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MARKED REDUCTION OF TYPE I KERATIN (K14) IN CISPLATIN-RESISTANT HUMAN LUNG SQUAMOUS-CARCINOMA CELL LINES

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Abstract—We have established two cisplatin-resistant human lung squamous-carcinoma cell lines, PC10-B3 and PC10-E5, from their original cell line PC10. To discover which proteins are associated with cisplatin resistance, we carried out a two-dimensional gel electrophoresis to analyze differences in protein alteration between PC10, PC10-B3 and PC10-E5. A protein spot M_r 50 kDa, pI5.3, was reduced markedly and a spot M_r 50 kDa, pI4.9 was increased when PC10-B3 and PC10-E5 were compared with PC10. A spot M_r 58 kDa, pI5.8 newly appeared only in PC10-E5. Cell fractionation showed that the M_r 50 kDa, pI5.3 (p50-5.3) and the M_r 50 kDa, pI4.9 fell within the nuclear fraction, while the M_r 58 kDa, pI5.8 was found among the cytosol and microsomal fractions. Microsequencing after *in situ* digestion of the dramatically reduced spot p50-5.3 revealed that it was identical to 50 kDa, type I keratin (K14). Moreover, a retinoic acid-mediated K14 reduction was concomitant with a 4.0-fold increase in cisplatin resistance in PC10. Our report is the first to suggest the possible association of marked K14 reduction and cisplatin resistance in PC10-B3 and PC10-E5.

Although cisplatin‖ has been used extensively against a broad range of human malignancies because of its high potency, complete remission is often hampered by a relapse of the residual tumors, probably owing to the development of cisplatin-resistant subpopulations. This eventual failure of cisplatin-based chemotherapy has urged many researchers and clinicians to pursue the mechanisms and means of circumventing cisplatin resistance. Mainly with the help of various *in vitro* established cisplatin-resistant cell lines, a multifactorial and complicated pattern of resistance to cisplatin has been postulated: (i) reduction of intracellular cisplatin accumulation [1–4], (ii) elevated expression of glutathione, its related enzymes [5–9] and metallothionein [10, 11], (iii) accelerated repair of cisplatin–DNA adducts [12–14], and (iv) overexpression or activation of oncogenes such as *ras*, *myc* or *fos* [15–17]. Thus, more than one factor is likely to contribute to cisplatin resistance in a single cell line [4, 18, 19]. Furthermore, we must consider other unknown factors and proteins in order to elucidate without contradiction a variety of phenomena related to cisplatin resistance [14, 20–23]. However, few studies have focused on the identification of these unknown candidates for modulating cisplatin resistance.

We have established previously a cisplatin-resistant human lung squamous-carcinoma cell line PC10-B3 and demonstrated that reduced platinum accumulation was one of the important factors contributing to cisplatin resistance in PC10-B3 [24]. In the present study, we established another cisplatin-resistant subline, PC10-E5, from its original cell line PC10. We used these cell lines to determine which protein alterations coincided with the acquisition of cisplatin resistance. Two-dimensional gel electrophoresis revealed a few apparently different proteins between PC10 and its cisplatin-resistant sublines. Moreover, we succeeded in identifying one of the proteins by microsequencing and discuss its possible manner of association with cisplatin resistance.

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‖ Abbreviations: cisplatin, *cis*-diamminedichloroplatinum(II); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; DMSO, dimethyl sulfoxide; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; DTT, dithiothreitol; PVP, polyvinylpyrrolidone; TFA, trifluoroacetic acid; RA, retinoic acid; and pI, isoelectric point.

MATERIALS AND METHODS

Chemicals

Cisplatin was obtained from the Nippon Kayaku Co., Ltd. (Tokyo), and retinoic acid (RA) was purchased from the Sigma Chemical Co. (St. Louis, MO).

Cell lines

A human lung squamous-carcinoma cell line PC10 (provided by Prof. Y. Hayata, Tokyo Medical College) was maintained at 37° in a humidified incubator of 5% CO_2 in RPMI 1640 medium,

supplemented with 10% heat-inactivated fetal bovine serum. Cisplatin-resistant sublines PC10-B3 and PC10-E5 were cultured in the same way, except that this was carried out in the presence of 0.5 and 1.0 μg/mL cisplatin, respectively, as described below.

Establishment of cisplatin-resistant sublines from PC10

At the outset, PC10 was exposed to 0.1 μg/mL cisplatin. By a stepwise increment of intermittent exposure, the concentration of cisplatin was increased by 0.1 μg/mL every few passages and reached 1.5 μg/mL over 6 months. Subsequently, 0.5 and 1.0 μg/mL cisplatin were exposed continuously. Cells in 0.5 μg/mL cisplatin were capable of growing persistently. PC10-B3 and PC10-E5 were cloned from different mass populations in 0.5 and 1.0 μg/mL cisplatin, respectively. The IC_{50} values measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay revealed that PC10-B3 and PC10-E5 were, respectively, 11.4- and 19.9-fold more resistant to cisplatin than PC10 [25]

Two-dimensional gel electrophoresis

Typically, 1–3 × 10^7 cells growing exponentially were harvested with scrapers and washed twice with cold phosphate-buffered saline (PBS). After low-speed centrifugation, cell pellets were sonicated (three bursts of 15 sec) by an ultrasonic disruptor (BH-200P, Tomy Seiko Co., Ltd., Japan) in ten times the volume of sonication buffer [10 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 50 μg/mL RNaseA, 1 mM phenylmethylsulfonyl fluoride (PMSF)] and kept on ice for 5–10 min. After 50 μg/mL DNase I had been added, the cell suspension was solubilized by a lysis buffer consisting of 9.2 M urea, 2% Nonidet P-40, 2% ampholytes (pH 5–7: pH 3.5–10 = 1.6:0.4), 5% mercaptoethanol. The concentration of each extracted protein was measured by the Bradford protein assay [26]. Two-dimensional gel electrophoresis was performed according to the method of O'Farrell [27]. Approximately 20- to 50-μL aliquots (equivalent to 100 μg protein) were applied to the first-dimensional isoelectric focusing tube gels (10 cm × 2.5 mm; i.d.), composed of 9.2 M urea, 4% polyacrylamide, 2% ampholytes (pH 5–7: pH 3.5–10 = 4:1), 2% Nonidet P-40. First-dimensional electrophoresis was performed at 200 V for 1 hr, 400 V for 12.5 hr and 800 V for the last 1 hr. After equalization in the sodium dodecyl sulfate (SDS) sample buffer for 30 min, the tube gel was topped on the SDS-PAGE gel composed of an upper 4% polyacrylamide stacking gel and a lower 10% polyacrylamide gel for the second-dimensional protein separation. Second-dimensional electrophoresis was carried out sequentially at 80 V for 1.5 hr and 120 V for 3.5 hr. Immediately after the end of the run, the gel was subjected to a silver-staining procedure to enable visualization of protein spots in the gel. The silver-staining procedure was based on the method originally described by Merrill *et al.* [28].

Cell fractionation

Cell fractionation was carried out according to the method of Hogeboom [29]. Exponentially growing cells (5–8 × 10^7) were harvested with scrapers and washed twice with cold PBS. Pellets were resuspended with 3–5 mL hypotonic solutions (5 mM $MgCl_2$, 10 mM Tris–HCl pH 7.5, 1 mM PMSF) and kept on ice for at least 30 min to make the cells swell and rupture. Cells were then homogenized by a homogenizer of the Potter–Elvehjem type. After centrifugation at 700 *g* for 5 min, cell pellets for the nuclear fraction were resuspended with 3 mL of 0.25 M sucrose solution (0.25 M sucrose, 5 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.5) and centrifuged again; the resultant pellets were subjected to the procedure of total protein extraction, as described above. Meanwhile, the supernatant was centrifuged at 5000 *g* for 10 min and the resultant pellets were dissolved by a lysis buffer to an appropriate volume for the mitochondrial fraction. The supernatant was further centrifuged at 54,000 *g* for 1 hr; the resultant pellet consisted of proteins of the microsomal fraction, while the last supernatant included proteins of the cytosol fraction.

Microsequencing

(i) *Electroblotting.* Following two-dimensional gel electrophoresis, proteins were electroblotted in a transfer buffer (9.0 g Tris, 43.2 g glycine, 3.0 g SDS, per 3 L dH_2O) onto polyvinylidene difluoride (PVDF) membrane (Problot, Applied Biosystems, Tokyo) at 70 V for 3 hr from the gel. Transferred proteins were stained with 0.1% Coomassie Blue R-250. Excess stain was removed by a destaining solution (10% acetic acid, 25% isopropanol) for the detection of protein spots.

(ii) *S-Carboxymethylation.* The protein spots of interest were cut off from PVDF membranes and transferred to Eppendorf tubes (1.5 mL). The filters in the Eppendorf tubes were washed twice with dH_2O for 4–5 min and stored wet at −20°. The above procedures were repeated, and forty pieces containing the protein of interest were collected in a single Eppendorf tube. Prior to *in situ* digestion, S-carboxymethylation was performed to enhance recovery of the protein from the membrane pieces. The membrane pieces were immersed in 300 μL of reducing buffer (6 M guanidine hydrochloride, 0.5 M Tris–HCl, pH 8.5, 0.3% EDTA, 2% acetonitrile) with 1 mg dithiothreitol (DTT) under nitrogen gas for at least 2 hr to ensure sufficient reduction of the protein. Then 2.4 mg of iodoacetic acid (in 10 μL of 0.5 N sodium hydroxide) was added and mixed in the dark for 20 min for S-carboxymethylation. After they had been washed three times with 2% acetonitrile, the pieces were mixed with 0.1% SDS for 5 min.

(iii) In situ *digestion* [30]. The membrane pieces were shaken for 30 min at 37° in 1.2 mL of 0.5% PVP-40 dissolved in 100 mM acetic acid in order to block absorption of trypsin during digestion. Excess PVP-40 was removed by washing 5–6 times with dH_2O. Filters were further cut into small pieces of approximately 1 mm × 1 mm and put back into the same tube. The protein on the PVDF membrane pieces was digested *in situ* with trypsin: 150 μL of 100 mM Tris–HCl, pH 8.2, and acetonitrile, 95:5 (v/v), at 37° overnight, while being shaken. The

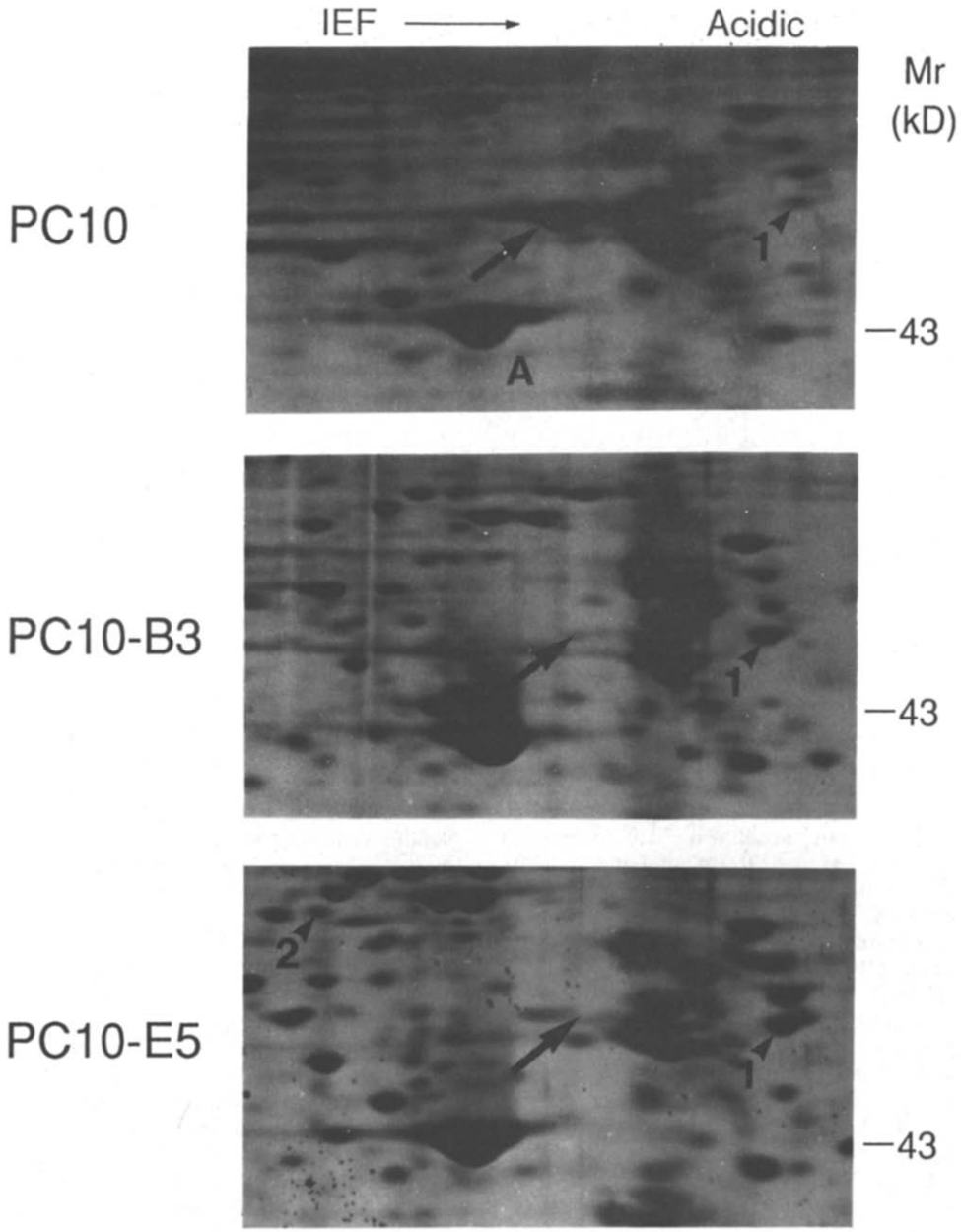

Fig. 1. Enlarged areas containing altered protein spots revealed by two-dimensional polyacrylamide gel electrophoresis. Arrows indicate that the spot M_r 50 kDa, pI5.3 was dramatically reduced in PC10-B3 and PC10-E5. Arrowheads (1) indicate that the spot M_r 50 kDa, pI4.9 increased in PC10-B3 and PC10-E5. Arrowhead (2) indicates the newly appeared spot in PC10-E5. Actin (A) is also indicated.

Table 1. Protein alterations between a human lung squamous-carcinoma cell line, PC10, and its cisplatin-resistant sublines, PC10-B3 and PC10-E5

Altered proteins	PC10	PC10-B3	PC10-E5	Cell localization
M_r 50 kDa, pI5.3	+++	±	±	Nucleus
M_r 50 kDa, pI4.9	+	++	++	Nucleus
M_r 58 kDa, pI5.8	−	−	+	Cytosol, microsome

Amount of protein spots detected by two-dimensional gel electrophoresis was indicated in large as − to +++.

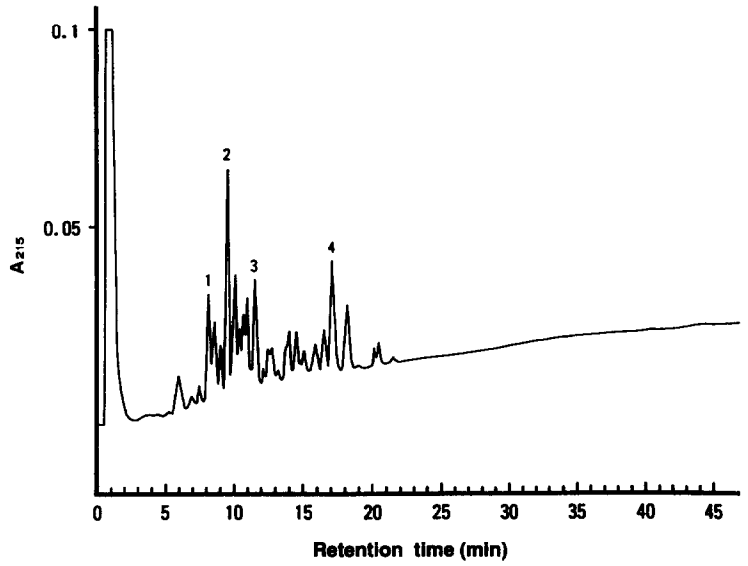

Fig. 2. Separation of p50-5.3 by reversed-phase HPLC. Forty spots of p50-5.3 were digested with trypsin [enzyme-to-substrate ratio 1:10 (w/w) in 100 mM Tris–HCl, pH 8.2, for 18 hr at 37°] and subjected to reversed-phase HPLC using a 2.1 × 30 mm Aquapore RP300 column (ABI). The peptides were eluted with a linear gradient of from 0% to 70% acetonitrile in 0.05% TFA.

enzyme-to-substrate ratio was kept between 1:10 and 1:20 (w/w). On the following day, the reaction mixture was stored at −20° or immediately loaded onto a reversed-phase HPLC column (model 130A Separation System, Applied Biosystems, Tokyo) after acidification with 10 μL trifluoroacetic acid (TFA):H_2O (10:90, v/v).

(iv) *Peptide mapping.* The peptide-containing solution was loaded onto a reversed-phase HPLC column (2.1 × 30 mm Aquapore RP300 column, Applied Biosystems) and the peptides were eluted with a linear gradient of from 0% to 70% acetonitrile in 0.05% TFA. Eluting peptides were collected manually in Eppendorf tubes based on their UV absorbance at 215 nm, and frozen immediately at −20°.

(v) *Amino acid sequencing.* Amino acid sequencing of collected peptides was performed by conventional Edman chemistry on a gas-phase protein sequenator (model 470A, Applied Biosystems) equipped with an on-line phenylthiohydantoin amino acid derivative analyzer (model 120A, Applied Biosystems). The peptide sequences were homology-searched on the basis of the protein sequence database (DNASIS, Hitachi Software Engineering, Tokyo).

MTT assay

An MTT assay modified by Mossman [25] was used to measure cellular survival fractions. The initial cell number in the 180 μL culture medium in a 96-well plate was optimized so as to be proportionate to MTT formazan product formation. Various doses of 20 μL cisplatin were added. After a 4-day incubation with cisplatin, 50 μL of MTT solution (2 mg MTT/mL PBS) was added to each well for 4 hr. The formazan crystals generated by viable cells were dissolved by 150 μL dimethyl sulfoxide (DMSO) and their spectrophotometric absorbances were assessed at 540 nm with an immunoreader (ImmunoReader NJ-2000, Nippon InterMed K.K., Tokyo).

Table 2. Determined sequence of five peptide peaks of p50-5.3 in PC10 revealed by reversed-phase HPLC, compared to that of authentic 50 kDa, type I keratin (K14)

Peak	Determined sequence	Authentic K14	Number of residues from NH_2-terminus
1	GSAGIGGGIGAGSSR	GSCGIGGGIGAGSSR	16–30
2a	APSTYGGGLSVSSS	APNTYGGGLSVSSS	42–55
2b	IPDWYQR	IRDWYQR	147–153
3	LLEGEDAHLSSSQFS	LLEGEDAHLSSSQFS	418–432
4	DAEEWFFTKTEELN	DAEEWFFTKTEELN	301–314

Amino acids different from those of authentic K14 are underlined.

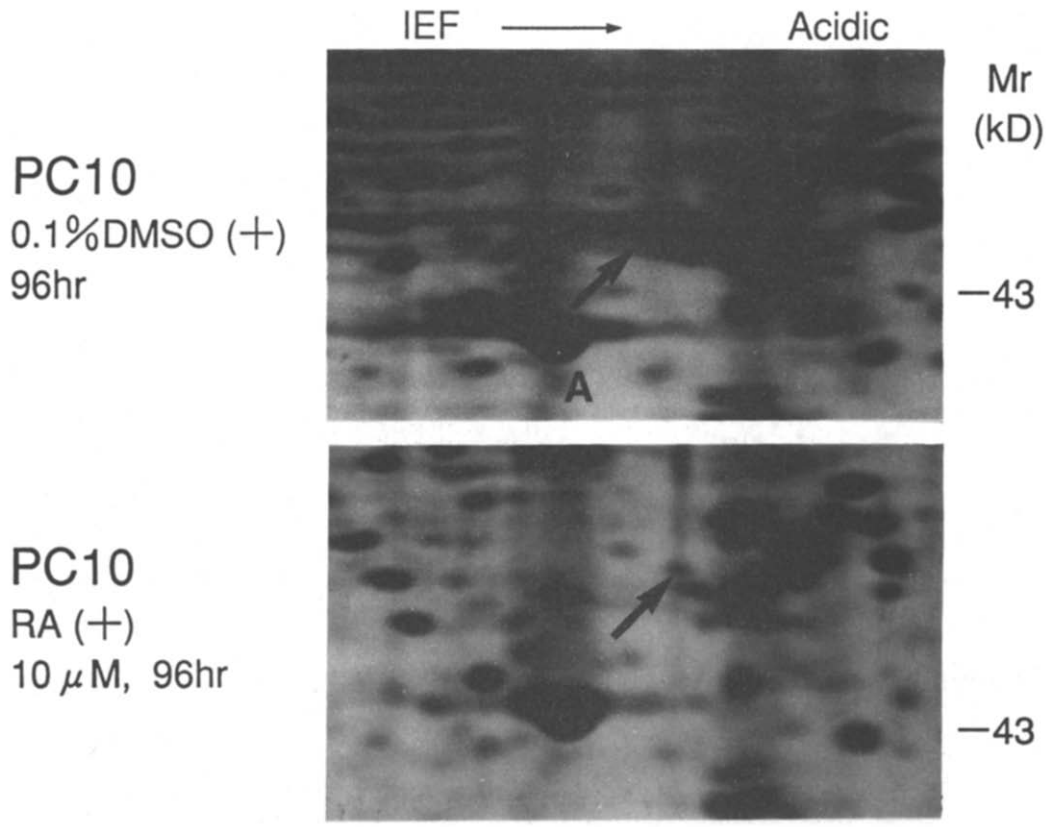

Fig. 3. RA-mediated reduction of type I keratin (K14) in PC10. Top panel: After treatment with 0.1% DMSO for 96 hr as a control. Bottom panel: After treatment with 10 μM RA for 96 hr. A indicates Actin.

RESULTS

Two-dimensional gel electrophoresis

We used two-dimensional gel electrophoresis to investigate differences in protein alteration between human lung squamous-carcinoma cell line PC10 and its cisplatin-resistant sublines PC10-B3 and PC10-E5. Figure 1 and Table 1 show that a protein spot M_r 50 kDa, pI5.3 (p50-5.3) was reduced markedly in PC10-B3 and PC10-E5, when compared to PC10.

When the mass population from which PC10-B3 had been derived was found to be resistant to continuous 0.5 μg/mL cisplatin exposure, the p50-5.3 was reduced markedly. The p50-5.3 was also reduced markedly upon the development of another cisplatin-resistant subline PC10-E5. Therefore, the acquisition of cisplatin resistance in PC10-B3 and PC10-E5 was apparently coincident with the marked reduction of the p50-5.3.

A spot M_r 50 kDa, pI4.9 was increased in PC10-B3 and PC10-E5, and a spot M_r 58 kDa, pI5.8 newly appeared only in PC10-E5. Other spots did not reproducibly change, or alterations, if any, were negligible. We fractionated proteins of PC10, PC10-B3 and PC10-E5 to locate the altered proteins. The p50-5.3 in PC10 was localized in the nuclear fraction. The M_r 50 kDa, pI4.9 in PC10-B3 and PC10-E5 was also localized only in the nuclear fraction, whereas the M_r 58 kDa, pI5.8 resided in both the microsomal and cytosol fractions (Table 1). The most dramatically changed spot p50-5.3 was analyzed thereafter to identify the p50-5.3 by microsequencing.

Microsequencing

Initially, we used a protein sequenator (model 470A) to determine the NH_2-terminal amino acid sequence of the p50-5.3 in PC10. No single amino acid peak, however, was detected, probably owing to an NH_2-terminal block in the p50-5.3. We therefore used the procedure of *in situ* digestion as described in Materials and Methods to analyze the internal amino acid sequences. We consequently obtained many cryptic peptide peaks from the peptide mapping of the p50-5.3 in PC10 on reversed-phase HPLC (Fig. 2). Among them, as shown in Fig. 2, the four most prominent peaks were selected and subjected to amino acid sequence analysis. The highest peak, 2, had two different peptide fragments (Table 2). According to the homology search on the protein sequence database, the two peptide fragments were 100% homologous to 50 kDa, type I keratin (K14) and 60 out of 63 residues were identical to K14 [31]. Since K14 also has the same M_r and pI

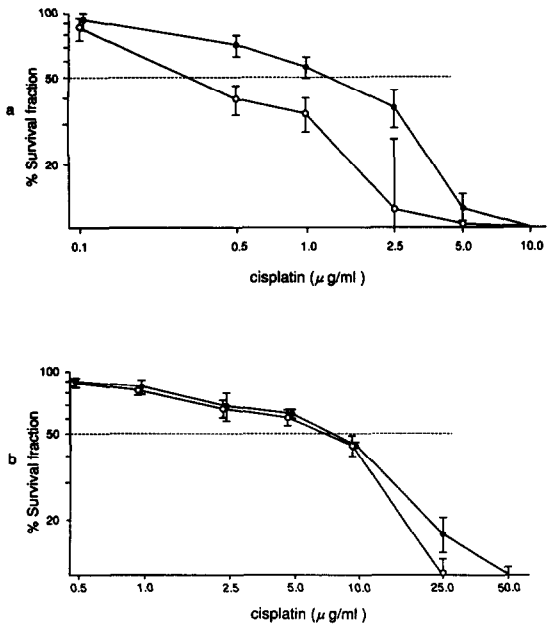

Fig. 4. Concentration–response curves of PC10 (a) and PC10-B3 (b) to cisplatin, previously treated with 10 μM RA for 96 hr (●) and treated in the same way with 0.1% DMSO (○). Points and bars represent means ± SD of three experiments performed in duplicate.

[32] as that of the p50-5.3, the p50-5.3 may be identical to 50 kDa, type I keratin (K14).

Retinoic acid-mediated reduction of K14 accompanied by cisplatin resistance in PC10

It has been reported that RA reduces K14 [33]. We have also been able to demonstrate that 10 μM RA reduces K14 in PC10 after 96 hr continuous incubation, although not to the level of its resistant sublines (Fig. 3). The RA-mediated reduction of K14 was reversible in PC10, because K14 began to reappear 48 hr after the removal of RA (data not shown). We performed an MTT assay on PC10 previously exposed to 10 μM RA/0.1% DMSO for 96 hr. Simultaneously, we prepared PC10 treated in the same way with 0.1% DMSO as a control. The RA-mediated reduction of K14 was consequently accompanied by a 4.0-fold increase in cisplatin resistance in PC10, whereas in the control sensitivity to cisplatin did not change at all (Fig. 4a). In contrast, RA lacked the effect on cisplatin cytotoxicity in PC10-B3 in which the K14 level was already very low (Fig. 4b). This result implies that K14 plays a role in cisplatin sensitivity in PC10 and that K14 reduction is associated with cisplatin resistance in PC10-B3 and PC10-E5.

DISCUSSION

The present study revealed that 50 kDa, type I keratin (K14) was reduced markedly in the cisplatin-resistant human lung squamous-carcinoma cell lines PC10-B3 and PC10-E5. Furthermore, the marked K14 reduction coincided with the acquisition of cisplatin resistance, and RA-mediated K14 reduction was concomitant with a 4.0-fold increase in cisplatin resistance in PC10. They imply the association of K14 reduction with cisplatin resistance in PC10-B3 and PC10-E5.

Keratin is a versatile operator. Type I keratin and type II keratin combine with each other through hydrophobic interaction to result in 8–10 nm intermediate filaments in cytoplasm, thereby influencing the state of differentiation, malignancy, and cytoskeletal structure. Recent studies have shown that the amount of keratin changes with cell differentiation in cyclophosphamide-resistant cell lines established *in vivo* [34] or clinically developed cisplatin-resistant small cell lung cancer [35]. K14 is one of the decisive markers of proliferating basal calls of epidermis and other stratified squamous epithelia [36]. As these cells differentiate, they generally down-regulate K14 at the transcriptional level. Thus, differentiation concomitant with K14 reduction could play an important role in cisplatin resistance in PC10-B3 and PC10-E5, although the influence of differentiation on cisplatin resistance has not been studied in detail, thus far. Alternatively, transcription factors which regulate K14 expression could modulate cisplatin resistance in PC10-B3 and PC10-E5 [37].

Cisplatin is supposed to form DNA cross-links which cause cell death [38, 39]. Cisplatin induces three major cross-links: intrastrand cross-links, interstrand cross-links and protein-DNA cross-links though their ranks of toxicity are controversial [40–43]. Protein-DNA cross-links have not been as well analyzed as other cross-links. Interestingly, Hnilica and his colleagues [44, 45] identified some types of keratins as one of the major components of cisplatin-mediated protein-DNA cross-links and showed that the number of keratin-DNA cross-links correlated with cisplatin cytotoxicity. Our study has shown that in PC10 K14 resides mostly in the nuclear fraction. Thus, K14 might be the major component of protein-DNA cross-links and contribute to cisplatin cytotoxicity in PC10. If so, PC10-B3 and PC10-E5 may be resistant to cisplatin as a result of the marked reduction of K14-DNA cross-links as one of the main protein-DNA cross-links. To test this hypothesis, we are undertaking studies to confirm the existence of K14-DNA cross-links in PC10, and to analyse the DNA repair capacity in PC10 and its resistant sublines.

Vernetti *et al.* [46] have demonstrated to date that a cyclosporin A-mediated alteration of keratin filament and a decrease of certain keratin proteins are lethal events. Their data make it possible to suppose that continuous K14 reduction exempts PC10-B3 and PC10-E5 from cisplatin-induced cell death, even if K14 were not to bind directly to cisplatin. Recently, Howell and his colleagues* have

* Jekunen AP, Jones JA, Christen RD, Shalinsky DR, Thiebaut FB and Howell SB, Molecular pharmacologic alteration of tubulin by cAMP and taxol influences cisplatin accumulation in human ovarian carcinoma cells. Molecular Oncology as a Basis for New Strategies in Cancer Therapy: The Second Joint Meeting of the American Association for Cancer Research and the Japanese Cancer Association. Abst. B-41, 1992.

shown that membrane-located β-tubulin is reduced concomitantly with the acquisition of cisplatin resistance in an ovarian carcinoma cell line. K14 reduction in our cisplatin-resistant cell lines is intriguing because both keratin and β-tubulin are associated with the cytoskeleton, although keratin and β-tubulin belong, respectively, to intermediate filaments and microtubules.

Although the present study has shown the association of K14 reduction with cisplatin resistance, the direct influence of K14 on cisplatin resistance is not yet determined. Thus, we are preparing to transfect K14 expression plasmid into PC10-derived cisplatin-resistant sublines.

We have demonstrated previously that reduced intracellular platinum accumulation may be one of the important contributors to cisplatin resistance in PC10-B3 [24]. Thus, we must also examine whether the marked K14 reduction may be independent of, or responsible for, the accumulation defect in PC10-B3. In any case, K14 can be a novel candidate to modulate cisplatin sensitivity, or a useful marker to reveal further the mechanisms of cisplatin resistance in PC10-B3 and PC10-E5. In addition, we need to explore the newly appeared spot M_r 58 kDa, pI5.8, since it could render PC10-E5 even more resistant to cisplatin than PC10-B3.

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