Element Array by Scanning X-ray Fluorescence Microscopy after Cis-Diaminedichloro-Platinum(II) Treatment

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Abstract

Minerals are important for cellular functions, such as transcription and enzyme activity, and are also involved in the metabolism of anticancer chemotherapeutic compounds. Profiling of intracellular elements in individual cells could help in understanding the mechanism of drug resistance in tumors and possibly provide a new strategy of anticancer chemotherapy. Using a recently developed technique of scanning X-ray fluorescence microscopy (SXFM), we analyzed intracellular elements after treatment with cis-diaminedichloro-platinum(II) (CDDP), a platinum-based anticancer agent. The images obtained by SXFM (element array) revealed that the average Pt content of CDDP-resistant cells was 2.6 times less than that of sensitive cells, and the zinc content was inversely correlated with the intracellular Pt content. Data suggested that Zn-related detoxification is responsible for resistance to CDDP. Of Zn-related excretion factors, glutathione was highly correlated with the amount of Zn. The combined treatment of CDDP and a Zn(II) chelator resulted in the incorporation of thrice more Pt with the concomitant down-regulation of glutathione. We propose that the generation of an element array by SXFM opens up new avenues in cancer biology and treatment. (Cancer Res 2005; 65(12): 4998-5002)

Introduction

Cis-Diaminedichloro-platinum(II) (CDDP) is an effective anticancer agent, but tumor cells can become resistant after CDDP-based therapy (1). Detoxification of CDDP, an increase in DNA repair, and excretion of CDDP have been implicated as major factors contributing to CDDP resistance (1). Incorporated CDDP is excreted by several molecules, such as overexpressed P-glycoprotein (2), a zinc-related defense system that is regulated by increased intracellular glutathione (GSH; ref. 3), and the ATP-dependent glutathione S-conjugate export pump (GS-X pump), which plays a role in the vesicle-mediated excretion of GSH-CDDP conjugates from resistant cells (4). Recent reports suggest that minerals such as zinc (Zn) and copper (Cu), important for normal cellular functions (5), are involved in CDDP resistance (6, 7). The simultaneous monitoring of multiple numbers of cellular elements would be helpful in identifying the mechanism of drug resistance in a malignant cell. The recently developed technique of scanning X-ray fluorescence microscopy (SXFM; refs. 8, 9) has made it possible to detect elements of interest by a single measurement and give a profile of these elements at the single-cell level (termed an element array). To examine the efficacy of element array analysis, we analyzed elements before and after treatment with CDDP and compared the element profiles of CDDP-sensitive and CDDP-resistant cells. We showed that the Zn content has an inverse correlation with Pt incorporation owing to a positive linkage with glutathione (GSH), a Zn-dependent detoxification factor. The combined treatment with CDDP and N,N,N,N-tetrakis-(2-pyridylmethyl)- ethylenediamine (TPEN), a Zn (II)-chelator (10), increased Pt uptake with a concomitant reduction of intracellular GSH. We propose that the element array is a versatile method suitable for obtaining information about metals involved in drug metabolism and could contribute to a novel strategy for anticancer chemotherapy.

Materials and Methods

Element array analysis by scanning X-ray fluorescence microscopy. SXFM was set up at an undulator beamline, BL29XU, of the SPring-8 synchrotron radiation facility (11) by combining a Kirkpatrick-Baez-type X-ray focusing system (12, 13), an XY-scanning stage for sample mounting, and an energy-dispersive X-ray detector (SDD, Röntec, Co., Ltd.). Monochromatic X-rays at 15 keV for Pt L-line excitation were focused into a 1.5 μm (f) × 0.75 μm (W) spot with a measured flux of 1 × 1011 photons/s. The focused X-rays simultaneously yielded the fluorescence of various chemical species in a small volume of sample cells, as shown in Fig. 1A. The fluorescence from each element was taken independently and did not overlap except for the PtLx signal, which was contaminated by ZnKβ (Fig. 1A). This was corrected by subtraction, as described previously (8). In this study, we could also measure Pt Lβ as a unique signal of Pt (Fig. 1A). After counts were collected for 4.0 to 8.5 seconds at each pixel of scanning, the detected counts were normalized by incident beam intensity. In addition to the mapping images, an elemental concentration per single cell was calculated from the integrated elemental intensity over the whole mapping image.10

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10 A. Saito et al, manuscript in preparation.
Chemicals and biochemical assays. TPEN (Sigma, St. Louis, MO; ref. 10), GSH (Calbiochem, La Jolla, CA), and CDDP (Daiichi Kagaku, Tokyo, Japan) were purchased. A GSH colorimetric assay kit (Calbiochem) and a BCA protein assay kit (Bio-Rad, Hercules, CA) were used for measuring intracellular GSH. About $3 \times 10^5$ to $4 \times 10^5$ cells were subjected to GSH measurement, and the data were normalized by cell number.

Cell lines. PC-9 cells (PC/SEN) and PC-9 cells resistant to CDDP (PC/RES), originally derived from a lung carcinoma cell line (14), were

Figure 1. Element array by SXFM. A, scheme of imaging cellular elements by SXFM. Coherent X-rays are focused on each area (pixel), and the X-ray fluorescence from each element is detected. Each pixel gives an elemental spectrum, as shown in the right panel, and an integrated intensity of the individual element was mapped to the corresponding area of analyzed cells. B, SXFM analysis after CDDP treatment. Cell morphologies obtained by Nomarski are shown at $\times 100$ magnification (left). Each field of view is equivalent to an area of $70 \times 70 \mu m$. Representative results are shown. Brighter colors indicate a higher signal intensity of each element. Results are shown for PC/SEN (top) and PC/RES cells (bottom). Note the high intensity of Pt L$_2$ in PC/SEN cells after CDDP treatment (second panel of the Pt column) and the higher signal intensity of Zn in PC/RES cells compared with that of PC/SEN cells. C, element array based on SXFM analysis. The mean signal intensity of each element obtained by SXFM analysis was calculated, and the fold increase of elements in PC/RES cells (red) was depicted by using the intensity in PC/SEN cells (blue) as a standard (left). A part of analyzed elements is shown. The fold increase of elements in PC/SEN (blue) and PC/RES cells (red) after CDDP treatment was also shown by using the intensity in PC/SEN before CDDP treatment as a standard (right).
maintained in DMEM (Nissui, Co., Tokyo, Japan) supplemented with 10% FCS (Sigma). The viability of PC/SEN cultured for 72 hours in the presence of 1 μmol/L CDDP was 40%, whereas that of PC/RES was ~90%. In this study, each cell line when treated with 1 μmol/L CDDP for 24 hours showed >85% viability.

Colony formation. After treatment, aliquots of PC/SEN and PC/RES were plated into culture dishes or soft agar, and the numbers of cell aggregates consisting of >50 cells were counted. Each number was normalized by plating efficiency, and the mean and SD of the number of formed colonies were calculated.

Sample preparation. Cells were plated on a silicon nitride base (NTT Advanced Technology, Tokyo, Japan) 1 day before the experiment. After incubation for 24 hours in the presence of 1 μmol/L CDDP, the cells were washed with PBS, fixed in 2% paraformaldehyde in PBS for 10 minutes at room temperature, and incubated in cold 70% ethanol for 30 minutes. The cells were then placed in a 1:3 solution of glacial acetic acid and methanol for 10 minutes, washed with 70% alcohol, and dried overnight at room temperature.

Measurement of cellular platinum and zinc. To measure Pt and Zn, ~5 × 10⁶ cells were subjected to inductively coupled plasma mass spectroscopy (ICP-MS; Toray Research Center, Shiga, Japan; ref. 15).

Statistical analysis. The Pearson product-moment correlation coefficient and Student’s t test were used to evaluate statistical significance (16).

Results and Discussion
Incorporation of platinum and element array after cis-diaminodichloro-platinum(II) treatment. We analyzed intra-cellular elements by SXFM after CDDP treatment (Fig. 1A). At 12 hours after treatment with 1 μmol/L CDDP, the level of Pt was increased in PC/SEN cells, whereas little increase in the Pt level was seen in PC/RES cells (Fig. 1B). The intensity of Pt in PC/RES cells was 2.6-fold less than that in PC/SEN cells, as confirmed by the results of ICP-MS, which indicated that the amount of Pt in PC/RES cells (5.5 fg/cell) was 3.6-fold less than that in PC/SEN cells (19.7 fg/cell). Therefore, the decreased accumulation of CDDP is likely to be responsible for resistance in PC/RES cells.

Based on the mean signal intensity obtained by SXFM, element array analysis was carried out (Fig. 1C). The element profile
The intracellular GSH levels in PC/SEN (black) and PC/RES cells (gray) were measured. GSH was significantly higher in PC/RES than in PC/SEN cells (t test, P < 0.05). The difference in the Zn contents of these cells was confirmed by ICP-MS (105 fg/cell for PC/SEN cells and 189 fg/cell for PC/RES cells, respectively). When 1 μmol/L CDDP was used for treatment, constitutive high Zn was observed in PC/RES cells (Fig. 1C, right). In PC/SEN cells, the amounts of all the elements were slightly increased, but the amount of Zn was increased most markedly.

We then analyzed the chronological changes in the levels of elements in PC/SEN cells following CDDP treatment. Representative results for S, Fe, Zn, Cu, and Pt are shown in Fig. 2A. Pt was clearly observed at 24 hours after treatment with 1 or 2 μmol/L CDDP (Fig. 2A). It was, however, barely detectable at 48 hours after the cells were treated with 1 μmol/L CDDP (Fig. 2A, top), suggesting that the cells excreted CDDP. In contrast, the cellular content of Pt gradually increased after treatment with 2 μmol/L CDDP (Fig. 2A, bottom), and apoptotic cells with high levels of incorporated CDDP were observed after 48 hours (Fig. 2A, bottom).

The element profile was plotted against the time after treatment with CDDP (Fig. 2B). When the cells were treated with 1 μmol/L CDDP, the Zn content increased remarkably and reached a peak at 24 hours (Fig. 2B, top, red line). In these cells, the Pt content was reduced after 48 hours. When the cells were treated with 2 μmol/L CDDP, the Zn content decreased within 24 hours (Fig. 2B, bottom), and the Pt content increased within 48 hours. In this analysis, Cu did not show significant changes. The results imply that the intracellular Zn content has an inverse correlation with the incorporated Pt content.

Cellular zinc and zinc-related detoxification. We studied Zn-related detoxification factors, such as metallothioneins (17), GSH (18), and the GSH-coupled excretory pump GS-X (4), and we observed that intracellular GSH was high in PC/RES cells (Fig. 3A). We then examined the possible correlation between the intracellular Zn content and GSH. As shown in Fig. 3B, the GSH levels showed a significant correlation with the levels of Zn detected by both ICP-MS and SXFM (Pearson product-moment correlation coefficient r = 0.794, P < 0.05 and r = 0.533, P < 0.05, respectively). The levels of Zn detected by SXFM may have less correlation with GSH than do the levels detected by ICP-MS because SXFM analyzed Zn in a small number of cells, whereas the analyses of GSH using ICP-MS were carried out on >10^5 cells.

Effects of zinc depletion and cis-diaminedichloroplatinum(II) uptake. To examine ways of increasing the sensitivity of PC/RES cells to CDDP, we used the Zn(II) chelator TPEN, as it was thought that CDDP uptake would increase when the GSH level was down-regulated by decreased Zn. Consistent with this hypothesis, treatment with 7.5 μmol/L of TPEN decreased cellular Zn to ~40 fg/cell at 30 hours after treatment in PC/SEN cells (Fig. 4A, left, solid line). The decrease seen in PC/RES cells owing to TPEN treatment was more rapid, with the Zn concentration being reduced to ~40 fg/cell within 7 hours (Fig. 4A, left, dashed line). The intracellular GSH also decreased with the reduction in intracellular Zn (Fig. 4A, right, dashed line).

To determine the effects of TPEN on the growth of PC/RES cells, the cells were pulse-treated for 2 hours with TPEN for 5 consecutive days and the growth was examined. Although treatment with 1 μmol/L CDDP did not induce apparent morphologic changes (Fig. 4B, bottom, left), the combined treatment with TPEN and CDDP caused prominent changes (Fig. 4B, bottom, right). A colony formation assay clearly showed that the combination of CDDP and TPEN, as well as single TPEN treatment, significantly impaired the growth of PC/RES cells (Fig. 4C). Consistent with these changes, ICP-MS indicated that the intracellular Pt content increased 3.5-fold after the combined treatment (from 0.38 to 1.35 fg/cell with TPEN treatment). It is important to note that the same dose of TPEN did not attenuate the growth of PC/SEN cells (Fig. 4C). These data indicate that the GSH level seems to be critical for resistance in PC/RES cells, consistent with previous reports that CDDP-resistant cells have high levels of GSH and that a decrease in GSH results in loss of resistance (3, 19). Our data also suggest that the high GSH content was maintained by the effects of Zn in PC/RES cells. Overall, our trial treatment with combined TPEN and CDDP suggests that this combination would be effective in eliminating tumors even if they include a CDDP-resistant population of cells with high Zn content.

We showed the use of element array analysis by SXFM to examine a mechanism of CDDP resistance. Based on element profiles, we successfully overcame CDDP resistance in PC/RES cells by using a Zn chelator that down-regulated the GSH level. Although it has been reported that Cu is a necessary factor for CDDP incorporation (7), the present work revealed that Cu was not involved in PC/RES cells. It is tempting to speculate that drug resistance is generated by various elements, and we propose that an element array can contribute to better understanding of cancer biology as well as other fields of medical science.
Acknowledgments

Received 2/3/2005; accepted 4/20/2005.

Grant support: Grant-in-aid for scientific research from the Ministry of Health, Labor, and Welfare of Japan and grant-in-aid for Center of Excellence Research (grant 08CE2004) from the Ministry of Education, Sports, Culture, Science, and Technology of Japan. The usage of BL29XU of the SPring-8 was supported by Riken.

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We thank Harumi Shibata and Yasunori Suzuki for technical assistance.

References

4. Ikeda K, Kondo A, Safaei R, et al. Impairment of cultured cell proliferation and colony formation after pulse treatment with CDDP with or without TPEN. After pulse treatment for 5 consecutive days, as described in (B), the cells were plated in soft agar and the colony formation assay was done. The means and SDs of colony numbers of PC/SEN (black columns) and PC/RES (gray columns) are shown. The experiments were carried out in triplicate.

Figure 4. Cellular Zn content and Pt uptake with TPEN, A, TPEN-induced depletion of cellular Zn and down-regulation of GSH. TPEN (7.5 μmol/L) was added to the culture medium for the indicated time periods, and cellular Zn was measured by ICP-MS (left). Intracellular GSH content was also monitored (right). The Zn contents in PC/SEN (solid lines) and PC/RES cells (dashed lines) are shown. B, morphologic changes after pulse treatment with TPEN and CDDP. The morphologies of untreated PC/RES cells (top, left) and of cells treated with TPEN (top, right), CDDP (bottom, left), and CDDP plus TPEN (bottom, right) are shown. The cells were exposed to 1.0 μmol/L CDDP with or without 7.5 μmol/L TPEN for 2 hours, and then the medium was replaced with fresh medium. Pulse treatment was carried out for 5 consecutive days. Magnification, ×200. Note that large cells are observed after treatment with TPEN alone, and larger cells with irregular shape are observed following the combination treatment. The data showed that TPEN caused cellular accumulation at G2-M phase with mitotic failure (data not shown), C. colony formation after pulse treatment with CDDP with or without TPEN. After pulse treatment for 5 consecutive days, as described in (B), the cells were plated in soft agar and the colony formation assay was done. The means and SDs of colony numbers of PC/SEN (black columns) and PC/RES (gray columns) are shown. The experiments were carried out in triplicate.

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