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Rapid Polymerase Chain Reaction Assay to Detect Variation in the Extent of Gene-specific Damage between Cisplatin- or VP-16-resistant and Sensitive Lung Cancer Cell Lines

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We previously established a rapid and facile polymerase chain reaction (PCR)-stop assay for quantitation of specific gene damage in very small numbers of cells. The present study investigated whether the PCR-stop assay was able to detect variation in the extent of DNA damage in transcribed active genes between cisplatin- or VP-16-resistant and sensitive cells. The assay demonstrated that about twice as much genetic damage occurs in PC-9 cells than in cisplatin-resistant PC-9/CDDP cells following cisplatin exposure and about 4.6 times more damage occurs in H69 than in VP-16-resistant H69/VP cells following VP-16 exposure. These results show that DNA damage, as detected by PCR-stop assay, correlates with cytotoxicity. In conclusion, the PCR-stop assay could be useful in detecting variation in DNA damage in specific genes.

Key words: Gene-specific damage — PCR

Cisplatin is one of the key drugs used in cancer chemotherapy. It kills cells as a consequence of covalent modification of DNA.[1)] A number of recent reports have demonstrated that the level of cisplatin-mediated DNA damage in the leukocytes of patients with a variety of tumors correlates with the response to chemotherapy.[2–6)] This could be due to pharmacokinetic differences among patients, resulting in the leukocytes and tumor cells becoming equally damaged. Alternatively, this correlation could represent a cellular parameter, common to both leukocytes and tumor cells, that affects either drug accumulation, DNA damage or DNA repair. Several studies have shown that results obtained in patients can be mimicked by incubation of leukocytes with drugs in culture, suggesting that this correlation reflects a cell-based phenomenon.[4, 5)] Accordingly, we have investigated assays that can predict potential therapeutic outcome by incubating leukocytes with relevant drugs before the patient is exposed to them.[7, 8)] Unfortunately, most assays are specific to an individual drug; DNA damage is frequently evaluated using a radioactive analogue, a damage-specific antibody or, in the case of cisplatin, atomic absorption.

The preferential repair of transcribed genes has been shown to correlate well with the sensitivity of cells to various DNA-damaging agents.[9, 10)] For DNA lesions produced by cisplatin, the uvrABC excinuclease complex has been used to produce initial cleavage, with recognition of about 30% of cisplatin adducts.[11, 12)] This assay is fairly time-consuming as it requires extensive purification of the DNA, quantification, various enzymatic digestion steps and complex procedures such as Southern transfer and hybridization.

The topoisomerase II inhibitor VP-16 is also very commonly used in cancer chemotherapy. The resistance mechanism to VP-16 in VP-16-resistant H69/VP small cell lung cancer cells has been shown to be decreased accumulation of VP-16, which is mediated by the *mdr* gene and leads to reduced DNA damage.[13)] Therefore, measurement of the variation in DNA damage between cells is important in analyzing their sensitivity to VP-16. Alkaline elution assay has been used to detect DNA damage induced by VP-16.[14, 15)] However, the extent of DNA damage and repair detected by this method in cells resistant to VP-16, where resistance was induced by overexpression of bcl-2, was no different to that detected in sensitive cells.[14)] This means that alkaline elution assay is only of marginal use in detecting DNA damage induced by VP-16. This assay is also very time-consuming and is not gene-specific.

The PCR[3]-stop assay has the advantages of being rapid and easy, as well as gene-specific. It has also been shown to detect most, if not all, of the lesions produced in DNA by cisplatin.[8)] Furthermore, this assay can quantify DNA damage in very small numbers of cells, and can be used for the analysis of DNA damage in freshly isolated human

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[3] Abbreviations used: PCR, polymerase chain reaction; HPRT, hypoxanthine phosphoribosyltransferase; kb, kilobase; dNTP, deoxynucleoside triphosphate; dCTP, deoxycytidine triphosphate; IC_{50}, concentration reducing cell growth by 50%.

blood.[7] In the present study, to examine the ability of the PCR-stop assay to detect differences in lesions between cells resistant and sensitive to various anticancer drugs, we compared the levels of DNA damage produced by either cisplatin or VP-16.

The cisplatin-resistant cell lines PC-9/CDDP and H69/CDDP and the VP-16-resistant cell line H69/VP were established by the exposure of PC-9, an adenocarcinoma cell line of the lung, and H69, a small cell lung cancer cell line, to increasing concentrations of cisplatin or VP-16. For drug sensitivity estimation, a colony-forming assay was used for PC-9 and PC-9/CDDP cells and a growth-inhibition assay was used for H69, H69/CDDP and H69/VP cells.

One million cells were incubated with drug at 37°C for 3 h, then the cells were washed and resuspended in 500 μl of K-buffer [10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.5% Tween 20, and 100 μg/ml proteinase K]. The cell suspension was incubated at 55°C for 1 h and then heated at 94°C for 10 min to inactivate proteinase K. These cell lysates were stored at −20°C until use. The PCR was carried out using the following primers derived from the appropriate human genes. A 2.7 kb fragment of the human HPRT gene was amplified with primers spanning the regions from (5′-TGGGATTACACGTGTGAACCAACC-3′) to (5′-TGTGACACAGGCAGACTGTGGATC-3′).[16] A typical 50 μl reaction mixture contained 50 m*M* KCl, 10 m*M* Tris-HCl (pH 8.3), 1.0 m*M* $MgCl_2$ 50 μM of each of the dNTPs, 1 μM of each primer, 10 μl of cell lysate and 2 μCi of [α-^{32}P]dCTP. The initial heating step was performed at 94°C for 5 min followed by 80°C for 4 min during which time 10 μl (2.5 units) of diluted *Taq* polymerase was added. The subsequent cycles were: 60°C for 1 min, 71°C for 2 min, and 94°C for 1 min 20 s. The final cycle was followed by an extra 1 min of annealing at 60°C, 2 min of polymerization at 71°C and cooling to 4°C. The reaction products (30 μl) were separated on a 1% agarose gel. The gel was dried and autoradiographed for 30 min to 3 h. Radioactive bands were counted by a Bio imaging analyzer, BAS 2000 (Fuji Film Co., Ltd., Tokyo).

To compare gene-specific damage between drug-resistant and sensitive lung cancer cell lines, the cisplatin-resistant PC-9/CDDP and H69/CDDP and VP-16-resistant H69/VP cell lines were used. The values of relative resistance of these three cell lines compared to their parental cell lines were 28.0, 10.9 and 9.4, respectively (Table I). Gene-specific damage in each cell line was analyzed by PCR-stop assay after 3 h cisplatin or 14 h VP-16 exposure. Initial experiments were designed to determine the number of cycles that would produce quantitative PCR amplification of the DNA fragment. The 2.7 kb HPRT fragment was amplified for 20–30 cycles and the products were determined by electrophoresis. At 26 cycles a faint band corresponding to the 2.7 kb fragment was observed (data not shown). To confirm that the extent of amplification remained directly dependent upon the amount of amplifiable sequence present, a control reaction with a two-fold dilution of undamaged DNA (C/2) was included in every experiment. In all cases this produced an amplification of close to half of the normal control amount of DNA. To determine the drug concentration causing 63% genetic damage, cells were treated with five different concentrations of each drug. As shown in Fig. 1, amplification of the 2.7 kb fragment was clearly inhibited by both drugs. The genetic damage in PC-9/CDDP, H69/CDDP and H69/VP cells was less than that in the respective parental cell lines. Each experiment was performed on three separate occasions, starting with different cell cultures. The mean values are shown in Fig. 2. These curves were used to calculate the concentration of each drug which reduced amplification by 63% (D63), which is defined by the Poisson distribution as the dose that produces an average of one lesion per single strand of 2.7 kb fragment. These values are presented in Table II and show that cisplatin produced about twice as many lesions in PC-9 as in PC-9/CDDP cells and 1.8 times as many lesions in H69

Table I. Characteristics of Anticancer-drug-sensitive and -resistant Human Lung Cancer Cell Lines

Cell line	Histology	Prior chemotherapy	Doubling time (h)	IC_{50} (μg/ml)	
				CDDP	VP-16
PC-9	Adeno	No	28±13	0.22±0.05	—
PC-9/CDDP	Adeno	—	30±14	6.2±0.4	—
H69	Small	Yes	60±10	0.076±0.002	0.52±0.15
H69/CDDP	Small	—	67±19	0.83±0.13	—
H69/VP	Small	—	61±10	—	4.9±0.4

Adeno: adenocarcinoma of the lung, Small: small cell carcinoma of the lung. IC_{50} is the drug concentration required to produce 50% growth inhibition by cisplatin (CDDP) or etoposide (VP-16). IC_{50} values were determined by colony-forming assay (continuous exposure) in PC-9 and PC-9/CDDP cells, and by growth-inhibition assay (continuous exposure) in H69, H69/CDDP and H69/VP cells.

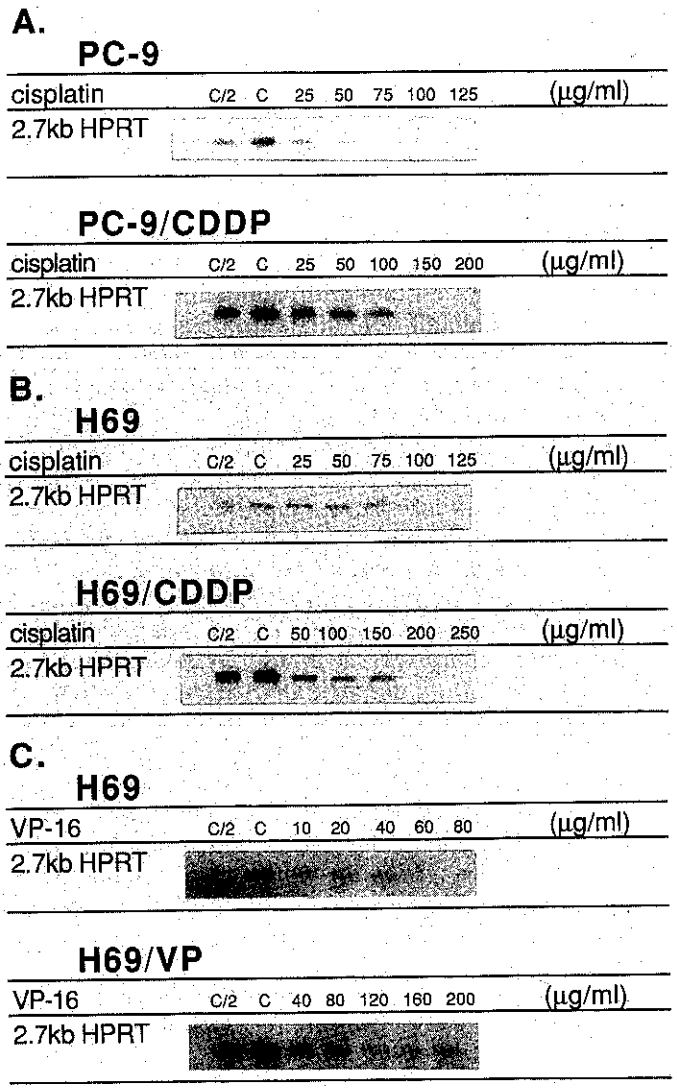

Fig. 1. Comparison of PCR amplification of DNA purified from drug-resistant and parental lung cancer cells. (A) PC-9 and PC-9/CDDP incubated with cisplatin for 3 h, (B) H69 and H69/CDDP incubated with cisplatin for 3 h, (C) H69 and H69/VP incubated with VP-16 for 14 h. [α-^{32}P]dCTP was included in the PCR step. The PCR products were separated by electrophoresis, then the gel was dried and autoradiographed. Lane C represents amplification of DNA from undamaged cells and C/2 represents half the amount of template DNA used in lane C.

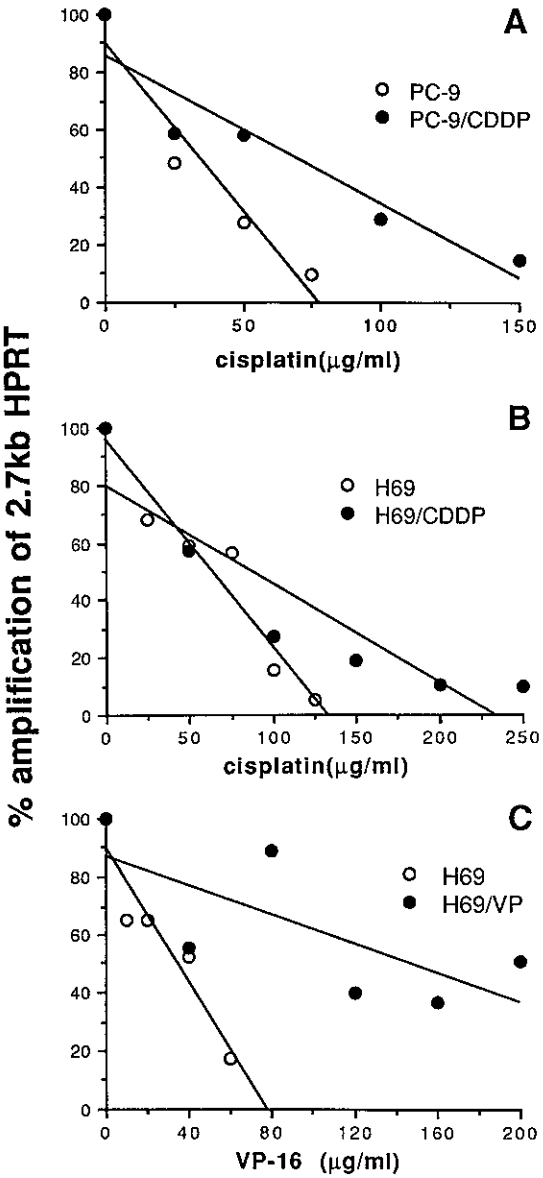

Fig. 2. Quantitation of PCR amplification products obtained from drug-resistant and parental lung cancer cells. (A) PC-9 (open circle) and PC-9/CDDP (closed circle) incubated with cisplatin for 3 h, (B) H69 (open circle) and H69/CDDP (closed circle) incubated with cisplatin for 3 h, (C) H69 (open circle) and H69/VP (closed circle) incubated with VP-16 for 14 h. The radioactivity from gels (as shown in Fig. 1) was counted and expressed as a percentage of the amplification obtained from undamaged DNA.

as in H69/CDDP cells, and that VP-16 produced about 4.6 times as many lesions in H69 as in H69/VP cells. These results demonstrate that PCR stop-assay can detect variation in the extent of DNA damage between cisplatin- or VP-16-resistant cells and sensitive cells.

We have previously reported that the PCR-stop assay is a rapid and facile method, capable of measuring DNA damage and repair in freshly isolated human cells.[7] An advantage of this method is that it can be used with very

Table II. Comparison of Genetic Damage Following Cisplatin or VP-16 Treatment

Cell line	D63 (μg/ml)		RD63/PD63
	CDDP	VP-16	
PC-9	49	–	–
PC-9/CDDP	105	–	2.14
H69	84	48	–
H69/CDDP	147	–	1.75
H69/VP	–	220	4.58

D63 is the cisplatin or VP-16 dose that reduces amplification of HPRT to 37% (i.e. 63% inhibition). RD63/PD63 is calculated as: D63 of resistant cell line/D63 of parental cell line.

low cell numbers. With various other techniques for assessing DNA damage, large numbers of cells are essential to obtain adequately measurable quantities of DNA damage. The majority of cisplatin-induced lesions are DNA intrastrand cross-links,[1] which arrest Klenow DNA polymerase at the same sites on a platinated DNA template.[17] The other method for measuring cisplatin-induced lesions in DNA involves incision at the lesion with the uvrABC excinuclease complex, followed by electrophoresis and Southern hybridization. However, it has been reported that this excinuclease cleaves at only about 30% of the cisplatin-induced lesions.[18] PCR-stop assay has been shown to detect about 2.5 times more damage than the uvrABC method.[19]

We performed the present experiments to investigate whether the PCR-stop assay could be used to detect differences in the extent of DNA damage between drug-resistant and -sensitive cells. The mechanisms of drug resistance differ between the two cell lines. PC-9/CDDP has a higher intracellular glutathione content and a reduced intracellular platinum level compared to the parental PC-9 cells.[20] The intracellular metallothionein content of H69/CDDP is higher than that of H69.[21] H69/VP has a typical multidrug resistance phenotype, and alteration of drug accumulation, mediated by P-glycoprotein, may play an important role in its resistance to VP-16.[13] Despite these differences, the final target of each drug is DNA, and decreased DNA damage and increased DNA repair are responsible for the drug resistance in each resistant cell line. As expected, the PCR-stop assay showed some difference in DNA damage between cisplatin-resistant and parental cells. The topoisomerase II inhibitor VP-16 is also a major anticancer drug, though the mechanism by which VP-16 induces cell death has not been completely elucidated. Alkaline elution assay has been used to detect DNA damage induced by VP-16 in most studies, but this assay is not gene-specific and is not very sensitive.[14] The present study is the first report to demonstrate that PCR-stop assay can detect gene-specific damage caused by VP-16 and that it can distinguish differences in DNA damage between VP-16-sensitive and resistant cells. We therefore conclude that the PCR-stop assay is a sensitive method which may be useful in detecting variations in DNA damage induced by cisplatin or VP-16, and that DNA damage assayed by the PCR method correlates to the cytotoxicity of cisplatin and VP-16. Furthermore, the PCR-stop assay may be able to detect variations in DNA damage cauced by other DNA-damaging agents. We are currently investigating whether genetic damage in human mononuclear cells in blood, assayed with PCR after incubation with cisplatin *in vitro*, correlates with the sensitivity to cisplatin of cancer cells in the same patient.

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