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Original Paper

Expression of Genes Involved in Nucleotide Excision Repair and Sensitivity to Cisplatin and Melphalan in Human Cancer Cell Lines

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DNA repair has been proposed to be an important determinant of cancer cell sensitivity to alkylating agents and cisplatin (DDP). Nucleotide excision repair (NER), which represents one of the most important cellular DNA repair processes able to remove a broad spectrum of DNA lesions, is involved in the recognition and repair of the crosslinks caused by DDP and melphalan (L-PAM). In this study, the mRNA levels of the different genes involved in NER (*ERCC1*, *XPA*, *XPB*, *XPC*, *XPD*, *XPF*) were examined in a panel of eight different human cancer cell lines, together with the overall DNA repair capacity using a host cell reactivation assay of a damaged plasmid. A statistically significant correlation was observed between the relative expression of XPA/XPC ($P < 0.05$) and ERCC1/XPC ($P < 0.05$) mRNAs. No correlation was found between the DDP and L-PAM IC_{50}s and the relative mRNA expression of the tested NER genes. When the overall cellular DNA repair capacity was studied, carcinomas seemed to have a higher repair activity than leukaemias; but this repair DNA activity correlated neither with the mRNA expression of the different NER genes nor with DDP and L-PAM IC_{50}s. These data seem to suggest that even if the NER pathway is an important determinant for the cytotoxicity of alkylating agents, as demonstrated by the extremely high sensitivity to alkylating agents in cells lacking this repair system, other factors have to play a role in regulating the cellular sensitivity/resistance to these antitumour drugs. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

SEVERAL FACTORS are involved in determining cell sensitivity to chemotherapeutic agents [1, 2]. It has been shown how the extent and the duration of DNA damage can activate two pathways: either halting the cell cycle to allow the cell to repair the damage or activation of apoptosis [3–5]. The predominant pathway will depend on the cell type and on its molecular environment (e.g. p53 status, the 'apoptotic competence', etc.). As long as repair is made possible, through a temporary halting of the cell cycle, the survival of a damaged cell would also depend on the cell's DNA repair capabilities.

DNA repair has been proposed to be a relevant determinant of cancer cell sensitivity to alkylating agents and cisplatin (DDP) [6, 7]. For chloroethylnitrosoureas and methyl nitrosoureas a correlation between the expression of O6 alkyltransferase and resistance has been reported [8, 9]. The nucleotide excision repair system (NER) is one of the major DNA repair systems and consists of a co-ordinated interplay between multiple proteins able to recognise and excise a broad variety of DNA damage, such as UV lesions and bulky chemical adducts [10–13]. Several studies on mutant human and hamster cell lines, defective in genes involved in NER, support the idea that the products encoded by them are important for the repair of the DNA lesions caused by the crosslinking agents DDP and melphalan (L-PAM) [14–16], two alkylating agents widely used in clinical practice. However, knowledge of the expression of the different NER genes in human tumours and their importance for the sensitivity and/or resistance to chemotherapy is limited and still controversial [17–22].

The present study examined the mRNA levels of different genes involved in the NER process in a panel of different human cell lines. The genes studied were *ERCC1*, *XPA*,

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XPB, *XPC*, *XPD*, *XPF* and *XPG*, all involved in the recognition/excision steps of the NER process that seems to be the rate limiting step. We also studied the DNA repair capacity of the different cell lines using the host cell reactivation assay of a damaged plasmid. The relationship among the DNA repair capabilities, the levels of the different mRNAs and the DDP and L-PAM cytotoxicities have been investigated.

MATERIALS AND METHODS

Cell cultures and drugs

The human ovarian carcinoma cell lines IGROV, OVCAR3, SKOV-3, the human leukaemia cell lines CEM, U937, K562 and JURKAT were maintained in RPMI-1640 (Gibco Europe, Glasgow, U.K.); the human colon carcinoma cancer cell line LoVo was maintained in F-12 (Biological Industries) media. All cell lines were supplemented with 10% fetal calf serum and grown at 37°C with 5% carbon dioxide.

DDP (generous gift from Bristol Myers-Squibb, U.S.A.) was dissolved in medium, and L-PAM (kindly provided by the Drug Synthesis and Chemical Branch, Division of Cancer Treatment, NCI, Bethesda, Maryland, U.S.A.) was dissolved in 0.3 N hydrochloric acid and diluted to the desired concentration in medium just before use.

Cytotoxicity

The cytotoxicities of DDP and L-PAM were determined using the 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [23]. The IC_{50}s were determined by extrapolation of the dose–response cytotoxic curves. The IC_{50} value for each drug is the ± standard error of the mean (SEM) of at least three different experiments.

Northern blot analysis

Total RNA from cell lines was separated from exponentially growing cells by the guanidium isothiocyanate/caesium chloride procedure [24]. After electrophoresis in a formaldehyde agarose gel, RNA (10 μg) was transferred to a Nylon membrane and hybridised separately to the random primed ^{32}P-labelled probes for 16 h at 42°C. The probes used to detect mRNA were: the 1.1 EcoRI/Hind III fragment from pE12-12 for ERCC1 (kindly provided by J.H.J. Hoeijmaker, Erasmus University, Rotterdam, The Netherlands); the 0.9 ecoRI fragment from pSLM for XPA (kindly provided by W. de Laat, Erasmus University); the pCDI plasmid for XPB (kindly provided by J.H.J. Hoeijmaker); the 3.5 Sfi1 fragment from pXPC-3 for XPC (kindly provided by C. Peterson); the 0.89 PvuII fragment from pER2-12 for XPD (kindly provided by C.A. Weber, Livermore, California, U.S.A.); the 1.9 XhoI fragment from pcDNA-XPF for XPF (kindly provided by W. de Laat); the PvuII fragment from XPG plasmid for XPG (kindly provided by S.G. Clarkson, Centre Medical Universitaire, Geneva, Switzerland); the 4.7 SaII fragment from pcBIsSe6 for ERCC6 (kindly provided by J.H.J. Hoeijmaker; 1.3 kb Pst insert of α actin.

The different mRNAs were quantified by scanning the autoradiographs with a densitometer and comparing the intensities of the different mRNAs with the intensity of the α actin mRNA which was used to normalise the amount of RNA loaded. The data are expressed relative to the amount of the CEM transcripts, which were assigned the arbitrary value of 1.

Host cell reactivation assay

Caesium chloride gradient purified pGL2 DNA was treated with 200 μM DDP in 1 mM Tris–HCl (pH 7.8), 10 mM NaCl and 1 mM ethylenediaminetetraacetic acid (EDTA) for 10 min at room temperature, as previously described [25]. The ovarian and colon carcinoma cell lines were transfected with 10 μg of the DDP damaged or undamaged pGL2 plasmid using the Ca/phosphate technique; leukaemic cells were instead electroporated using the Bio-Rad gene pulser (Bio-Rad, Milan, Italy) with a capacitance setting of 960 μF and a voltage setting of 260 V. After electroporation, the cells were incubated on ice for 10 min then transferred to fresh culture medium. As an internal control, 1–2 μg of β-galactosidase plasmid was included in the transfection. Luciferase activity was assayed 48 h after transfection with a Promega kit, while the activity of β-galactosidase was determined spectrophotometrically as previously described [26]. The results are expressed as a percentage of the reported activity of control undamaged pGL2 plasmid, normalised by the β-galactosidase values and are ± SEM of three different experiments, carried out in triplicates.

RESULTS

The DDP and L-PAM IC_{50}s in the eight human cell lines selected for this study are shown in Table 1. Figure 1 shows a representative Northern blot analysis of the transcripts of the NER genes analysed and Table 2 summarises the mRNA levels in the different cell lines. All mRNA data were normalised to the α actin densitometric absorbance in order to eliminate loading differences and are expressed relative to normalised CEM transcripts, which were assigned the arbitrary value of 1. Except for ERCC6/CSB, the transcript of which could not be detected in any of the cell lines tested (data not shown), all the cell lines expressed the tested genes. For ERCC1 mRNA the 1.1 kb band was evaluated, whereas XPF hybridisation revealed two major bands (approximately 7.7 kb and 4.4 kb bands) and only the former one was considered. Reproducible statistically significant correlations were observed in different experiments between the relative expression of XPA and XPC ($P < 0.05$) and between those of ERCC1 and XPC ($P < 0.05$). A representative experiment is shown in Figure 2. No correlation was found between the DDP and L-PAM IC_{50}s and the relative expression of the tested NER genes (data not shown).

In order to determine whether the detection of the transcripts of the genes involved in the NER process could be related to the overall DNA repairability of the cell, the capacity of the different tumour cell lines to reactivate a pGL2–

Table 1. *Cisplatin (DDP) and melphalan (L-PAM)* IC_{50}*s in the different cell lines*

Cell line	DDP (μM)	L-PAM (μM)
CEM	9 ± 2	1 ± 0.2
JURKAT	8 ± 2	6 ± 1
K562	68 ± 5	111 ± 20
U937	23 ± 3	10 ± 0.3
IGROV	28 ± 2	151 ± 33
OVCAR-3	48 ± 3	115 ± 3
SKOV-3	83 ± 12	102 ± 3
LOVO	99 ± 14	88 ± 6

The data are ± standard error of the mean of three different experiments.

luciferase plasmid, previously damaged with DDP was assessed. Figure 3 shows the relative luciferase activity measured in the different cell lines after transfection with pGL2–luciferase treated or not with DDP. The CEM cell line could not be easily electroporated, so that the data concerning luciferase activity were not reliable and were not included in the figure. Although the numbers are too small for a statistical comparison, the repair activity seemed to be higher in carcinoma cell lines than in the leukaemic cell lines (JURKAT, K562, U937). No correlation was found between DDP and L-PAM cytotoxicities or mRNA expression of the different genes and the relative luciferase activity.

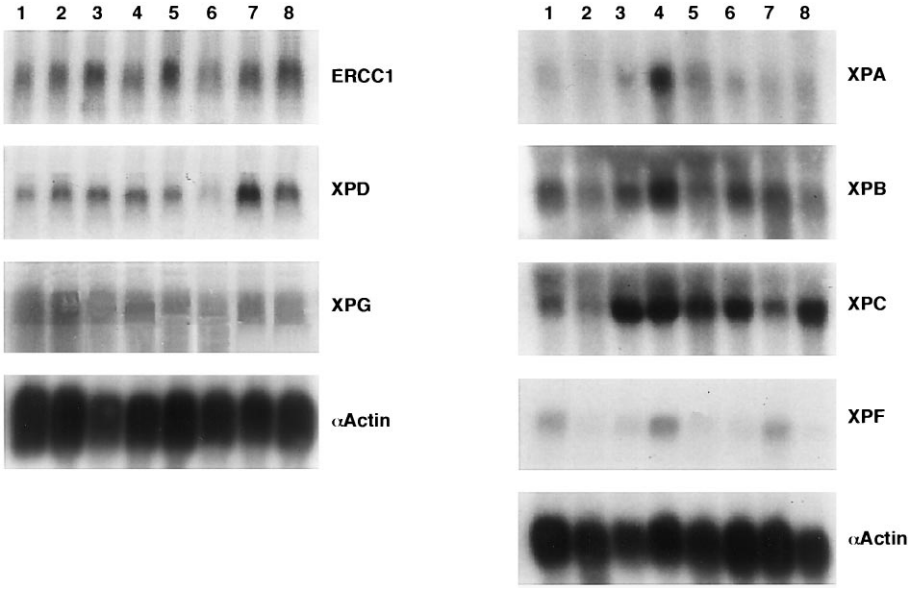

Figure 1. Representative Northern blot analysis of the different genes involved in nucleotide excision repair in CEM (lane 1), JURKAT (lane 2), K562 (lane 3), U937 (lane 4), IGROV (lane 5), OVCAR-3 (lane 6), SKOV-3 (lane 7) U937 and LoVo (lane 8) cell lines.

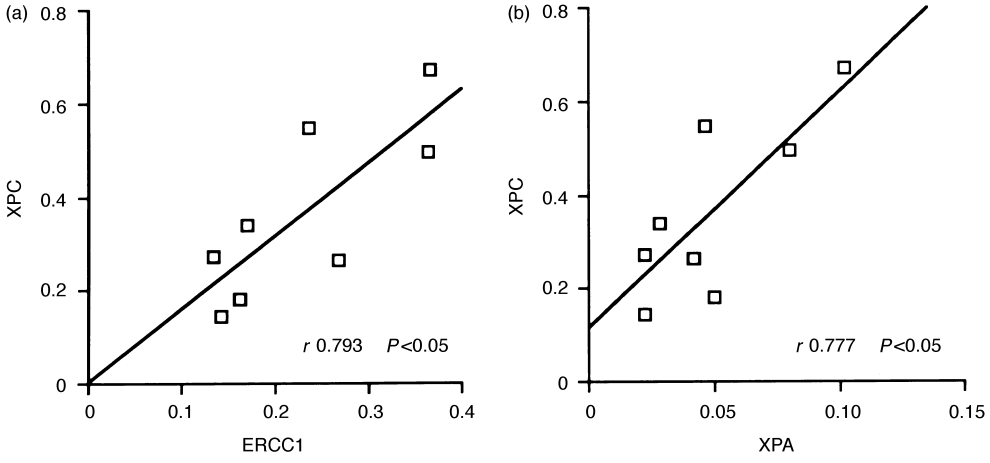

Figure 2. Correlations between the mRNA expression of XPC and ERCC1 (a) and between XPC and XPA (b) in the different cell lines. A representative experiment is shown.

Table 2. Relative mRNA expression of genes involved in nucleotide excision repair in the different cell lines

Cell line	ERCC1	XPA	XPB	XPC	XPD	XPF	XPG
CEM	1	1	1	1	1	1	1
JURKAT	1.4 ± 0.3	1.8 ± 0.3	1.5 ± 0.8	1.4 ± 0.4	2.1 ± 0.2	0.8 ± 0.2	1
K562	3 ± 2	3.6 ± 0.5	1.4 ± 0.4	6.2 ± 0.9	3.8 ± 1.2	1.4 ± 0.4	1.6 ± 1
U937	2.3 ± 1	2.9 ± 1.7	1.4 ± 0.4	2.8 ± 0.8	2.3 ± 0.3	0.9 ± 0.3	1.3 ± 0.4
IGROV	3 ± 1	3 ± 0.6	1 ± 0.1	5.9 ± 2.5	1.5 ± 0.5	0.8 ± 0.3	0.9 ± 0.3
OVCAR-3	1.5 ± 0.6	2 ± 0.9	0.8 ± 0.2	1.9 ± 0.4	0.9 ± 0.4	0.6 ± 0.4	0.9 ± 0.2
SKOV-3	2.3 ± 0.3	1.4 ± 0.2	0.6 ± 0.2	1 ± 0.4	3.7 ± 1.6	0.6 ± 0.3	0.8 ± 0.1
LOVO	3 ± 0.5	2.5 ± 1	0.8 ± 0.2	3.8 ± 0.4	3.3 ± 0.6	0.2 ± 0.06	0.9 ± 0.2

All the data have been normalised to the α actin densitometric absorbance and are expressed relative to normalised CEM transcripts. The data are the mean ± standard error of three different experiments.

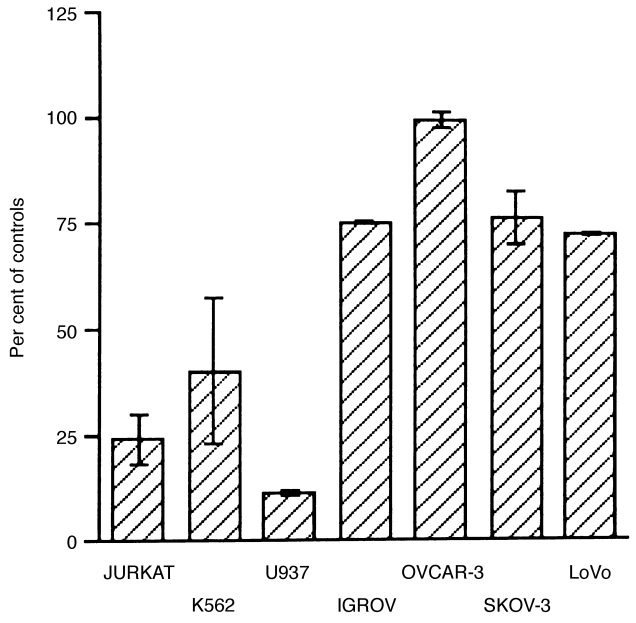

Figure 3. Luciferase activity expressed as a percentage of the reported activity of control undamaged pGL2 plasmid, normalised by the β-galactosidase value. Leukaemia (U937, JURKAT, K562) and carcinoma cell lines were transfected with either cisplatin (DDP) damaged or undamaged pGL2 and β-galactosidase plasmids. After 48 h, the luciferase activity was determined. Each value is the mean ± standard error of three different experiments, carried out in triplicate.

DISCUSSION

NER represents one of the most important DNA repair processes, able to correct the majority of bulky lesions in DNA, including UV-induced photoproducts and bulky chemical adducts, such as those derived from DDP and mitomycin treatments [12]. All the human repair genes involved in the process have been cloned and now named with the XP (xeroderma pigmentosum, a rare disorder in which the patients are totally or partially deficient in NER) complementation group with that specific gene defect, with the exception of the *ERCC1* gene, the first human cloned gene, for which no human phenotype or disease has so far been identified [10, 27].

The eight human cancer cell lines we selected for this study did express mRNA of *ERCC1*, *XPD*, *XPB*, *XPA*, *XPC*, *XPF* and *XPG* genes. When these data were analysed for a correlation between the relative expression of the different genes, we found a statistically significant positive correlation only between ERCC1/XPA ($P < 0.05$) and XPA/XPC ($P < 0.05$) mRNAs. These findings could be interesting considering that XPA and XPC proteins are involved in the very early steps of DNA damage recognition and recruit the ERCC1/XPF complex and XPG to the site of damage [28–30]. In fact it has been reported that RPA (replication protein A) in combination with XPA acts as a damage recognition complex directing the nuclease subunits to the proper incision sites by its specific interaction [16, 31–33]. XPC is considered to be involved in global genome NER (GGR), that deals with the repair of bulky DNA, including the non-transcribed strand of active genes, and seems to have an important role in preventing carcinogenesis [34]. We did not find a statistically significant correlation between the relative expression of ERCC1 and XPA, as reported by others in malignant and non-malignant brain tumours [19]. Discordance of the relative levels of XPD mRNA with the levels of ERCC1 mRNA has already been described in ovarian cancers [35], in brain tumours [19] and in lymphoblasts of patients suffering from chronic lymphocytic leukaemia [36]. No correlation was found among the other NER genes, but this might not be surprising, as almost all the proteins involved have a role in different processes of cell metabolism, such as transcription and cell cycle regulation [37–39].

DDP IC_{50}s of the different cell lines do not correlate with ERCC1 mRNA levels. Some authors did find a correlation between ERCC1 mRNA levels and the response to a platinum-based therapy in ovarian cancer patients [17], but others were not able to find any increase in ERCC1 mRNA levels in ovarian cancer patients resistant to therapy [22], and DDP sensitivity in human lung cancers was not related to the level of ERCC1 mRNA [40]. An explanation for these data could be that the levels of ERCC1 mRNA do not reflect the levels of a functional endonuclease ERCC1/XPF complex. It has, in fact, been recently demonstrated that in order to function as an endonuclease, ERCC1 has to be complexed with XPF, and ERCC1 mutants interfering with complex formation result in rapid degradation of ERCC1 [27]. It might be possible that a minimal amount of ERCC1 protein is sufficient to carry out the DNA repair process and a higher amount of the protein would not increase the efficacy of repair of DNA induced DNA damage. Again, it is interesting to note that an extreme sensitivity to crosslinking agents has been reported in cell lines that are defective in a functional ERCC1/XPF complex (rodent mutants belonging to ERCC1 and ERCC4 groups) [41], strongly suggesting that in some cases it is the absence rather than the relative content of a functional protein which is important for cellular sensitivity to a certain drug. The same holds true for other repair proteins, such as 3-methyladenine-DNA glycosylase, O6-alkylguanine-alkyltransferase and XPG protein, whose inactivation render the cells very sensitive to alkylating agents [42–44]. Recent data from the literature suggest that a lack of mismatch repair is associated not only with resistance to methylating agents, but also to a low level DDP resistance [45, 46]. When considering the integrity of the mismatch repair system in the cell lines selected for this study, data from our and other laboratories demonstrate that LoVo, SKOV-3, OVCAR-3 and CEM cell lines do not express the mismatch repair proteins, while K562, JURKAT, U937 and IGROV cell lines seem to be proficient in this repair system [47–49]. These data would suggest no correlation between the DDP sensitivity and mismatch repair status in the cell lines used (i.e. CEM and JURKAT cells display a similar DDP sensitivity despite their different mismatch repair status).

When the overall DNA repair capacity of the cell lines was investigated, we found that carcinomas seemed to have a higher repair activity than leukaemias. However, the relative luciferase activities correlated neither with the RNA expression of the different NER genes nor with DDP and L-PAM IC_{50}s. While a higher capacity to repair damaged pRSV-CAT positively correlated with *in vitro* DDP cytotoxicity in human lung cancers [50], no differences in reactivation of a damaged plasmid were observed in the human ovarian cancer cell (2008) and its DDP resistant subline (C13) [51]. The CHO-UV96 cell line (a hamster cell line mutant for ERCC1) that is 100-fold more sensitive to DDP treatment than the

CHO-AA8 cell line (parental cell line) is only approximately 3.5-fold less efficient in reactivating a DDP damaged plasmid (data not shown from this laboratory) and this probably reflects a limitation of the assay.

In conclusion, we have shown in a panel of different human cancer cell lines the pattern of expression of different genes involved in NER. These data provide evidence that human cancer cell lines do express the genes coding for the proteins involved in NER and that these cells are 'functionally' able to process a DNA lesion through NER machinery, as demonstrated by their ability to reactivate a DDP damaged pGL2 plasmid. When, however, the cellular sensitivities to DDP and L-PAM were correlated with the mRNA expression of different NER genes, no statistically significant correlations were found. Several recent studies have established that the biological response to a similar DNA damage can vary in different cells depending on their p53 status and to various other factors which regulate the cell cycle checkpoints and cell death [3, 5]. Therefore, even though the efficiency of DNA repair mechanisms is likely to be an important determinant in the cytotoxicity of DNA damaging agents, the ultimate effects appear to be highly dependent on downstream events activated by the presence of DNA damage.

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