Enhancement of Cisplatin Sensitivity in High Mobility Group 2 cDNA-transfected Human Lung Cancer Cells

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To elucidate the role of high mobility group 2 protein (HMG2) in cis-diamminedichloroplatinum (II) (cisplatin, CDDP) sensitivity, we constructed a human HMG2-transfected human non-small cell lung cancer cell line, PC-14/HMG2. The HMG2 mRNA expression level was approximately twice those of parental PC-14 and mock-transfected PC-14/CMV. Gel mobility shift assay revealed a CDDP-treated DNA-protein complex in the nuclear extract of PC-14/HMG2, which was not found in the extracts of PC-14 and PC-14/CMV. This complex formation was subject to competition by CDDP-treated non-specific salmon sperm DNA, indicating that ectopic HMG2 recognizes CDDP-damaged DNA. PC-14/HMG2 showed more than 3-fold higher sensitivity to CDDP than PC-14 and PC-14/CMV. The intracellular platinum content of PC-14/HMG2 after exposure to 300 µM CDDP was 1.1 and 1.5 times that of PC-14 and PC-14/CMV, respectively. Cellular glutathione levels were not different in these cell lines. Repair of DNA interstrand cross-links determined by alkaline elution assay was decreased in PC-14/HMG2. These results suggest that HMG2 may enhance the CDDP sensitivity of cells by inhibiting repair of the DNA lesion induced by CDDP.

Key words: HMG2 — Cisplatin — Interstrand cross-link-DNA repair

High mobility group (HMG) proteins are a family of non-histone proteins found in nuclei of higher eukaryotes,1 among which HMG1 and HMG2 are the most abundant. There is more than 85% identity of HMG1 and HMG2 proteins in the amino acid sequences of the “HMG box” regions, which are thought to be involved in binding to DNA. Studies in yeast have shown that HMG-domain proteins bind to DNA repair sites.2,3 Most of these proteins have high homology in the HMG domain, but have different functions.4 There are reports indicating that HMG1 and HMG2 play roles in replication and transcription.5–8 A recent study showed that antisense HMG2 repressed cell cycle progression and, consequently, cell growth.9 Pil and Lippard found another property of HMG1: its specific binding to DNA damaged by the anti-neoplastic drug cis-diaminedichloroplatinum (II) (cisplatin, CDDP).10 Another study showed that both HMG1 and HMG2 bind with high affinity to CDDP-damaged DNA (CDDP-DNA).11

There are only a few reports, however, which describe the role of these proteins in CDDP sensitivity. Huang et al. showed that HMG1 protein inhibited nucleotide excision repair of the CDDP-DNA adduct in vitro.12 Proteins reacting with anti-HMG1 and HMG2 monoclonal antibody were detected in nuclear extracts of several human lung cancer cell lines, and their expression was increased after exposure to CDDP.13 The amounts of these proteins were spontaneously increased in CDDP-resistant sublines. Therefore, the precise role of HMG proteins in CDDP sensitivity remains to be established. We have constructed a human HMG2-transfected human non-small cell lung cancer cell line in order to reveal the involvement of this gene in CDDP sensitivity. The transfected cells showed higher sensitivity to CDDP. The possible mechanisms of the increased sensitivity to CDDP in the HMG2-transfectants are discussed.

MATERIALS AND METHODS

Cells and culture A human non-small cell lung cancer cell line, PC-14, was a gift from Dr. Y. Hayata, Tokyo Medical College. The cells were cultured in RPMI 1640 medium containing 100 µg/ml streptomycin and 100 U/ml penicillin G (Nikken Biomedical Laboratory, Kyoto) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (RPMI-FBS) at 37°C in humidified air with 5% CO₂.

Transfection with HMG2 gene The human HMG2 sequence was taken from articles by Majumdar et al.14

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and Shirakawa and Yoshida. The polymerase chain reaction (PCR) was performed to obtain 630-bp HMG2 cDNA with oligonucleotide primers and a human thymus 5'-stretch cDNA library (Clontech Lab., Palo Alto, CA) as a template. Sequences of the primers were as follows: 5'-GGCAAGCTTCCAACATGGGTTAAAAGGAGAACCCTAA
3' and 5'-TTAAAGCTTCTAGATTTATCTCTCATCTTCACT
ATCCTCTTCTC-3'. We used rather long primers because of the sequence similarity between HMG1 and HMG2 genes, and an overlap and repeat of about 40 tandem nucleotide sequences in 70 3'-end bases in the HMG2 gene. The product was subcloned and further amplified with a TA Cloning Kit (Invitrogen Corp., San Diego, CA). The amplified DNA was cut with HindIII and ligated into the expression vector pRc/CMV (Invitrogen Corp., San Diego, CA). The amplified DNA was cut with HindIII and ligated into the expression vector pRc/CMV (Invitrogen Corp., San Diego, CA). The amplified DNA was cut with HindIII and ligated into the expression vector pRc/CMV (Invitrogen Corp., San Diego, CA).

We designated the cell MO) to RPMI-FBS and transfected clones were selected by the addition of 1 mg/ml G418 (Sigma Chemical Co., St. Louis, MO) to RPMI-FBS and transfected clones were selected by the limiting dilution method. We designated the cell lines transfected with the vector containing HMG2 and with the vector only as PC-14/HMG2 and PC-14/CMV, respectively. The cells were cultured in G418-free RPMI-FBS for at least 24 h before use.

Northern blot analysis Total RNA of each cell line was extracted by the guanidinium thiocyanate-phenol-chloroform extraction method using Isogen (Nippon Gene, Tokyo). Northern blot analysis was performed as previously reported with the full-length cDNA of the HMG2 gene as a probe because of sequence similarity between HMG1 and HMG2. The probe was radiolabeled with [α-32P]deoxyxycytidine triphosphate (dCTP) using the Rediprime DNA labeling system (Amersham International plc, Buckinghamshire, UK). The full-length cDNA of the human HMG1 gene was also used as a probe. A 400-bp human β-actin probe (Wako Pure Chemical Industries, Osaka) was used as a control. The bands were quantified densitometrically using ImageMaster DTS (Pharmacia Biotech, Tokyo). The intensities of the HMG2 and HMG1 bands were corrected on the basis of those of the corresponding β-actin bands for quantification.

Gel mobility shift assay We performed a gel mobility shift assay to detect proteins in nuclear extracts that react with CDDP-damaged DNA as previously reported. Nuclear extracts of the cells were obtained by a small-scale procedure as previously described. The protein content of the nuclear extracts was measured by the Lowry method with some modifications. The extracts were stored at ~80°C until just before use. A HindIII- and EcoRV-cut product of pBR322, 158-bp fragment, was used as a probe. This fragment was gel-purified and 3'-end labeled with Klenow fragment and [α-32P]dCTP. Unincorporated radiolabeled nucleotide was removed with CHROMA SPIN-10 columns (Clontech Lab.). The nucleotide (50 nM) was allowed to react with 16.7 μM CDDP in 3 mM NaCl and 1 mM sodium phosphate (pH 7.4) at 37°C for >12 h. Unbound CDDP was removed by ethanol precipitation. One hundred femtomols of the platinated fragment was mixed with 10 μg of nuclear extract and 2 μg of poly[dI-dC]-poly[dI-dC] (Sigma Chemical Co.) in binding buffer (25 mM Hepes-KOH pH 7.9, 0.5 mM ethylendiaminetetraacetic acid (EDTA), 50 mM KCl, 10% (v/v) glycerol, 0.5 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride). The mixture was incubated at room temperature for 30 min with or without CDDP-treated salmon sperm DNA (Stratagene, La Jolla, CA) as a competitor. The reaction mixture was electrophoresed at 30 mA in a 4% (w/v) polyacrylamide gel in TGE buffer (50 mM Tris, 380 mM glycine and 2 mM EDTA) at room temperature following a 30-min pre-run. The gel was dried and autoradiographed at ~80°C.

Growth-inhibitory effect CDDP and carboplatin were purchased from Bristol-Myers Squibb K.K. (Tokyo). Growth-inhibitory effects of these drugs were determined by the modified tetrazolium dye assay originally described by Mosmann. Each experiment was performed in 6 replicate wells. Percent cell survival was calculated as follows: [(mean absorbance of wells containing the drug) – (mean absorbance of cell-free control wells)] × 100/ [(mean absorbance of drug-free wells) – (mean absorbance of cell-free control wells)]. IC50 values were expressed as the concentration of the drug that inhibits the cell growth by 50%. The IC50 ratio was calculated as (IC50 for the parental cells)/IC50 for the transfectants).

Cellular CDDP accumulation Cellular platinum contents were measured by atomic absorption spectrophotometry. Cells (3 × 105/ml) were seeded into 150-mm culture dishes and incubated for 24 h. The cells were then exposed to 300 μM CDDP for 3 h. The drug was discarded, and the cells were washed 3 times with ice-cold phosphate-buffered saline (PBS), then collected by centrifugation. The cell pellets were dried and digested with 60% (v/v) nitric acid at 80°C for 24 h. The platinum content of the extracts was determined with an atomic absorption spectrophotometer, Spectra AA-40 (Varian Instruments, Palo Alto, CA), by Sumitomo Metal Bio-Science (Tokyo).

Intracellular glutathione (GSH) content Total cellular GSH was measured using the enzyme recycling assay with some modifications. Briefly, 1×106 cells were suspended in 300 μl of phosphate-EDTA solution (125 mM KH2PO4, 6.3 mM EDTA, pH 7.5) and homogenized, then
100 µl of 12% (w/v) 5-sulfosalicylic acid was added, and protein-free lysates were obtained by centrifugation. The enzyme reaction was carried out at 25°C using a reaction mixture comprising 125 mM KH₂PO₄, 6.3 mM EDTA, 0.21 mM NADPH, 0.6 mM 5,5'-dithio-bis(2-nitrobenzoic acid) and 0.5 U/ml glutathione reductase. The optical density (OD) at 412 nm was monitored for 3 min and the GSH content was calculated on the basis of the standard curve. The cellular protein concentration was determined as described.

Alkaline elution assay  To determine CDDP-induced interstrand cross-link (ICL) formation and its repair, we performed an alkaline elution assay with some modifications. Exponentially growing cells (2×10⁷/ml) were radiolabeled with 0.2 µCi/ml [methyl-¹⁴C]thymidine for 12 h, then washed with ice-cold PBS, incubated for 3 h with 5 µg/ml CDDP, washed with ice-cold PBS, and incubated in RPMI-FBS for various periods to induce repair. The cells were then γ-irradiated for 10 min at a rate of 0.5 Gy/min. Before and after the irradiation, cells were kept on ice to prevent repair. The cells were diluted with ice-cold PBS, deposited onto a polycarbonate filter (Nucleopore, Costar, Cambridge, MA), and lysed for 1 h with lysis solution (2% (w/v) SDS, 25 mM Tris, 50 mM glycine and 0.5 mg/ml proteinase K, pH 10), which was then allowed to flow through the filter by gravity. Following this step, the filter was rinsed 3 times with 20 mM disodium EDTA (pH 10) to remove cell protein and RNA. The remaining DNA was eluted with eluting buffer (2% (w/v) tetrapropylammonium hydroxide, 20 mM tetrahydroxy EDTA, pH 12.1) for 15 h with a constant flow. The filter was removed from the filtration funnel and incubated in 500 µl of 1 N HCl for 1 h at 70°C. It was then replaced on the filtration funnel and the tube were washed with 20 ml of 0.4 N NaOH. Each sample was mixed with 10 vol. of liquid scintillator Clear-sol I (Nacalai-Tesque, Kyoto) and radioactivity was counted with an LS 6000TA liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA). The relative retention value for each sample indicated the relative amount of [¹⁴C]DNA retained on the filter and was calculated as (radioactivity detected on the filter)/(total radioactivity). The cross-link index was defined as: [(1−Rcontrol)/(1−R CDDP)]¹/²−1, where Rcontrol was the relative retention of control cells and R CDDP was that of CDDP-treated cells.

Statistical analyses  The IC₅₀ values, intracellular platinum content and intracellular GSH of the various cell lines were compared by one-way factorial ANOVA. Levels of ICL repair were compared by two-way repeated-measures ANOVA.

RESULTS  

Characteristics of the HMG2-cDNA transfectants (Table I)  Doubling times of PC-14, PC-14/HMG2 and PC-14/CMV were 25.6±0.9, 25.1±1.3 and 26.2±4.1 h, respectively, in 3 independent experiments. There was no significant difference of cell growth between these cells. Cell size and protein content did not differ among these cells (data not shown). In order to confirm the cDNA transfection, the expression of HMG2 mRNAs was compared in these cell lines by northern blot analysis and higher HMG2 mRNA expression was observed in PC-14/CVM.
HMG2 than in its parental PC-14 or mock-transfected PC-14/CMV (Fig. 1). Upon adjustment of the intensity of the bands with respect to those of β-actin, the HMG2 expression in PC-14/HMG2 was 2.0-fold that of PC-14, whereas that in PC-14/CMV was 1.1-fold, which was statistically significantly different (P<0.01, by Student’s t test in 3 independent experiments). On the other hand, the amounts of HMG1 mRNA in PC-14/HMG2 and PC-14/CMV were 0.9- and 1.5-fold that of PC-14. These results indicate the effective introduction of the HMG2 gene into PC-14/HMG2.

**Gel mobility shift assay of CDDP-DNA complex with nuclear extracts** We then performed a gel mobility shift assay to detect proteins bound to CDDP-treated DNA. Several bands were detectable in all 3 cell lines (Fig. 2). There was an additional band in the PC-14/HMG2 lanes, which was competed out by the addition of CDDP-treated salmon sperm DNA, indicating the existence of another protein or overproduction of a certain protein recognizing CDDP-treated DNA, probably HMG2 protein, in PC-14/HMG2. The proteins seen in common in all the cell lines might be HMG1, SSRP1 or other unknown proteins which can bind to the CDDP-DNA complex.20)

**Growth-inhibitory effect of CDDP for transfectants** We determined the growth inhibitory effect for CDDP to HMG2-transfectants by 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide (MTT) assay. As shown in Fig. 3, the IC₅₀ for PC-14/HMG2 was 1.2±0.3 μM (mean±SD). PC-14/HMG2 was more sensitive to CDDP than PC-14 or PC-14/CMV, for which the IC₅₀ values were 4.2±0.3 μM and 3.6±0.9 μM, respectively (summarized in Table I). The IC₅₀ for PC-14/HMG2 was significantly different from those for PC-14 or PC-14/CMV. The IC₅₀ ratio for PC-14/HMG2 was 3.50, while that for PC-14/CMV was 1.17. These phenomena were also observed for carboplatin, with IC₅₀ ratios of 2.25 and 0.88 for PC-14/HMG2 and PC-14/CMV, respectively. These results indicate that overexpression of the HMG2 gene leads to increased sensitivity to platinum compounds, particularly to CDDP.

**Intracellular platinum content** We measured the intracellular platinum content of the cells because it correlates with CDDP sensitivity.24) The intracellular platinum contents of PC-14, PC-14/HMG2 and PC-14/CMV after
exposure to 300 \( \mu M \) CDDP for 3 h were 497.7 \( \pm \) 40.1, 567.5 \( \pm \) 25.9 and 371.5 \( \pm \) 55.0 ng/mg cellular protein, respectively (Table I). The value for PC-14/HMG2 was 1.5 times higher than for PC-14/CMV, while the ratio of PC-14/HMG2 to PC-14 was 1.1. The level of intracellular platinum in PC-14/CMV was significantly lower than that in the other cell lines.

**Cellular GSH content** Cellular GSH content was measured because it is one of the factors determining CDDP sensitivity and it is increased in some CDDP-resistant cell lines.25, 26) The total cellular GSH levels were 125.0 \( \pm \) 17.4, 91.3 \( \pm \) 7.4 and 95.8 \( \pm \) 1.5 nmol/mg cellular protein for PC-14, PC-14/HMG2 and PC-14/CMV (Table I). There was no significant difference among the cell lines.

**Alkaline elution assay** To determine whether HMG2 inhibits repair of ICL, we performed an alkaline elution assay. In PC-14 and PC-14/CMV, the cross-link index was maximum after a repair time of 6 h as reported previously,22, 23) and the cross-link lesion was repaired thereafter (Fig. 4). On the other hand, PC-14/HMG2 showed a higher cross-link index even after a 12-h repair period, and had about a 5-fold higher cross-link index 24 h after the removal of CDDP. Differences among the cell lines analyzed by two-way repeated-measures ANOVA were significant \((P=0.0234)\), and the contrast method revealed that the differences between PC-14/HMG2 and PC-14 or PC-14/CMV were significant \((P=0.0099)\) or \((P=0.0031)\), whereas that between PC-14 and PC-14/CMV was not \((P=0.5472)\). These results indicate that HMG2 inhibits ICL repair.

**DISCUSSION**

Several proteins have been found to recognize CDDP-damaged DNA.17, 26–28) These include HMG1 and HMG2.29) However, the exact role of these proteins in CDDP sensitivity has not yet been elucidated.

HMG1 and HMG2 are highly conserved cellular proteins which have functions in cell growth. HMG2 may have essentially the same functions as HMG1 because of the similarity in amino acid sequence. HMG1 binds specifically to DNA that has been damaged by CDDP,10) and a central HMG box domain is critical for binding to CDDP-modified DNA.30) Another study indicated that HMG2 as well as HMG1 binds to CDDP-treated DNA in a cell-free system.11) SSRP1, a protein containing an HMG box domain with 47% amino acid homology to that of HMG1, also recognizes CDDP-damaged DNA.31) However, there remains a controversy as to whether binding of these proteins to CDDP-damaged DNA results in enhancement or reduction of CDDP sensitivity. Brown et al. reported that a yeast strain with an inactivated IXR1 gene, an SSRP1 homologue, was twice as resistant to CDDP and accumulated one-third as many platinum lesions after CDDP treatment as its parental strain.32) These results indicate that IXR1 inhibits repair of a
CDDP-DNA lesion and confers CDDP sensitivity. Furthermore, Huang et al. reported inhibition of human excision nuclease activity in vitro by HMG1.22)

In contrast, we previously demonstrated overproduction of proteins reacting with anti-HMG antibody in CDDP-resistant cells.23) We demonstrated in this study that the introduction of the HMG2 gene into human lung cancer cells increased CDDP sensitivity more than 3-fold (Fig. 3). A similar result was obtained with carboplatin (Table I), as expected, since carboplatin is known to form the same DNA adducts as CDDP. Sensitivity to other platinum compounds may also be enhanced. The determinants of CDDP sensitivity are intracellular platinum levels after CDDP exposure, intracellular detoxication mechanisms such as GSH, and DNA repair of CDDP lesions.25) The augmented sensitivity of PC-14/HMG2 may result from increased intracellular platinum content in the cell line because cellular platinum after CDDP exposure correlates well with CDDP sensitivity.24) However, it seems unlikely that the 1.1-fold difference in intracellular platinum accumulation is solely responsible for the 3.5-fold difference in CDDP sensitivity between PC-14/HMG2 and PC-14 (Table I). The cellular GSH content, which contributes to CDDP resistance in some CDDP-resistant cell lines, was not different in our cell lines (Table I). We conclude from these results that intracellular platinum content or GSH level is not the determinant of increased CDDP sensitivity in PC-14/HMG2. Gel mobility shift assay revealed an additional protein bound to CDDP-modified DNA in PC-14/HMG2 (Fig. 2). We thus consider that the ectopic HMG2 recognizes CDDP-damaged DNA. What roles, then, does HMG2 play in CDDP sensitivity? We hypothesize that HMG2 inhibits the pathway of repair of the DNA lesion and renders cells sensitive to CDDP, since DNA repair is one of the possible mechanisms of CDDP resistance.25, 26, 33)

HMG2 may recognize DNA interstrand lesions because HMG1 was reported to bind to CDDP-induced ICL with the same affinity as ICL. We demonstrated in this study that overexpression of HMG2 resulted in increased ICL repair (Fig. 4). Although ICL is a minor lesion formed by CDDP in a cell-free system,34) ICL is important for inducing cell death since there was more ICL formation in the cells sensitive to CDDP.35) Levy et al. established ras-transformed human epithelial cells which showed resistance limited to CDDP without any difference in intracellular platinum levels compared with their parental cells. These cells formed a smaller amount of ICL and exhibited increased nucleotide excision repair activity.36) Zhen et al. stated that there was decreased genomic ICL formation and a marked increase in gene-specific ICL repair in acquired CDDP-resistant cells, while no difference was observed in intrastand cross-link repair.37) Based on these reports, ICL and its repair are considered to be major factors determining CDDP sensitivity. We thus speculate, on the basis of our results, that the inhibition of ICL by HMG2 contributes to the increased sensitivity of PC-14/HMG2 to CDDP. Further study is required to determine the precise role of this protein in DNA repair of the CDDP lesion.

We examined the sensitivities of the cell lines to other anticancer agents. There was no significant difference of sensitivity to vindesine, paclitaxel, or etoposide between PC-14, PC-14/HMG2, and PC-14/CMV. On the other hand, PC-14/HMG2 cells showed higher sensitivity to mitomycin C, which is a DNA-interacting agent. If the DNA repair mechanisms are common with CDDP- and mitomycin C-damaged DNA, the higher sensitivity of PC-14/HMG2 to CDDP and mitomycin C seems consistent with the speculation that HMG2 acts on the DNA-repair system. Further studies should be performed to clarify the relationship between DNA-repair, HMG2 protein, and cellular sensitivity to DNA-attacking agents.

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