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Correlation of Gene-specific Damage with Cisplatin between Human Adenocarcinoma Cells and Peripheral Blood Mononuclear Cells Analyzed by Polymerase Chain Reaction-stop Assay

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We investigated gene-specific damage in adenocarcinoma cells, obtained from pleural effusions of 9 primary lung cancer patients, induced by incubation with cisplatin for 3 h *in vitro*. The 2.7 kb fragment of the hypoxanthine phosphoribosyltransferase (HPRT) gene was amplified by the polymerase chain reaction (PCR) to quantify the DNA damage. A 7-fold difference in the extent of gene-specific damage among the patients was observed. Mononuclear cells (MNC) were obtained from freshly isolated blood from the same patients before they received chemotherapy. These cells were also incubated with cisplatin *in vitro*, and PCR amplification of the HPRT gene was carried out. A 4-fold variation of DNA damage among the patients was observed. Moreover, there was a linear correlation between the extents of the DNA damage in the tumor cells and MNCs ($R^2=0.676$, $P=0.0016$). These results suggest that the PCR-stop assay could be used to detect interindividual variations in the extent of gene-specific damage in both tumor cells and MNC from the same patients induced by cisplatin treatment. In conclusion, MNC could be used to analyze cisplatin-induced gene-specific damage in cancer patients whose tumor cells are inaccessible.

Key words: Gene-specific damage — Cisplatin — PCR — Human adenocarcinoma cell — Human mononuclear cell

Cisplatin is one of the major drugs used in cancer chemotherapy. It kills tumor cells as a consequence of its covalent modification of DNA.[1] A number of studies have demonstrated that the extent of cisplatin-mediated DNA damage in leukocytes from patients with a variety of tumors correlates with their response to chemotherapy,[2-6] which may be due to pharmacokinetic differences between patients such that the leukocytes and tumor cells become equally damaged. Alternatively, this correlation may represent a cellular characteristic common to leukocytes and tumor cells that is affected by drug accumulation, DNA damage and/or DNA repair. Several workers have shown that results obtained in a patient can be mimicked by incubating the patient's leukocytes with cytotoxic drugs in culture, which suggests this correlation reflects a cell-based phenomenon.[4,5] Accordingly, we have been investigating assays that will enable the therapeutic outcome of chemotherapy to be predicted by incubating leukocytes with anticancer drugs before the patients have been treated with any such drug.[7,8] A further goal of these experiments was to develop an assay that will enable the DNA damage induced by many different DNA-damaging agents to be quantified rapidly. Unfortunately, most assays are specific to an individual drug: DNA damage is frequently evaluated by using a radioactive analogue, a damage-specific antibody or, in the case of cisplatin, atomic absorption. A PCR[4]-stop assay that has the potential to quantify most types of DNA damage has been developed.[8]

All damage in DNA is not equal. It has been established that cells exhibit different repair mechanisms for different types of lesions, as well as preferential repair of certain genomic domains. The preferential repair of transcribed genes, in particular, has been shown to correlate well with the sensitivity of cells to various DNA-damaging agents.[9,10] In order to quantify cisplatin-induced DNA lesions, the uvrABC excinuclease complex has been used to produce the initial cleavage, and this resulted in recognition of about 30% of the cisplatin adducts.[11,12] This assay is fairly time-consuming, as extensive DNA purification, quantitation, various enzymatic digestion steps and complex procedures, such as Southern transfer and hybridization, are required. The PCR-stop assay has the advantage of being rapid and facile for quantitation of gene-specific damage in very small numbers of cells.[7] This assay has interexperimental accuracy and can detect different extents of anticancer agent-induced DNA damage in sensitive and resistant

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[4] Abbreviations used: PCR, polymerase chain reaction; HPRT, hypoxanthine phosphoribosyltransferase; kb, kilobase; dNTP, deoxynucleoside triphosphate; dCTP, deoxycytidine triphosphate; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; MNC, mononuclear cells; LSM, lymphocyte separating medium.

cells.[7,13] Furthermore, it has been shown to recognize most, if not all, cisplatin adducts in DNA. Therefore, this assay may be clinically useful to predict therapeutic responses.

We hypothesized that the extent of the DNA damage in individual tumor cells incubated with cisplatin *in vitro* differs and that the interindividual differences reflect different therapeutic responses. In this study, we investigated the quantitative variations of DNA damage in human tumor cells isolated from pleural effusions of lung cancer patients induced by cisplatin *in vitro*. A parallel study on fresh MNC from peripheral blood isolated from the same patients was also carried out. We compared the DNA damage induced in these cell types and assessed whether MNC could be used to analyze cisplatin-induced DNA damage and to predict tumor sensitivity to cisplatin.

MATERIALS AND METHODS

Chemicals Cisplatin was supplied by Nippon Kayaku Co., Ltd., Tokyo, [α-^{32}P]dCTP was purchased from Amersham Japan, Taq polymerase was obtained from Perkin-Elmer Cetus, Norwalk, CT, and all other chemicals and enzymes used were purchased from Sigma Chemical Company, St. Louis, MO.

Adenocarcinoma cells and MNC collection Adenocarcinoma cells were obtained from pleural effusions of untreated primary lung cancer patients. The effusion fluid was drawn into a heparinized syringe, layered over an equal volume of LSM (density 1.077–1.080) and centrifuged at 1,800 rpm for 20 min at room temperature. The mononuclear cell layer was recovered, diluted with 25 ml of PBS, layered over a Percoll gradient (3 layers; density 1.030, 1.050 and 1.080), and centrifuged at 1,800 rpm for 20 min. The 1.030 and 1.050 layers were recovered, washed twice with PBS and resuspended with 25 ml of complete medium [RPMI 1640 medium, supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml)]. The cells were counted with a Coulter counter. MNC (88% lymphocytes and 12% monocytes) were obtained from 5 ml of peripheral blood from the same patients. The blood was drawn into a heparinized syringe, layered over an equal volume of Histopaque (density 1.077) and centrifuged at 1,400 rpm for 30 min. The MNC layer was recovered, washed with 25 ml of complete medium, and centrifuged at 1,200 rpm for 10 min. The cell pellet was resuspended in 20 ml of complete medium and the cells were counted with a Coulter counter.

Cisplatin treatment and cell lysate One million cells were pelleted at 1,000 rpm for 5 min, resuspended in 1 ml of serum-free medium containing the required cisplatin concentration (0 to 125 μg/ml), incubated at 37°C for 3 h, then collected by centrifugation at 1,000 rpm for 5 min. The cells were resuspended in 5 ml of serum-free medium then a 250 μl aliquot (5×10^4 cells) was transferred to a 1.5-ml microfuge tube, and centrifuged at 10,000 rpm for 5 min. The resulting supernatant was discarded. Five hundred 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] was added to the pellet, and the cell suspension was incubated at 55°C for 1 h, then heated at 94°C for 10 min to inactivate the proteinase K. The resulting cell lysates were stored at −20°C until required for use.

PCR The following primers derived from the appropriate human genes were synthesized. A 2.7-kb fragment of the human HPRT gene was amplified with primers spanning (5′-TGGGATTACACGTGTGAACCAACC-3′) to (5′-TGTGACACAGGCAGACTGTGGATC-3′).[14]

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 each dNTP, 1 μM each primer, 10 μl of cell lysate and 2 μCi of [α-^{32}P]dCTP. The initial heating step was carried out at 94°C for 5 min, followed by 80°C for 4 min, during which 10 μl (2.5 units) of diluted Taq polymerase was added, followed by 27 cycles of 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 annealing at 60°C, 2 min polymerization at 71°C and cooling to 4°C.

Gene-specific damage analysis The reaction products (35 μl) were separated on a 1% agarose gel and visualized by ethidium bromide staining using *Bst*E II-digested lambda DNA as a molecular weight standard. The gel front containing unincorporated radioactivity was cut off, and the gel was washed twice with water for 20 min to remove any background radiation, then dried and autoradiographed for 30 min to 3 h, after which the radioactive bands were counted using a Bio imaging analyzer BAS 2000 (Fuji Film Co., Ltd., Tokyo). Differences between samples were analyzed for significance using the two-tailed Student's *t* test. The criterion of significance was taken to be $P<0.05$.

RESULTS

In order to study the gene-specific damage induced by cisplatin *in vitro* in tumor cells from primary lung cancer patients, adenocarcinoma cells in malignant pleural effusions were obtained using the Percoll gradient method before the patients received chemotherapy. The resulting purified preparation contained 80–90% adenocarcinoma cells. The gene-specific damage to the cells induced by incubation with cisplatin for 3 h was analyzed using the PCR-stop assay. Previous experiments demonstrated that amplification of the 2.7 kb HPRT fragment for 27 cycles produced the best conditions for gene-damage quantita-

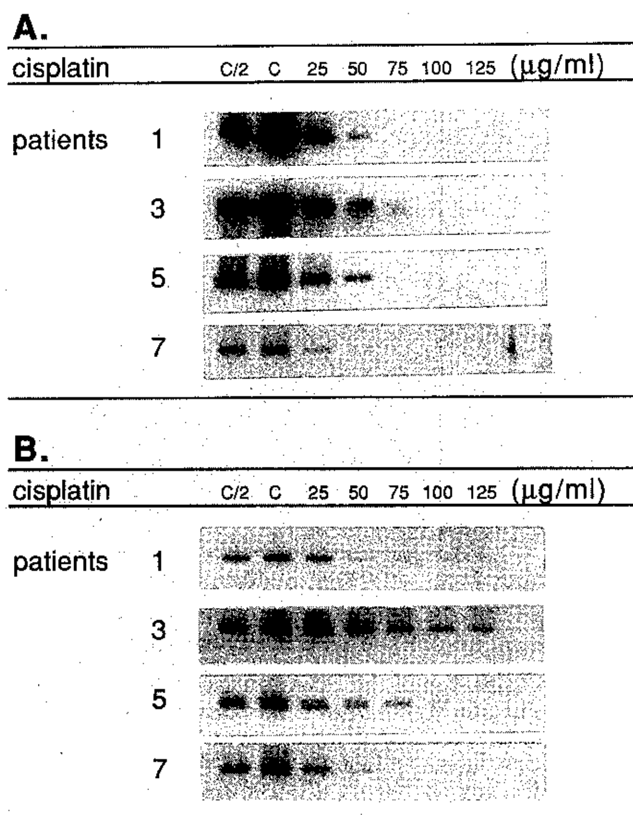

Fig. 1. The PCR amplification, using [α-^{32}P]dCTP, of DNA obtained from (A) adenocarcinoma cells in pleural effusions and (B) mononuclear cells in peripheral blood after incubation with cisplatin for 3 h *in vitro*. The PCR products of 2.7 kb HPRT were separated by electrophoresis and the gel was dried and autoradiographed. The lane marked C represents amplification of DNA from undamaged cells and C/2 represnts half of the amount of template DNA as used in the C lane.

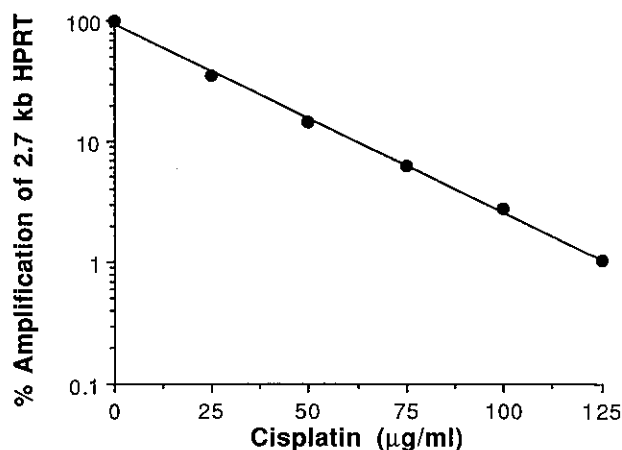

Fig. 2. Quantitation of the PCR amplification products from human adenocarcinoma cells obtained from pleural effusions after incubation with cisplatin for 3 h *in vitro*. The radioactivity on the gel of patient 1 (shown in Fig. 1) was counted and expressed as a percentage of the amplified product obtained from undamaged DNA.

tion (data not shown). In order to ensure that the extent of amplification remained directly dependent upon the amount of amplifiable sequence throughout, a control reaction with a 2-fold dilution of undamaged DNA (C/2) was included in every experiment, and in all cases this resulted in amplification of virtually half the normal control amount of DNA. In order to determine the cisplatin concentration that would cause 63% gene-specific damage, the cells were treated with 5 different concentrations of cisplatin. Amplification of the 2.7 kb fragment was clearly inhibited by cisplatin (Fig. 1A). In order to compare the gene-specific damage among the patients, cisplatin concentration-response relation curves were drawn for each one (Fig. 2) and used to calculate the concentration of cisplatin that reduced amplification by 63% (D63), which is defined, according to a Poisson distribution, as the dose that produces an average of one lesion per single strand of the 2.7 kb fragment. These values are presented in Fig. 3A. Large variations of the adenocarcinoma cell D63 values were observed among the lung cancer patients; a 7-fold difference between the most sensitive and most resistant tumor cells was found. A parallel study on freshly isolated MNC in peripheral blood obtained from the same patients was carried out. Amplification of the 2.7 kb fragment was clearly inhibited by cisplatin (Fig. 1B) and DNA damage variations among patients were observed (Fig. 3B), with a 4-fold difference between the most sensitive and most resistant cells. We analyzed the relationship between DNA damage of the tumor cells and MNC (Fig. 4) and found they were linearly correlated [(MNC D63) = 14.735 + 1.772 × (tumor cell D63), $R^2=0.676$, $P=0.0016$].

DISCUSSION

The experiments reported here from a part of our ongoing program to develop a rapid and facile assay that will enable DNA damage and repair in freshly isolated human cells to be measured. Such information would be valuable in predicting whether a patient is likely to respond to cancer chemotherapy. The current PCR-stop assay measures DNA damage in specific genes. However, the repair of gene-specific damage has been found to correlate well with the sensitivity of cells to various agents[9, 15] and, therefore, may represent a better predictor of therapeutic efficacy.

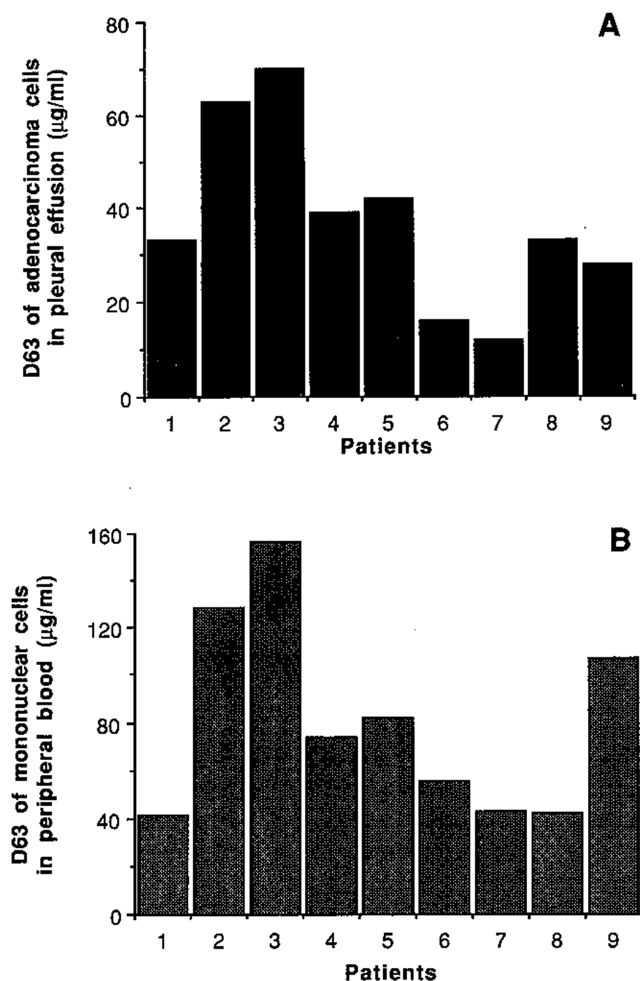

Fig. 3. Interindividual variations in the amount of damage induced in the HPRT gene of (A) human adenocarcinoma cells in pleural effusions and (B) human mononuclear cells in peripheral blood after incubation with cisplatin for 3 h *in vitro*. The cells were from the same patients, denoted by numbers in (A) and (B). The results represent the D63 value of each cell type from each patient. Interindividual differences were observed with both cell types.

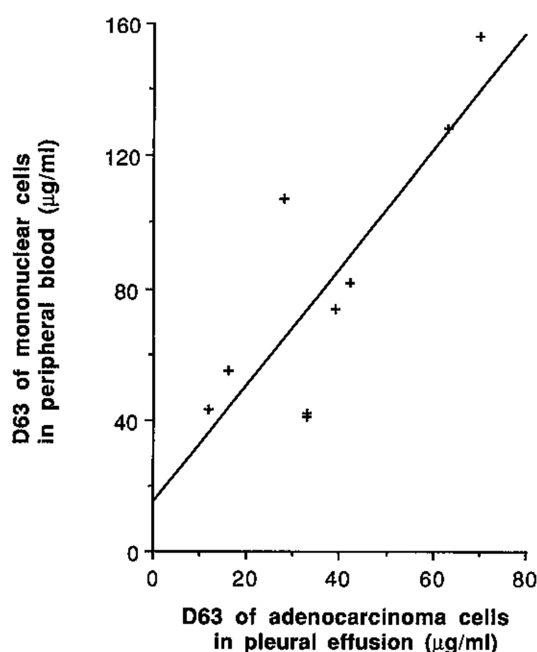

Fig. 4. Comparison of the DNA damage induced by incubation with cisplatin for 3 h *in vitro* in adenocacinoma cells from pleural effusions and mononuclear cells from peripheral blood. The adenocarcinoma and mononuclear cell D63 values correlated significantly ($R^2=0.676$, $P=0.0016$).

At present, chemotherapy for metastatic non-small cell lung cancer is not particularly effective. This malignant disease cannot be cured, even with the most effective cisplatin-based combination chemotherapy regimens, although responders to chemotherapy may have a better prognosis than nonresponders.[16] Accordingly, it is believed to be very important to predict likely responders before subjecting patients to chemotherapy. Unfortunately, no predictor of the response to chemotherapy prior to administration has been found until now. Platinum-DNA adducts in leukocyte DNA have been reported to correlate with the response of cancer patients receiving cisplatin-based chemotherapy.[2,3,5,6] This correlation may represent a cellular parameter common to leukocytes and tumor cells that affects drug accumulation, DNA damage and/or DNA repair. These adducts were analyzed by an ELISA using only leukocytes obtained from peripheral blood after chemotherapy. Therefore, whether chemotherapy-induced DNA damage in leukocytes correlates with that of tumor cells remains to be elucidated.[2,5]

In this study, we examined variations in the extent of the *in vitro* cisplatin-induced DNA damage in adenocarcinoma cells obtained from pleural effusions from 9 lung cancer patients prior to chemotherapy. In a previous study, we showed that PCR-stop assay has interexperimental accuracy.[7] Three different PCR-stop assay experiments were performed to detect DNA damage using HL-60 cells or different samples of MNC obtained on different days from five persons. These experiments demonstrated that there was almost no difference in DNA damage to MNC with cisplatin, ormaplatin or UV light on different days in the same individuals, or in HL-60 cells. Therefore, the interindividual variation was considered to be due to factors intrinsic to the patient, and not to interexperimental variability in the PCR-stop

assay. These differences of the DNA damage in adenocarcinoma cells were thought to reflect varying degrees of natural resistance to cisplatin. Unfortunately, the 9 patients analyzed in this study received various types of chemotherapy, so we could not analyze the correlations between DNA damage in tumor cells and the clinical response to and side effect severity of chemotherapy. Three patients received cisplatin-based combination chemotherapy, of whom one responded and the others had progressive disease. The adenocarcinoma cell D63 value of the responder was 33 μg/ml, and those of the non-responders were 63 and 70 μg/ml. The adenocarcinoma cells formed about twice as many cisplatin adducts as the human MNC from the same patients. This phenomenon may occur if cisplatin accumulation by these cell types differs. However, the reason remains to be elucidated, although possible plausible explanations are that the cell size influences cisplatin accumulation and/or its accumulation by organ tissues is subject to interindividual variation.

Our previous analysis of the total DNA platination in MNC isolated from 5 normal volunteers found no significant interindividual differences,[7] but in this study on lung cancer patients, a 4-fold variation in the cisplatin-induced DNA damage in MNC was observed. It is possible that higher susceptibility to DNA-damaging agents in cancer patients favored tumor development. We found a linear correlation between the extent of DNA damage in tumor cells and MNC induced by incubation with cisplatin *in vitro*. A tumor usually forms different subclones during its development. We have no data indicating that different subclones show different degrees of gene-specific damage by cisplatin, and it was shown that gene-specific damage of MNC reflects the mean gene-specific damage in tumor cells. This suggests that the genetic basis relevant to drug pharmacology in cells is common to tumor cells and MNC. Moreover, a linear correlation was reported between the number of cisplatin-DNA adducts in *in vitro* cisplatin-exposed human leukocytes from patients prior to chemotherapy and in cells collected immediately after *in vivo* exposure to it.[4, 5] Therefore, the extent of gene-specific damage in MNC induced by incubation with cisplatin *in vitro* is believed to reflect the extent of DNA damage in tumor cells in patients treated with cisplatin *in vivo*.

In summary, interindividual variations in the extent of gene-specific damage in both tumor cells and MNC from the same patients induced by cisplatin treatment *in vitro* were found, and the degrees of damage in these cell types correlated significantly. In concluion, MNC can be used to detect cisplatin-induced DNA damage *in vitro* and is expected to reflect the response or side effects, such as bone marrow suppression or renal dysfunction in cisplatin-based combination chemotherapy regimens. We are conducting a prospective study to determine whether gene-specific damage, assayed using PCR in human MNC in blood incubated with cisplatin *in vitro*, reflects the clinical outcome in patients treated with cisplatin-based combination chemotherapy.

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