Activating Transcription Factor 4 Increases the Cisplatin Resistance of Human Cancer Cell Lines

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Abstract

Resistance to cisplatin is a major problem in the treatment of solid tumors. To investigate the determinants of cisplatin resistance, we have identified cisplatin-inducible genes by differential display of mRNA. One of the cisplatin-inducible genes was identified as activating transcription factor 4 (ATF4). Northern blot analysis demonstrated that expression of ATF4 is inducible at the transcriptional level. Its expression is also up-regulated in two cisplatin-resistant cell lines. We tested whether cellular levels of ATF4 are responsible for cisplatin sensitivity by examining 11 human lung cancer cell lines. Expression of ATF4 was found to correlate with cisplatin sensitivity (P = 0.01). We also evaluated the cisplatin sensitivity of two stable transfectants overexpressing ATF4. Both were less sensitive to cisplatin than the parental cells but equally sensitive to vincristine. Our findings suggest that levels of ATF4 expression could help to predict cisplatin sensitivity.

Introduction

Cisplatin is a potent antitumor agent that has been used successfully to treat various solid tumors (1, 2). However, development of cisplatin resistance is a major obstacle in clinical treatment (1). Resistance is thought to involve several mechanisms, including decreased drug accumulation (3), increased levels of cellular thiol (4), and increased DNA repair activity (5, 6). We have identified previously Y-box binding protein-1 (YB-1), a transcription factor that binds to DNA intranadr, as cisplatin cross-links (7). We showed that human cancer cells that overexpressed YB-1 were resistant to cisplatin and that transfection of such cells with a YB-1 antisense expression plasmid increased their drug sensitivity (8). Using differential display, we showed that cisplatin induced one proton pump subunit gene and that several pump subunit genes were up-regulated in cisplatin-resistant cell lines (9). Cellular pH is also one of the critical parameters affecting cisplatin sensitivity (10).

Cisplatin induces a complex response in cancer cells. Activation of the tumor suppressor gene products p53/p73 by DNA damage signaling can result in cell cycle arrest and apoptosis. Loss of p53 function confers resistance in some human cancer cell lines (11), whereas overexpression of p73 is associated with resistance to cisplatin (12). The relationship between cisplatin sensitivity and damage-induced expression of p53/p73 remains unclear. Several signaling pathways are thought to contribute to resistance to cisplatin. In addition to p53/p73, transcription factors activated in response to cisplatin might be involved in drug sensitivity (11). However, little is known about the expression or activation of such factors. Therefore, the identification of cellular determinants of cisplatin sensitivity could be important for clinical practice. In the current study, we have isolated cisplatin-inducible genes using differential display and report here that activating transcription factor 4 (ATF4) is one of these genes and that it is up-regulated in cisplatin-resistant cell lines. We show also that the expression of ATF4 correlates with cisplatin resistance.

Materials and Methods

Cell Culture. The cisplatin-resistant cell line KB/CP4 derived from human KB epidermoid cancer cells and the cisplatin-resistant cell line P/CDP6 derived from PC3 prostate cancer cells have been described previously (9). They were cultured in Eagle’s MEM (Nissui Seiyaku, Tokyo, Japan) containing 10% fetal bovine serum. KB/CP4 and P/CDP6 were found to be 63- and 23-fold, respectively, more resistant to cisplatin than their parental cells (3, 13). Eleven lung cancer cell lines were cultured as described (14).

Drugs and Antibodies. Cisplatin, vincristine, and etoposide were from Sigma (St. Louis, MO). An anticyclic adenosine monophosphate-responsive element binding-2 antibody to ATF4 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-high mobility group 1 (HMG1), anti-thioredoxin (TRX) -1, and anti-YB-1 antibodies were made as described previously (8, 15, 16).

Differential Display. Total RNA was isolated by the method described previously (17). For differential display, we used a differential display kit from Takara Shuzo (Kyoto, Japan). In brief, reverse transcription was carried out using 9 anchored primers, and 10-mer primers were used with the appropriate anchored primers. Untreated and cisplatin-treated KB cells were analyzed simultaneously. Gels were dried and autoradiographed for 1–2 days, and DNA fragments were eluted from the gels by boiling and were reamplified by PCR. cDNA fragments were then cloned into pGEM-Teasy (Promega, Madison, WI) and sequenced.

Northern Blot Analysis. Northern blot analysis was carried out on total RNA extracted from the indicated cells. The ATF4 cDNA fragment was labeled with random primers using the Megaprime DNA labeling kit (Amersham, Aylesbury, United Kingdom) and hybridized at 42°C in Ultra-hyb solution (Ambion, Austin, TX). Signal intensity was quantified using a bio-imaging analyzer (BAS 2000; Fujix, Tokyo, Japan).

Western Blot Analysis. Whole cell extracts were analyzed by 10% SDS-PAGE. Protein fractions were transferred onto polyvinylidene fluoride membranes, and the membranes were incubated with antibodies against ATF4 (1:1000), YB-1 (1:500), HMG1 (1:2500), and TRX1 (1:2000) for 1 h at 25°C and visualized by chemiluminescence with the enhanced chemiluminescence protocol (Amersham Biosciences, Piscataway, NJ).

Cytotoxicity Assays. Cells were seeded in 96-well tissue culture plates at 2 × 103 cells/well, and drugs were added the following day. After 72 h, surviving cells were assayed with TetraColar ONE (Seikagaku Corporation, Tokyo, Japan) for 2 h at 37°C according to the protocol provided, and absorbance was measured at 450 nm.

Statistical Analysis. Cellular levels of ATF4 were assessed numerically with the NIH image system. The Pearson correlation was used for statistical analysis, and significance was set at the 5% level.

Construction of an Expression Plasmid. A plasmid containing a full-length cDNA fragment of human ATF4 was generated by reverse transcrip-
tion-PCR using total RNA from KB cells, and the cDNA was cloned into pGEM-Teasy (Promega). To construct a mammalian expression plasmid, the EcoRI fragment of ATF4 cDNA was cloned into pcDNA3 (Invitrogen, Carlsbad, CA). The following oligonucleotides were used to construct the ATF4 cDNA (GenBank accession no. NM001675): 5’-ATGACCCGAAATGAGCTTCCTGAGC-3’ and 5’-CTAGGGGACCTTTCTTCCCC-3’.

Stable Transfection. A549 cells were seeded into 12-well tissue culture plates at a concentration of 5 x 10⁴. The following day they were transfected with 0.3 μg of either pcDNA3 or the pcDNA3-ATF4 expression plasmid using 6 μl of effectene transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. After 24 h, the cells were transferred to fresh medium in a new plate. The following day they were challenged with selection medium containing 300 μg/ml of geneticin (Life Technologies, Inc., Rockville, MD) and incubated for 3–4 weeks. Several transfectants that overexpressed the ATF4 gene were selected. These transfectants, referred to as A549-ATF4, were maintained in the presence of 300 μg/ml of geneticin.

Results and Discussion

ATF4 Expression Is Cisplatin-Inducible and Up-Regulated in Cisplatin-Resistant Cell Lines. To isolate cisplatin-inducible genes, we performed differential display on total RNA from paired untreated and cisplatin-treated KB cells (9). Sequence analysis of the cisplatin-inducible cDNA clones showed that one was identical to the ATF4/CREB2 gene, and Northern blot analysis demonstrated that this ATF4 mRNA was induced ~5-fold by cisplatin treatment after 24 h (Fig. 1A). ATF4 (Mr, 39,000) protein levels also increased (Fig. 1B). In addition, ATF4 mRNA was up-regulated >2-fold in two independently isolated cisplatin-resistant cell lines, and ATF4 protein was ~3- to 5-fold higher (Fig. 1, C and D). These data suggest that ATF4 is involved in cisplatin resistance.

ATF4 Expression and Cisplatin Sensitivity in 11 Lung Cancer Cell Lines. We analyzed ATF4 expression and cisplatin sensitivity in 11 human lung cancer cell lines. All of the cell lines have a similar growth rate except A529L, which is shown in Fig. 2A by the doubling time. Western blot analysis revealed various levels of ATF4 expression (Fig. 2A): A549 and B203L cells had the strongest expression, and PC1 cells had the weakest. To determine whether ATF4 expression was correlated with cisplatin sensitivity, these cell lines were examined for sensitivity to cisplatin, vincristine, and etoposide using cytotoxic assays. There appeared to be a correlation between levels of ATF4 and cisplatin resistance (coefficient of correlation = 0.711; P = 0.0119; Fig. 2B) but not with resistance to vincristine (coefficient...
of correlation = −0.087; \( P = 0.8043 \)) or etoposide (coefficient of correlation = 0.053; \( P = 0.8798 \)). As an additional control, we established that TRX1 expression did not correlate with cisplatin sensitivity (coefficient of correlation = −0.140; \( P = 0.6907 \)). There was no significant correlation between growth rate and sensitivity to cisplatin (data not shown).

**Overexpression of ATF4 Increases Resistance to Cisplatin.** To determine whether ATF4 is directly involved in cisplatin sensitivity, we established two stable transfectant derivatives of A549 cells. Unfortunately, because of low DNA transfection efficiency, we were unable to establish the stable transfectants from the cell lines with low expression of endogenous ATF4. The expression of endogenous ATF4 is high in A549 cells among 11 lung cancer cell lines. Therefore, it is possible that stable transfectants provide a minor effect against drug sensitivity. The growth rate of these derivatives, A549/ATF4−3 and A549/ATF4−4, was slightly higher than controls transfected with empty vector, and ATF4 expression was increased by 2.5–4.5-fold (Fig. 3). Both derivatives showed increased resistance to cisplatin, with a 3- to 5-fold higher IC\(_{50}\) (Fig. 4, top). Their sensitivity to vincristine was unaffected (Fig. 4, bottom). To our knowledge, this is the first report that ATF4 is DNA-damage inducible and that it is a
ATF4 EXPRESSION AND SENSITIVITY OF CISPLATIN

determinant of the cisplatin resistance of human cancer cells. We did not detect any preferential binding of a purified ATF4-GST-fusion protein to cisplatin-modified DNA (data not shown).

ATF4 is a member of the ATF/cyclic adenosine monophosphate-responsive element binding family of transcription factors, and is widely expressed in a variety of tissues and tumor cell lines. It has been shown to form a homodimer in vitro that binds to the cyclic adenosine monophosphate responsive element (18). It has also been shown to interact with nuclear factor-erythroid 1- and 2-related factors, suggesting that it modulates their expression. Nuclear factor-erythroid 1- and 2-related factors are recruited to the antioxidant response element, which plays an important role in the regulation of antioxidant genes (19). We propose that either ATF4 itself or its target genes, Nrf1 and Nrf2, are involved in cisplatin sensitivity. Many genes, including DNA repair genes, contain cyclic adenosine monophosphate response element and/or antioxidant response element in their promoter regions (20). Activating transcription factor 2, another member of the ATF/cyclic adenosine monophosphate-responsive element binding family, is activated by DNA damage and plays a substantial role in drug resistance by promoting DNA repair (21). ATF4-null cells have been shown recently to be impaired in expressing genes involved in glutathione biosynthesis and resistance to oxidative stress (22, 23). Transcription profiling by cDNA arrays has shown that ATF4 is up-regulated in drug-resistant cells that are inducible by genetic suppressor elements (24). Additionally, these data imply that ATF4 expression is involved in drug resistance. Protein kinases that phosphorylate eukaryotic initiation factor 2 are activated by endoplasmic reticulum stress signals and repress translation. These protein kinases selectively increase translation of ATF4, resulting in the induction of target genes (25). Because ATF4 mRNA is increased by cisplatin treatment, it is possible that anticancer agents induce endoplasmic reticulum stress and activate these kinases, increasing ATF4 levels. Further study of the expression of ATF4 target genes may provide insight into the relation between ATF4 expression and cisplatin resistance.

References
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