to mutation or cell death. Among the cellular factors involved in survival and mutagenesis following genotoxic treatments, DNA repair activities play a major role [1]. Nucleotide excision repair (NER) represents the most versatile DNA repair mechanism since it recognizes and processes all the lesions tested, at least in vitro [1,2]. Comparison of DNA repair activities of different tissues requires: (i) the determination of the rate of disappearance of DNA adducts or (ii) the expression of repair proteins or (iii) the direct measurement of repair activities.

The rates of disappearance of lesions such as cisplatin or benzopyrene DNA adducts have been measured in various rodent tissues [3–6]. Slight variations between tissues were observed that could reflect differences in the NER activity.

The determination of repair protein expression has been conducted in the case of O-6 guanine methyl transferase (OGMT) [1]. The expression of OGMT evaluated in different tissues and more recently in various cell types of specific tissue showed differences between individual cell types, either normal or tumoral [7,8]. In contrast, since in the NER process a multienzymatic protein complex is involved, it might be hazardous to estimate the global repair activity from the differential expression of a limited number of repair proteins.

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The NER repair mechanism has been dissected into two broad stages: first recognition, incision and excision of the lesion and then DNA polymerization through the gap followed by ligation to restore the strand continuity [1,2]. Since the whole reaction has been reproduced in vitro with protein extracts from cell lines incubated in the presence of damaged plasmid DNA [9], it should be feasible to use this approach in order to compare the NER activity between rat tissues. In this bioassay, repair reactions are detected via radiolabelled repair patches. NER activity is determined by the quantification of nucleotide incorporation during the repair synthesis step. One drawback is due to the fact that radiolabel incorporation could reflect both the numbers of incisions and the repair patch size, so that all stages of the repair reaction contribute to the final repair signal. The incision step appears to be slow and rate limiting while gap filling and ligation probably proceed very rapidly [10]. Therefore a derivative assay has been set up to measure directly the incision activity of cell extracts [11,12].

We report here some modifications in the preparation of protein extracts from rat tissues compared to cell lines, allowing specific quantification of DNA repair synthesis and incision on damaged plasmid DNA. The biochemical parameters of the NER assay have been optimized. Under these experimental conditions, NER activity has been measured in protein extracts from liver, kidney, lung and brain of rat.

2. Materials and methods

2.1. Animal experiments

Male Sprague-Dawley rats weighting about 250 g were fed on a normal standard diet and kept fasting for about 12 h before sacrifice. They were killed by decapitation by an experienced technician and the cardiovascular system was rinsed with about 300 ml of 4°C cold isotonic phosphate-buffered saline (PBS). Organs were rapidly removed and kept on ice. All procedures were subsequently performed at 0°C.

2.2. Extract preparation

Liver, brain, lung and kidney were sliced, finely chopped, then frozen in liquid nitrogen to pulverize tissue with a pestle and mortar. Of each tissue 0.4 to 1.2 g was used to prepare protein extracts. In order to obtain extracts proficient in DNA repair synthesis the Manley procedure used in the in vitro repair assay [13] has been modified as follows: (1) the hypotonic lysis was for 1 h under gentle rotation; (2) centrifugation speed was $358\,000 \times g$ (liver and brain) or $300\,000 \times g$ (kidney, lung); (3) final ammonium sulphate concentration was 45% instead of 63%. All these modifications were done in order to minimize the activity of non-specific nuclease tested on plasmid DNA. AHH1 cell extracts were performed as previously reported [9]. Protein concentration was determined using the Bio-Rad assay and values were in the range of 10 to 18 mg/ml. At least three independent extractions were performed in order to determine the repair synthesis activity in vitro.

2.3. Preparation of plasmids and treatments

pBS (pBluescript KS$^+$ from Stratagene) and pHM, a 3738 bp derivative plasmid (gift from Dr R.D. Wood, ICRF, UK), were pre-

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pared as reported [14]. pBS plasmid was either treated with cisplatin (R. Bellon Co.) or UVC irradiated at 254 nm [9,15].

2.4. In vitro repair reaction

(1) The repair synthesis assay was performed in a total volume of 50 μl which contained 300 ng damaged pBS, 300 ng untreated pHM, 40 mM HEPES-KOH (pH 7.8), 0.5 mM dithiothreitol, 4 μM dATP, 20 μM of each of dGTP, dCTP and dTTP, 40 mM of phosphocreatine, 18 μg bovine serum albumin, 2.5 μg creatine phosphokinase (type I Sigma), 2 mM ATP, 7 mM $MgCl_2$, 2 mM EGTA, 100 mM KCl except when mentioned and 200 μg protein in cell extract. After 3 h of incubation at 30°C with 0.2 μCi of [α-^{32}P] dATP (>37 TBq/mmol, ICN) and treatment by 200 μg/ml proteinase K (56°C, 30 min) and 0.5% SDS, plasmid DNA was purified by two extractions with phenol-chloroform-isoamyl alcohol (25/24/1), ethanol precipitated (−20°C, 20 min), washed in 70% ethanol, dried and then linearized with 0.5 U *Hin*dIII. The reaction was stopped by adding 3 μl of loading buffer with 1% SDS and plasmids were loaded onto a 1% agarose gel containing 0.5% ethidium bromide. The running buffer was Tris (pH 8) 90 mM/borate 90 mM/EDTA 2 mM.

(2) In the incision assay, deoxyribonucleotides were omitted and 4.5 μM aphidicolin was added [12]. After 2 h at 30°C, the reaction was stopped by the addition of 25 mM EDTA. Plasmids were treated by gentle manual agitation with 200 μg/ml proteinase K (56°C, 30 min) and 0.5% SDS, purified by phenol-chloroform-isoamyl alcohol extraction, ethanol precipitated (−20°C, 20 min), washed in 70% ethanol, dried and redissolved in TE buffer. After a rapid incubation (10 min at ambient temperature) in a mixture of 2 mM dithiothreitol, 90 mM HEPES-KOH (pH 6.6), 0.2 μCi of [α-^{32}P] dATP (>37 TBq/mmol, ICN), 20 μM of each of dGTP, dCTP and dTTP and 1 U of *E. coli* DNA polymerase I large fragment, the reaction was stopped by EDTA 50 mM. DNA was extracted, precipitated, washed before its linearization by *Hin*dIII and its electrophoresis on a 1% agarose gel containing 0.5 μg/ml ethidium bromide.

(3) Data were quantified by autoradiography, scintillation counting of the excised DNA bands and densitometry of the photographic negative of the gel to normalize for plasmid DNA recovery (Scanning Laser Densitometer Biocom, France).

3. Results

3.1. Preparation of protein extracts

The extraction procedure used previously for cell lines [9], was not suitable for tissues. First, different dissociation procedures either mechanical or enzymatic (trypsin, collagenase solutions) were tested, the highest repair activity being obtained by a pulverization step of frozen tissue in liquid nitrogen with a pestle and mortar after a fine chopping step. Second, by evaluating the extent of repair synthesis in protein extract preparations, a lack of repair activity was observed due to the presence of non-specific nuclease. Therefore, the extraction procedure (ammonium sulphate precipitation, rate

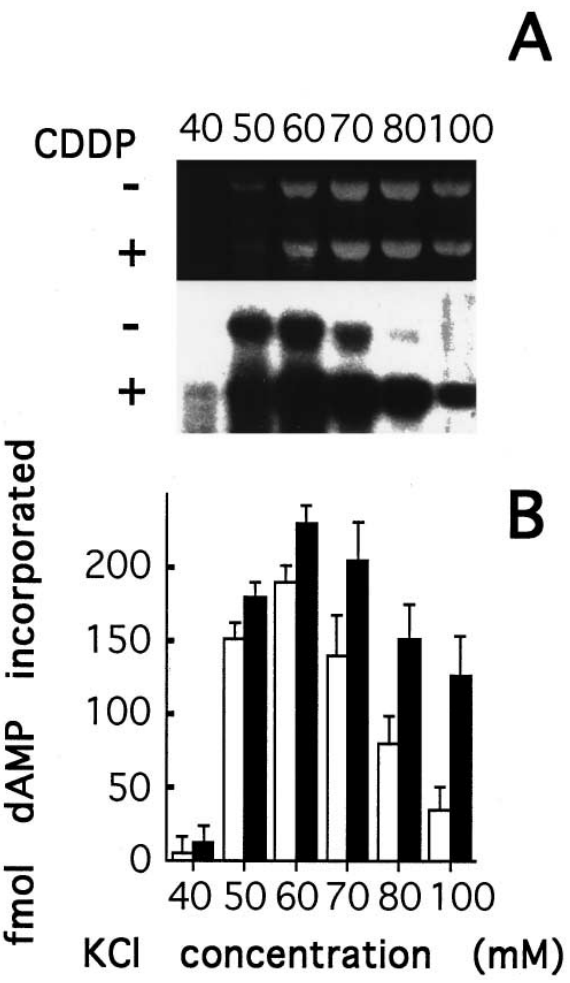

Fig. 1. Effect of KCl concentration on repair synthesis. Protein extract from rat liver (200 μg per assay) was incubated 3 h at 30°C in the presence of CDDP-treated pBS and untreated pHM with different KCl concentrations from 40 to 100 mM. The repair synthesis assay was performed as reported in Section 2. A: Upper: photograph of the ethidium bromide-stained agarose gel; lower: autoradiograph of the dried gel. B: Quantification of [α-^{32}P] dAMP incorporation into CDDP-damaged pBS (■) and undamaged pHM (□); the extent of incorporation was normalized to the amount of recovered DNA and the results were the mean ± S.D. of three independent experiments.

Table 1
Repair synthesis activity in different tissue extracts

	DNA damaging treatment					
	UV light			Cisplatin (CDDP)		
	+	−	*n*	+	−	*n*
Liver	241 ± 70	121 ± 26	4	235 ± 58	81 ± 13	6
Kidney	128 ± 15	61 ± 7	2	155 ± 32	56 ± 20	4
Brain	149 ± 27*	79 ± 5	2	127 ± 13*	64 ± 8	3
Lung	137 ± 12*	70 ± 6	5	140 ± 21**	77 ± 9	5

Reaction mixtures contained 300 ng each of untreated or treated (UVC light or CDDP) plasmid DNA with 200 μg protein extract from each organ tested. Incorporation of [α-^{32}P] dAMP in plasmids is determined and normalized with respect to plasmid DNA recovery. Values are mean ± S.D. (n = 2–6).

* and ** indicate significant differences ($P < 0.05$ and $P < 0.01$, respectively) of absolute [α-^{32}P] dAMP specific incorporation in damaged plasmids obtained with kidney, brain and lung from values with liver extracts (Mann-Whitney's U test).

of centrifugation) had to be modified in order to minimize the non-specific nuclease activities (see Section 2).

3.2. In vitro conditions to monitor DNA repair synthesis

Once the protein extraction procedure was set up, the effect of salt concentration (KCl) on repair synthesis was undertaken (see Fig. 1). No repair activity could be measured below 50 mM KCl, because of DNA degradation. At higher KCl concentration (80–100 mM) specific repair activity was measured while at 120 mM the extent of incorporation was low. Therefore, the repair synthesis was measured with 100 mM KCl in the reaction mixture which corresponds to a higher concentration than that generally used in the repair assay (50–70 mM). Moreover, EGTA (2 mM) was added to the reaction mixture in order to inhibit any residual DNAse activity without any variation in the extent of repair synthesis as checked with AHH1 cell extracts (data not shown).

3.3. NER activity in different tissue extracts of rat

The extent of repair synthesis was determined with UVC damaged plasmid DNA (Fig. 2). First, DNA repair synthesis value was higher with cell lines (350–460 fmol dAMP incorporated, data not shown) than with tissue extracts. Second,

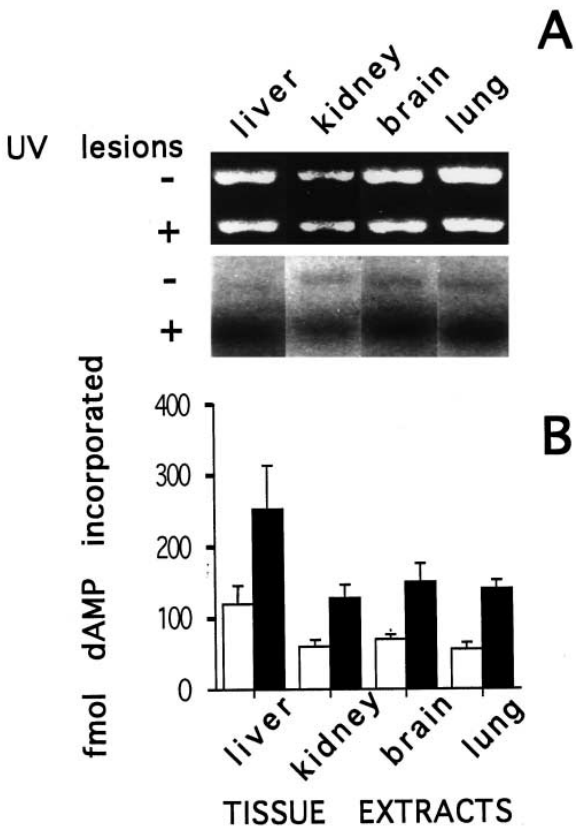

Fig. 2. DNA repair synthesis activity of extracts from different rat tissues. UVC-treated pBS and untreated pHM (300 ng each) were incubated 3 h at 30°C with 100 mM KCl concentration, 2 mM EGTA and 200 μg of extract from liver, kidney, lung and brain. The repair synthesis assay was performed as reported in Section 2. A: Upper: photograph of the ethidium bromide-stained agarose gel; lower: autoradiograph of the dried gel. B: Quantification of [α-^{32}P] dAMP incorporation into UVC damaged pBS (■) and undamaged pHM (□); the extent of incorporation was normalized to the amount of recovered DNA and the results were the mean ± S.D. of at least three independent extract preparations.

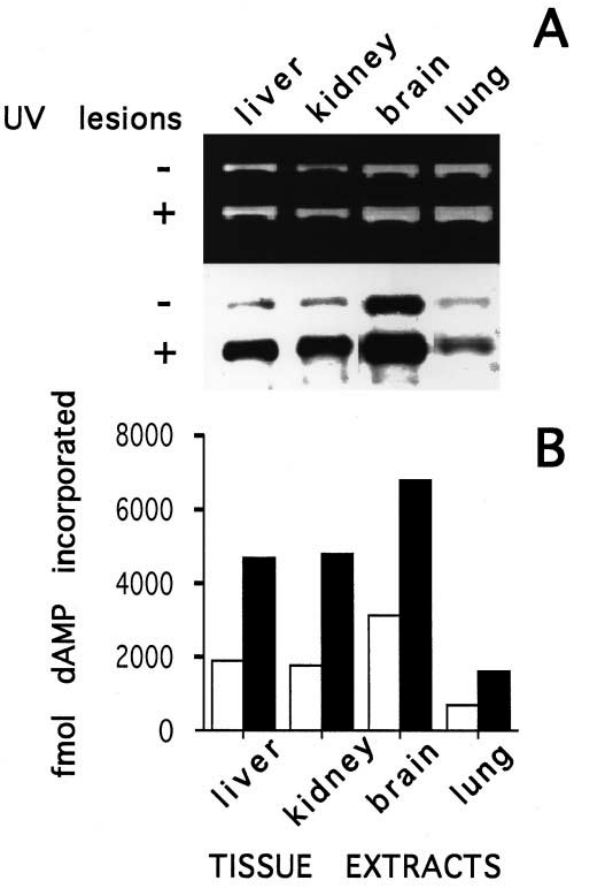

Fig. 3. DNA repair incision activity of extracts from different rat tissues. UVC-treated pBS and untreated pHM (300 ng each) were incubated 2 h at 30°C in the absence of added dNTP and with 4.5 μM aphidicolin, 100 mM KCl, 6 mM EGTA, 6 mM $MgCl_2$ and extracts (200 μg each) from liver, kidney and brain. After purification, plasmids were radiolabeled with [α-^{32}P] dATP by Klenow polymerase as reported in Section 2. A: Upper: photograph of the ethidium bromide-stained agarose gel; lower: autoradiograph of the dried gel. B: Quantification of [α-^{32}P] dAMP incorporation into UVC-damaged pBS (■) and undamaged pHM (□); the extent of incorporation was normalized to the amount of recovered DNA.

slight variations in NER activity were found between tissue extracts with higher values found for liver than for kidney, brain or lung extracts (Table 1). Non-specific incorporation (undamaged plasmid DNA) was higher in liver extract indicative of variations in nuclease activity between tissue extracts. The same result was obtained with cisplatin-damaged plasmid DNA (Table 1). In order to eliminate potential artifacts due to post-mitotic cells with differences in DNA polymerisation activity, the incision activity was determined with tissue extracts. As with the repair synthesis assay, a lower incorporation was observed in the lung extract compared to the other extracts, but no significant variation was found between the value of the incision ratios that were equal to 2–2.7-fold (Fig. 3).

4. Discussion

This study was aimed at determining the NER activity in various tissues of rat monitored by in vitro biochemical assays [9,12]. The bioassays allowed measurement of both the repair synthesis and the incision activity in protein extracts from cell

lines incubated in the presence of damaged plasmid DNA. However, the extraction procedure and the biochemical parameters of the assays had to be adapted to tissue extracts in order to determine the NER activity. The main difficulty in obtaining a specific repair activity signal was due to non-specific nuclease activity which was overcome by a modification of the extraction procedure in order to increase the total protein recovery. In addition the biochemical conditions were optimized by increasing the KCl concentration (100 mM) and the addition of EGTA (2 mM) to the reaction mixture. This leads to a 1.8–2.9-fold improvement in the specific incorporation level (Table 1). The extent of repair synthesis in tissues was lower than that in cell lines which reflect, among other possibilities, on variations related to the purification procedure, or intrinsic differences in DNA repair capacity between cells from tissue or culture in vitro. It has been previously reported that protein extracts from leukemia mice L1210 cells grown in vivo as ascitic fluid led to a lower signal of repair synthesis than the same cell line adapted to in vitro culture [16].

Slight variations in repair synthesis between extracts were found with higher values in the case of liver than the other tissue extracts. Such a difference has been previously reported for the rate of disappearance of benzopyrene-DNA adducts [3,4,6]. However, a higher repair capacity of liver extract did not appear when incision activity was measured by comparison with repair synthesis activity. Such difference may be due to higher level of non-specific nuclease activity in liver extract which could lead to differences in the length of the repair synthesis patch in vitro. Alternatively, lung extract showed a lower incision activity compared to the other tissue extracts which support the conclusions of Jones et al. who have previously reported a higher repair synthesis activity measured in brain, kidney and liver than in lung tissue extract [17].

Variations in repair synthesis and or incision between tissue extracts measured in vitro might in part be related to: (i) variations in the preparation of tissue extract, easier to perform with liver and kidney than with brain and lung, (ii) the low efficacy of the repair reaction in vitro (about 7%) [18] which did not allow subtle comparison between protein extracts. Comparison between in vivo and in vitro assays is nonetheless hazardous since (i) preferential repair [19] cannot be estimated with the in vitro assay and (ii) the in vitro repair activity is quantified with protein extracts of non-treated cells whereas the disappearance of lesions by definition is determined after treatment which may induce repair activities.

The correlation between the repair ratios determined in the repair synthesis assay and the incision assay is consistent with a rate limiting incision step in the NER reaction [10,20]. Variations in DNA replication activity between tissues do not interfere with the extent of repair synthesis measured. Moreover, despite the low extent or the absence of cell proliferation in tissues, the residual DNA polymerisation activity was sufficient to measure the repair synthesis. This implies that the replication activity was not limiting.

In conclusion, with the exception of peripheral blood lymphocytes that exhibit a very low incision activity [21], the sensitivity of different mammalian cells in tissue to DNA damaging agents may not be due to variable global capacity of repair but rather depends on other mechanisms not taken into account by this test, concerning the drug, its metabolism and biodistribution.

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