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Reversal of cisplatin resistance in human ovarian cancer cell lines by a c-*jun* antisense oligodeoxynucleotide (ISIS 10582): evidence for the role of transcription factor overexpression in determining resistant phenotype

Bin Pan[a], Kang-Shen Yao[a], Brett P. Monia[b], Nicholas M. Dean[b], Robert A. McKay[b], Thomas C. Hamilton[c], Peter J. O'Dwyer[a,*]

[a]*University of Pennsylvania, Cancer Center, 51 No. 39th Street, MAB Suite 103, Philadelphia, PA 19104, USA*
[b]*ISIS Pharmaceuticals, Carlsbad, CA 92008, USA*
[c]*Fox Chase Cancer Center, Philadelphia, PA 19111, USA*

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Abstract

Human ovarian cancer cell lines derived from A2780 by stepwise exposure to increasing cisplatin concentrations show progressive resistance to cisplatin. Previous studies have shown increased cellular glutathione and elevated steady-state expression of γ-glutamylcysteine synthetase (γ-GCS) and of the transcription factor c-Jun, all in proportion to the level of resistance in the resistant cells. We hypothesized that c-Jun was an important locus of control of the detoxicating enzymes mediating resistance, and that resistance reversal would be achieved by specific inhibition of this mechanism. A2780 (sensitive) and C30 (resistant) cells were treated with a 20-mer c-*jun* phosphorothioate antisense oligodeoxynucleotide (ISIS 10582, 1 μM), and a decrease in steady-state c-*jun* mRNA was demonstrated in the resistant cells. The expression of γ-GCS mRNA was down-regulated and the cellular level of glutathione was decreased in C30 cells. No change in γ-GCS expression occurred in A2780 cells. Using the microtetrazolium (MTT) cytotoxicity assay, we determined that the c-*jun* antisense decreased the IC_{50} value for cisplatin in C30 cells from 18.2 to 3.7 μM, and had a substantially smaller effect in A2780 cells. To determine if c-*jun* overexpression alone could confer resistance to the sensitive cell line, we transiently transfected A2780 cells with a c-*jun* expression vector. The transfected cells exhibited a 10.7-fold elevation of glutathione (GSH) content, a 9.2-fold increase in c-Jun protein content, and a 2-fold increase in the IC_{50} for cisplatin. These data suggest that altered regulation of transcription factor expression contributes to the acquired resistance phenotype in these ovarian cancer cells, and provide a novel potential target for therapeutic intervention.

Keywords: Antisense; ISIS 10582; c-Jun; Drug resistance; Ovarian cancer

1. Introduction

The use of platinum-based chemotherapy has markedly improved the outcome of advanced ovarian cancers [1,2]. However, though patients survive longer with this treatment, recurrence is usual, and the recurrent disease is more resistant to chemotherapy. An understanding of the basis of platinum drug resistance has been afforded by the study of ovarian cancer cell lines selected through stepwise exposure to increasing cisplatin concentrations [3]. These cell lines span a degree of resistance from 27.5- to 500-fold [3]. Resistance is associated with a proportional increase in cellular GSH concentrations, implicating cellular detoxication mechanisms in the resistant phenotype [3]. Further investigation revealed a direct association with progressive increases in the expression of γ-GCS, the rate-limiting enzyme in GSH synthesis [4]. We previously analyzed the basis of γ-GCS expression and found a direct relationship of steady-state γ-GCS mRNA content with the rate of the γ-GCS mRNA transcription [5]. In turn, this was

* Corresponding author. Tel.: +1-215-662-8632; fax: +1-215-243-3268.
E-mail address: peter.odwyer@uphs.upenn.edu (P.J. O'Dwyer).

Abbreviations: GSH, glutathione; γ-GCS, γ-glutamylcysteine synthetase; MTT, microtetrazolium; CMV, chloramphenicol acetyltransferase; MMC, mitomycin C; AP-1, activator protein-1; DMEM, Dulbecco's Modified Essential Medium; JNK, c-Jun-N-terminal kinase; ARE, antioxidant response element; bZIP, basic leucine zipper; Nrf, nuclear factor E2 p45-related factors.

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associated with stable elevation of c-*jun* expression, and of c-Jun binding to the activator protein-1 (AP-1) element in extracts of nuclear protein [5].

The similarity between adaptive responses to exogenous toxins and the established resistant phenotype has been pointed out [6]. We have demonstrated previously the critical role of c-*jun* in the adaptive response to mitomycin C (MMC) in colon adenocarcinoma cells [7]. Others have demonstrated induction of c-*jun* and activation of c-Jun-N-terminal kinase (JNK) by cisplatin in human glioblastoma and leukemia cells [8,9] and murine fibroblasts [10] and keratinocytes [11]. The induction of this transcription factor is also responsible for the resistance of HT29 cells under hypoxic conditions [12]. Therefore, we selected the AP-1 transcription factor as a potential therapeutic target for the modulation of drug resistance.

The development of antisense technology has made possible the application to human cancer of a therapy specific to a gene of interest [13–15]. Antisense oligodeoxynucleotides are designed to bind to a target mRNA by complementary base pairing, and are believed to result in mRNA cleavage through the action of RNase H upon the RNA:DNA duplex [16]. Enhanced stability of the antisense construct is provided by modification of the sugar–phosphate backbone [17]. A c-*jun* antisense oligodeoxynucleotide directed to position 2277–2258 of the c-*jun* coding region has been modified as a phosphorothioate (ISIS 10582), and is under development as a potential anticancer agent.

In this study, we sought to determine the potential role of the c-*jun* antisense in reversing cisplatin resistance in human ovarian carcinoma cell lines, to demonstrate evidence of selectivity for the resistant phenotype, and to confirm that this phenotype could be recapitulated by overexpression of c-*jun* in sensitive cells.

2. Materials and methods

2.1. Cells and reagents

The A2780 human ovarian carcinoma cell line (cisplatin IC_{50}: 3.5 μM) and the A2780-derived C30 cisplatin-resistant cell line (IC_{50}: 18.2 μM) were grown at 37° in 5% CO_2/95% air in Dulbecco's Modified Essential Medium (DMEM), with 10% fetal bovine serum added. Cisplatin was obtained from Bristol-Myers Squibb. The chloramphenicol acetyltransferase (CMV)-c-Jun mammalian expression vector was a gift of T. Curran. Other reagents were purchased from the Sigma, unless stated otherwise.

2.2. Synthetic oligodeoxynucleotides

The following synthetic phosphorothioate oligodeoxynucleotides were obtained from ISIS Pharmaceuticals: c-*jun* antisense ISIS 10582 (TCA GCC CCC GAC GGT CTC TC) and the scrambled control construct ISIS 11563 (CAC CTC CAC GCG CTT CTG GC). These reagents were provided as a 1 mM stock solution and diluted with serum-free DMEM for cell treatment. The oligonucleotides were stored at −20°.

2.3. Antisense treatment

The lipofectin method was employed for antisense transfection [18]. Briefly, subconfluent growing cells were trypsinized and seeded in complete medium. The cells were incubated at 37° in 5% CO_2/95% air for 24 hr at which time they were about 60–80% confluent. At the time of transfection, cells were washed with serum-free medium. Lipofectin solution (Gibco BRL) was prepared as follows: 1 μL oligodeoxynucleotide stock (1 mM) was diluted to 100 μL with serum-free medium; 7.5 μL of lipofectin reagent (1 mg/mL) was diluted to 100 μL with serum-free medium and allowed to stand at room temperature for 30–45 min; these two solutions were mixed and incubated at room temperature for an additional 10–15 min, when 0.8 mL of serum-free medium was added to produce a final volume of 1 mL. After a 4-hr incubation in this mixture of reagents, the cells were washed once and reincubated in complete growth medium. The cells were harvested at the indicated times following the end of treatment.

2.4. c-jun Expression vector transfection

The calcium phosphate procedure [19] was used for c-*jun* expression vector transfection. A2780 cells (80% confluent) were trypsinized and collected by centrifuging at 4° for 5 min at 500 *g*. The cells were resuspended in 5 mL of complete culture medium, counted, and divided in aliquots of 3×10^5 cells per tube. The cells were again centrifuged at 4° for 5 min at 500 *g*, and the pellet was resuspended in 1 mL of calcium phosphate/DNA solution (25 mM HEPES, pH 7.05, 0.75 mM Na_2HPO_4, 5 mM KCl, 140 mM NaCl, 6 mM glucose, 125 mM $CaCl_2$, 15 μg/mL of plasmid DNA) at room temperature for 15 min. The samples were diluted with complete culture medium, plated, and incubated at 37° in 5% CO_2/95% air.

2.5. MTT cytotoxicity assays

Cisplatin cytotoxicity was determined using the MTT assay as described previously [20]. The cells were trypsinized, and aliquots (2000 cells per well) were placed in 96-well culture plates in complete medium and incubated for about 5 hr at 37° to allow attachment. At the end of this time, the medium was aspirated, and the cells were washed and incubated with fresh complete medium overnight. Cisplatin was added to the wells at various concentrations and incubated for 4 hr. Then the medium was aspirated and replaced with fresh complete medium, and the plates were

returned to the incubator for 3–5 days. The cells were then processed for the MTT assay as described and read with a plate reader (Elx800; BioTek, Inc.). The IC_{50} values were obtained from the results of triplicate determinations of at least two independent experiments.

2.6. Northern blot analysis

Total RNA was isolated using TRIzol reagent (Gibco BRL). Briefly, 10^6 to 10^7 cells were lysed in 1 mL of TRIzol reagent by shearing through repetitive pipetting, and then were incubated for 5 min at room temperature. After adding 0.2 mL of chloroform, the tube was shaken vigorously by hand, and incubated at room temperature for 2–3 min. The sample was centrifuged at 12,000 *g* for 15 min at 4°, and the aqueous phase was transferred to a fresh tube. The RNA was precipitated from the aqueous phase by mixing with 0.5 mL isopropyl alcohol, and centrifuged at 12,000 *g* for 10 min at 4°. The RNA pellet was washed once with 70% ethanol, and dissolved in diethyl pyrocarbonate (DEPC)-treated water. RNA was separated in 1% agarose gels containing formaldehyde. The RNA was transferred to nylon membranes, and hybridized to probes as follows: (i) a 1.2 kb c-*jun* fragment excised by *Xho*I from pUC18 [21]; (ii) a 1.4 kb γ-GCS fragment excised by *Bam*HI from human γ-GCS in pUC19 [5]; and (iii) a β-actin probe [22]. Autoradiography was carried out at −70° for 1–3 days. The blot was subsequently stripped and reprobed. The intensity of β-actin labeling was used in normalizing values to provide a measure of the relative expression of the mRNA of interest. The quantification was done by densitometric analysis of autoradiograms.

2.7. Western analysis

Proteins were separated in 10–12% SDS-polyacrylamide gels essentially according to Laemmli [23]. For immunoblotting analysis the proteins were transferred to nitrocellulose overnight in the presence of 25 mM Tris, 192 mM glycine, and 20% methanol [24]. Nonspecific binding was blocked by incubating the blots with 5% non-fat milk powder in TBST (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). The nitrocellulose blots were incubated with primary polyclonal antibody directed to c-Jun (SC-045, Biotechnology, Inc.) 1:500 in 1% non-fat milk TBST for 1 hr at room temperature. After three washes in TBST, the membranes were incubated with a peroxidase-coupled secondary antibody (SC-2004). The blots were developed using the ECL Western blotting detection system (Amersham Life Science).

2.8. GSH quantitation

GSH was measured by a modification of the method of Griffith [25], in which the rate of formation of a GSH conjugate of 5,5′-dithio-bis(2-nitrobenzoic acid) was determined spectrophotometrically. GSH concentrations were determined by reference to a standard curve (GSH, 0.05–5 nmol/mL), which was run with each batch of samples. Samples were precipitated by the addition of 12% 5-sulfosalicylic acid in a 1:3 ratio. The reaction was conducted in 0.2 mM NADPH–0.6 mM 5,5′-dithio-bis(2-nitrobenzoic acid), at pH 7.5. Absorbance was monitored at 412 nm for 3 min at 25°, and results were derived from the standard curve and expressed per milligram of protein.

3. Results

Our previous work documented the clear association of c-*jun* steady-state mRNA levels with γ-GCS expression in resistant ovarian cancer cell lines [5]. The level of γ-GCS mRNA is proportional to the degree of cisplatin resistance [4]. In this study, we have focused on the cisplatin-sensitive cell line A2780 and its cisplatin-resistant derivative C30. We have established optimal conditions for antisense oligodeoxynucleotide transfection in these cell lines using the lipofectin technique. A lipofectin concentration of 7.5 μg/mL was found to be optimal to maintain cell survival and to maximize the pharmacodynamic effect (data not shown).

The C30 cell line has a substantially higher level of c-*jun* mRNA than does the A2780 cell line, which showed no visible band on conventional Northern blots (data not shown). Transfection of the C30 cell line with the c-*jun* antisense oligodeoxynucleotide ISIS 10582 dramatically decreased c-*jun* mRNA expression in a time- and concentration-dependent manner, while cells transfected with lipofectin alone or with a scrambled-sequence control phosphorothioate oligodeoxynucleotide ISIS 11463 had a c-*jun* mRNA content similar to that of untreated C30 cells (Fig. 1A). Following treatment with 1 μM c-*jun* antisense, depletion was evident by the end of 4 hr, and reached a maximum at 24 hr, when the content was 7.9% of the starting value. By the 48-hr point, recovery was evident, particularly at the lower concentration of the antisense construct. Concentration-dependency of the pharmacologic effect was evident in that 1 μM produced more pronounced depletion of c-*jun* mRNA than 0.3 μM ISIS 10582. These concentration–response relationships and time-courses are consistent with the effects of antisense constructs directed to other targets also [26].

The relationship of altered c-*jun* mRNA content to c-Jun protein levels was investigated by Western analysis. A2780 and C30 cells were exposed to antisense treatment for 4 hr, and harvested at 24 hr. Treatment with the antisense construct, but not with the scrambled oligodeoxynucleotide control, resulted in depletion of c-Jun protein (Fig. 1B). The functional consequences of c-Jun depletion were examined by electrophoretic mobility shift assays of

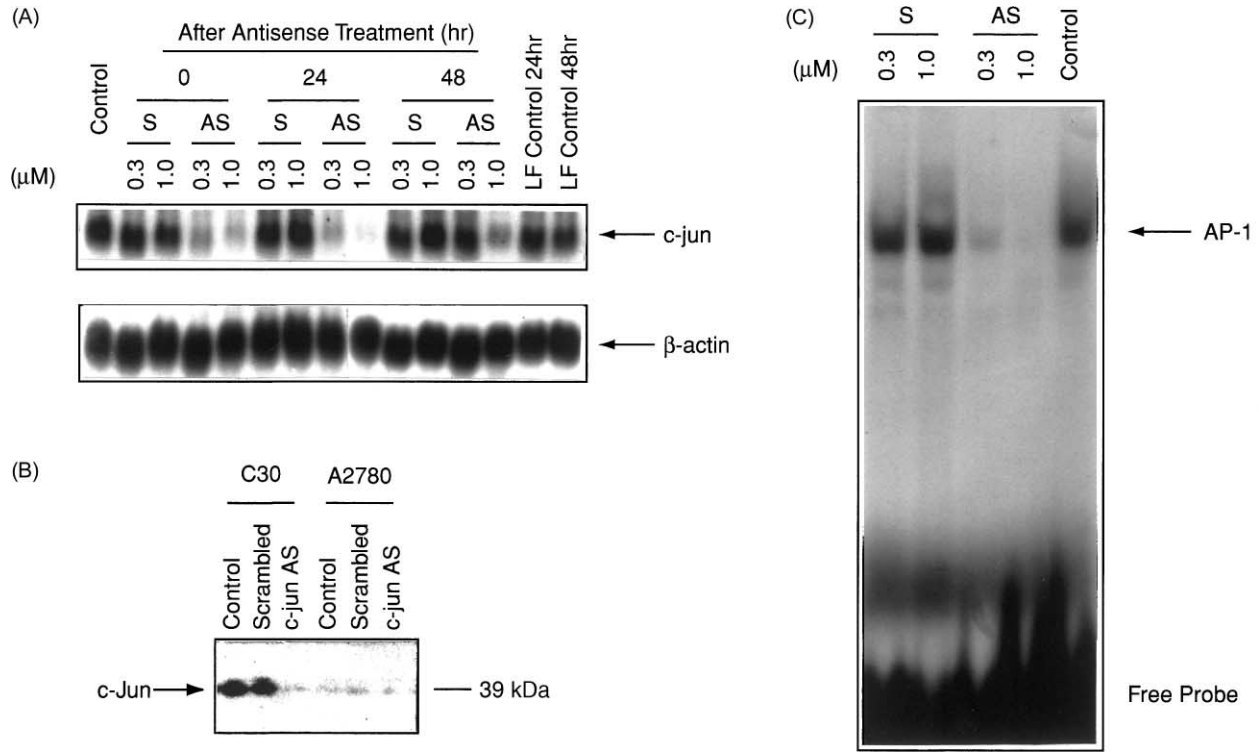

Fig. 1. Functional analysis of cisplatin-sensitive A2780 and cisplatin-resistant C30 cell lines after c-*jun* antisense treatment. (A) Northern analysis of c-*jun* mRNA content. The C30 cells were transfected with 0.3 and 1 μM c-*jun* antisense (AS) oligonucleotide along with a scrambled (S) oligonucleotide for 4 hr as described in Section 2. Total RNA was isolated from the cells at 0, 24, and 48 hr after antisense treatment. The blots were probed for c-*jun* and β-actin as described; LF = lipofectin. (B) Detection of c-Jun in antisense- and control-treated cells by Western blot analysis. After treatment with oligonucleotide for 4 hr, cells were maintained in drug-free medium for 24 hr, harvested, and lysates separated on polyacrylamide gels. Blots were probed using c-Jun antibody. (C) Electrophoretic mobility shift assay to demonstrate inhibition of AP-1 binding after c-*jun* antisense treatment. Cells were treated as described for panel B, and nuclear protein (10 μg) was incubated with a ^{32}P-labeled synthetic γ-glutamylcysteine synthetase AP-1-binding element oligonucleotide.

nuclear proteins isolated from cells treated similarly (Fig. 1C). Nuclear factor binding to a synthetic oligonucleotide with the γ-GCS AP-1 sequence was selectively inhibited in the c-*jun* antisense-treated C30 cells. There was no detectable factor binding to the oligodeoxynucleotide probe in treated or untreated A2780 cells (data not shown [22]).

We previously showed that γ-GCS mRNA expression is elevated in cisplatin-resistant cells, to a degree proportional to the level of resistance [26], and proportional to c-*jun* expression [5]. To determine if c-*jun* antisense oligodeoxynucleotide treatment could inhibit γ-GCS expression, mRNA from treated cells was subjected to Northern analysis. The results (Fig. 2A) show that c-*jun* antisense treatment inhibited γ-GCS mRNA expression in C30 cells in a time- and concentration-dependent manner. Following treatment with 1 μM c-*jun* antisense, γ-GCS mRNA expression was decreased progressively out to the 24-hr point, at which time it was 5.9% (1.0 μM) of the starting value. The results demonstrated that 0.3 μM antisense is sufficient to decrease c-*jun* mRNA, and this concentration also has an effect upon γ-GCS mRNA expression. The γ-GCS mRNA expression was decreased to 24.9% of the starting value at 24 hr. The similar time-course of c-*jun* mRNA depletion compared with that of γ-GCS further indicates that the transcriptional activity of c-Jun protein regulates γ-GCS gene expression since the γ-GCS promoter contains two AP-1 elements. Since γ-GCS catalyzes the rate-limiting reaction in GSH synthesis, the cellular GSH content should change in accord with γ-GCS mRNA content. Spectrophotometric analysis shows that the cellular GSH content of C30 cells was 45-fold that of A2780 cells and was decreased by a factor of 3.0 by c-*jun* antisense oligodeoxynucleotide treatment (Fig. 2B).

The demonstration that an antisense construct directed to a key control element of γ-GCS transcription could down-regulate this important detoxication pathway prompted the investigation of transcription factor inhibition on cisplatin sensitivity. We asked if these pharmacologic effects of c-*jun* antisense treatment could restore C30 cell sensitivity to cisplatin. The cells were treated for 4 hr with antisense oligodeoxynucleotides as described previously, and incubated with various concentrations of cisplatin for an additional 4 hr. A colorimetric assay for cell number (MTT) was utilized as a measure of cell survival (Fig. 3). The use of c-*jun* antisense but not scrambled control antisense decreased the IC_{50} of C30 cells for cisplatin from 18.2 to 3.7 μM (4.9-fold) (Fig. 3B). A substantial effect was also found in the sensitive A2780 line with a 1.8-fold sensitization to cisplatin (from 3.5 to 1.9 μM) (Fig. 3A). Therefore, disruption of transcription

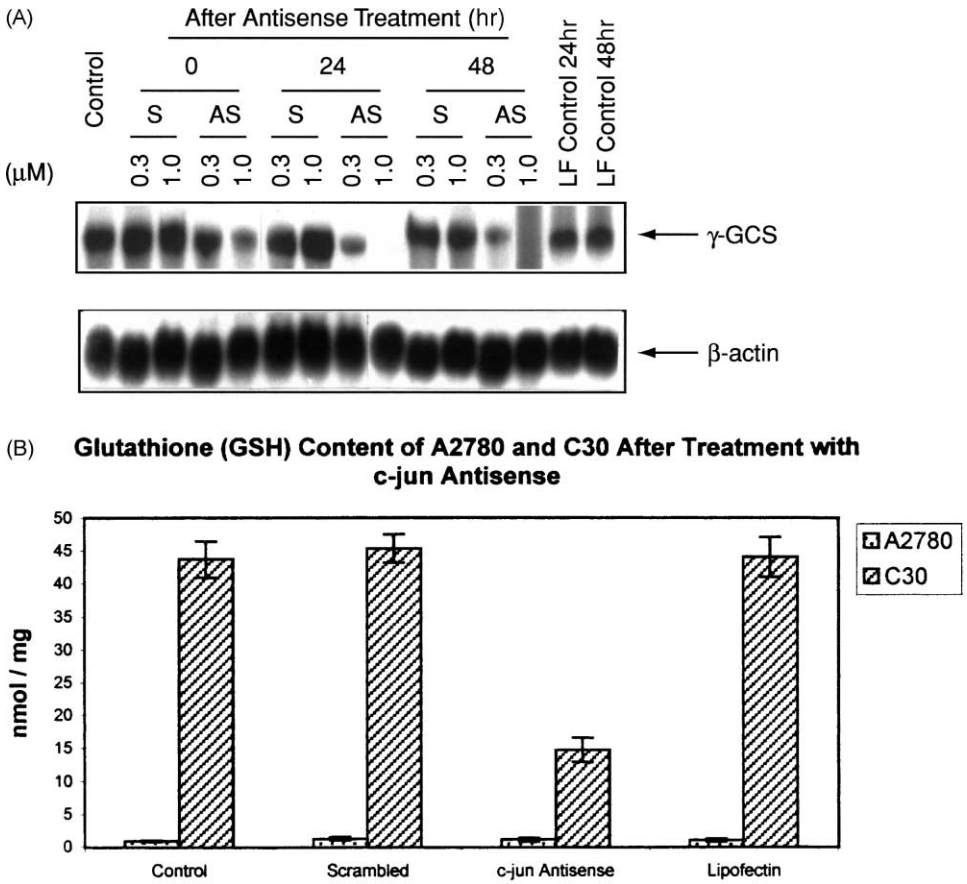

Fig. 2. Effect of c-*jun* antisense on γ-GCS mRNA and glutathione content. Cells were treated as described in the legend of Fig. 1B, and total RNA and glutathione were isolated. Shown are the results of a Northern analysis of C30 cells (A) and glutathione determination in both cell lines (B). Values are means ± 1.8, $N = 3$.

factor function was demonstrated to alter sensitivity to cisplatin.

To confirm the role of c-Jun in conferring a resistant phenotype, we used a c-*jun* mammalian expression vector in the sensitive cell line. We transiently transfected a c-*jun* cDNA construct under the control of the CMV promoter into A2780 cells, and found a 9.2-fold increase in the expression of c-Jun protein (Fig. 4A). This was accompanied by a

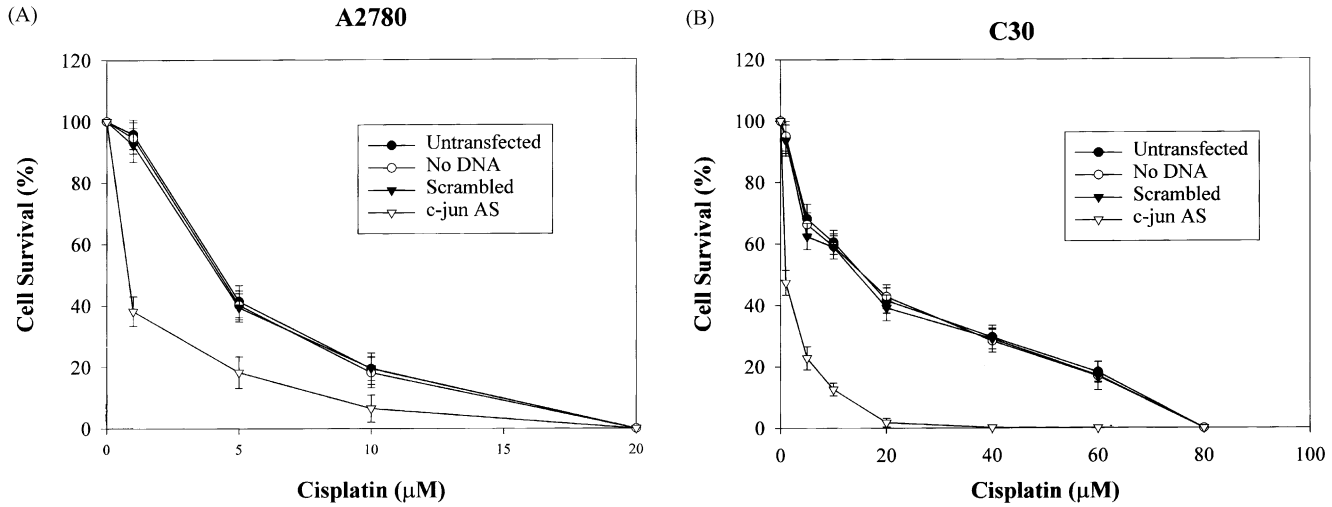

Fig. 3. Cisplatin cytotoxicity assay. A2780 and C30 cells were transfected for 4 hr with the c-*jun* antisense or scrambled oligonucleotide. The transfection control consisted of cells treated with lipofectin alone (no DNA), while the control cells were untransfected. Cells in all groups were exposed to various concentrations of cisplatin for an additional 4 hr. Cells were incubated in fresh medium, and analyzed using the MTT assay as described. The experiments were repeated three times, and the beginning number of cells in the MTT assay was 3000 cells per well.

10.7-fold increase in cellular GSH (Fig. 4B). These findings implicated up-regulation of detoxication pathways by overexpression of c-Jun. To determine the influence of these biochemical effects on cisplatin sensitivity, we again used the MTT assay. We found that the IC_{50} of A2780 transfected by CMV-c-Jun was 7.4 μM, which was 2.0-fold higher than that of both the wild-type A2780 and CMV-c-Jun vector transfected cell lines (IC_{50}: 3.7 μM) (Fig. 4C).

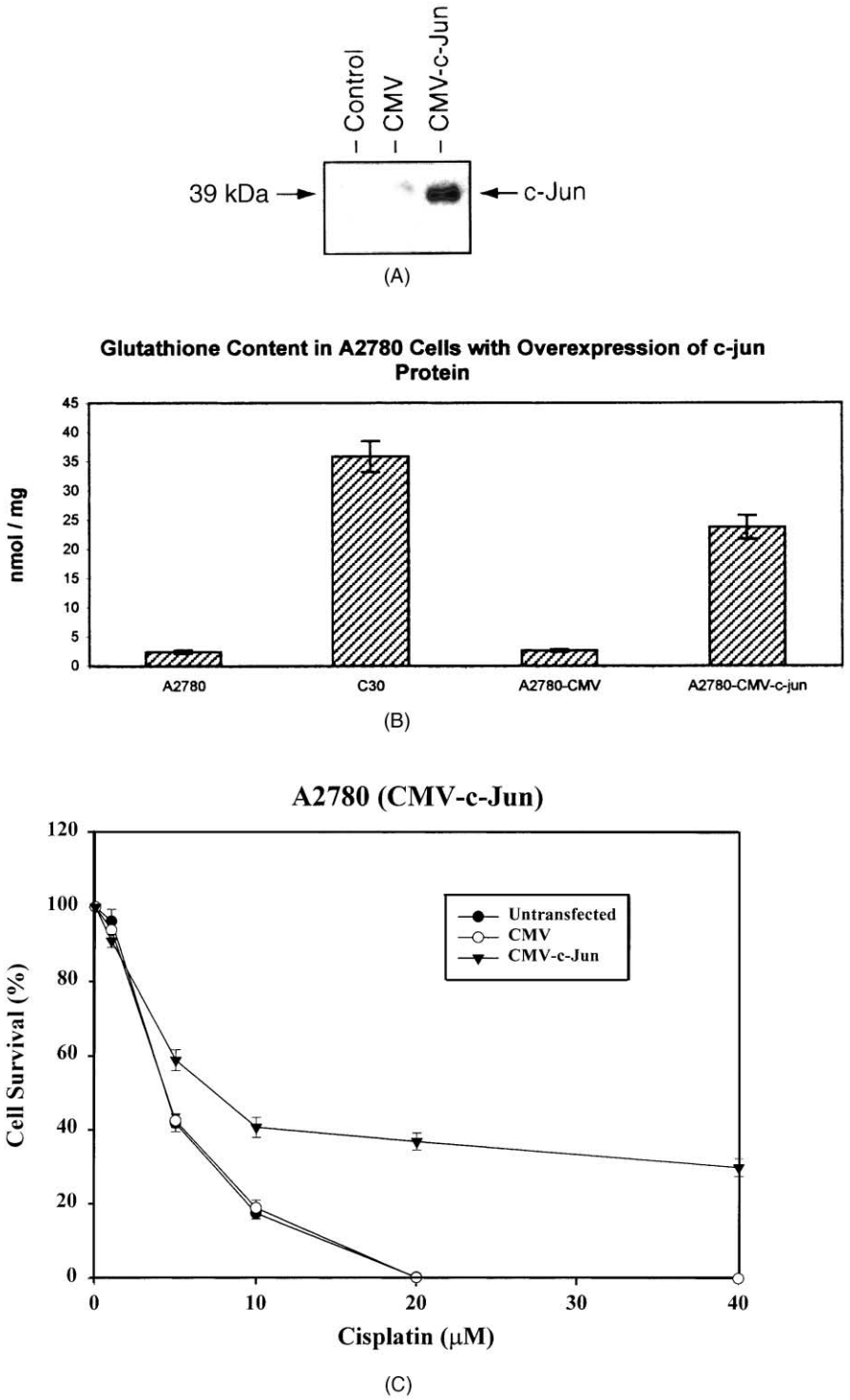

Fig. 4. Effects of overexpression of c-Jun protein in the cisplatin-sensitive A2780 cell line. Cells were isolated after transfection with the CMV-c-Jun plasmid as described in Section 2 and c-Jun expression was assessed by Western blotting (A). The effect of c-Jun overexpression on glutathione content (B) and cytotoxicity (C) was also determined. The glutathione content and cytotoxicity assays were repeated three times (values are means ± 2.4). The beginning number of cells in the MTT assay was 3000 cells per well. C30 cells were included in panel B for comparison purposes.

4. Discussion

The c-*jun* proto-oncogene is one of the genes activated rapidly and transiently in response to a variety of extracellular stimuli [27,28], including growth factors, interleukins, hormones, various tumor promoters such as tetradecanoylphorbol acetate (TPA), thapsigargin and okadaic acid, and ultraviolet light [29–32]. The nuclear proteins Jun and Fos are important components of AP-1, a direct regulator of gene expression. Fos can *trans*-activate AP-1 containing promoters only in the presence of Jun [33–35], while Jun can control gene transcription on its own by forming homodimers [28]. c-Jun has the capacity to form functional heterodimers with multiple transcription factors, and these partners can alter the affinity of c-Jun for DNA-binding sites and, subsequently, determine the downstream target genes controlled by c-Jun [36–38]. c-Jun not only stimulates transcription but also represses transcription of certain genes (e.g. human chorionic gonadotropin α and β genes [39]). c-Jun mediates changes in the gene expression program of the cell in response to proliferative, toxic, and in some cells, differentiating signals in the environment [37]. Activation of c-Jun (principally through the JNK pathway) has been demonstrated to mediate hypoxic cell resistance [40,41]. Induction of c-*jun* expression has been associated with drug resistance in several settings: teniposide-resistant human leukemic lymphoblasts [42], etoposide-resistant human K562 cells [43], multi-drug-resistant human prostate cancer [44], and retinoid and tamoxifen resistance in breast cancer cells [45,46]. c-Jun may also act to mediate apoptotic signals through caspase activation [47].

Cisplatin is a widely utilized antineoplastic agent, efficacious in the treatment of ovarian, testicular, and head and neck cancer [2]. However, the rapid development of resistance requires strategies to potentiate cisplatin toxicity in resistant cells. Previous work has shown that cisplatin resistance is accompanied by increasing cellular context of GSH and of its critical regulatory enzyme γ-GCS [5,20]. We have shown in a series of ovarian cancer cell lines with acquired resistance to cisplatin that overexpression of c-*jun* is associated with several biochemical changes that permit the resistant cells to detoxicate a number of cytotoxic drugs more efficiently [8]. To determine if a causal relationship exists between c-*jun* overexpression and elevated detoxicating capacity, we treated C30 cells (about mid-range of resistance in the series of cell lines) with an antisense construct directed to c-*jun*. As a consequence of the decrease in c-*jun* mRNA and protein, the expression of γ-GCS declined and the cell content of GSH was diminished. The decline in γ-GCS expression pursued a more gradual time-course than that of c-Jun: the same phenomenon occurs with hypoxic stress, where the increase in γ-GCS is observed later than that of c-Jun [40]. In part, this reflects the rather varied repertoire of c-Jun: in addition to immediate early responses, c-Jun expression (unlike that of c-Fos) often remains elevated to exert its effects over prolonged periods [11,12,48]. Similar findings were obtained in two ovarian cancer cell lines (A2780 and Caov-3) by Hayakawa *et al.* [49]: resistance reversal was achieved by transfection with dominant negative Jun, and similar levels of sensitization were found.

Jun family homo- and heterodimers have the capacity to bind AP-1 sites in the promoters of various genes. That c-Jun itself is the factor most critical to the activation of γ-GCS is supported by the current experiments. The AP-1 site in the promoters of γ-GCS and DT-diaphorase is a component of a larger regulatory element, the antioxidant response element (ARE) [50–52]. Analysis of these AREs has demonstrated that they contain AP-1 or AP-1-like elements as reverse or direct repeats [53]. Mutation of the consensus AP-1 element abolishes transcriptional responses to the antioxidant β-naphthoflavone [54], but fine regulation of the antioxidant response requires additional flanking sequences [55–57]. Two novel basic leucine zipper (bZIP) transcription factors, nuclear factor E2 p45-related factors 1 and 2 (Nrf-1 and -2), have been demonstrated to bind to AP-1-like elements in the ARE [58,59]. These factors have been shown to regulate antioxidant-induced expression of DT-diaphorase and of γ-GCS [60,61]. Venugopal and Jaiswal [62] have shown that Nrf and Jun family proteins interact in this response. It will be of interest to determine if these transcription factors participate in the resistant phenotype of ovarian cancer cell lines, and if so, whether disruption of their function may provide additional potency or specificity in its reversal.

The decrease in GSH (67.3%) following antisense treatment was less pronounced than the effect upon γ-GCS (94.1% decrease at 48 hr). In part, these differences may be accounted for by the GSH measurement, which represents a steady-state between synthesis and degradation, in a stable environment in which little degradation is likely. A more detailed examination, which is in progress, of the effects of the antisense construct upon cisplatin cellular pharmacology will elucidate the functional consequences of this degree of GSH depletion more clearly.

These effects were sufficient, however, to result in a marked decrease in the IC_{50} of cisplatin towards the resistant cell line. Whereas the sensitive A2780 cell line was rendered only slightly more susceptible to cisplatin cytotoxicity, a 4.9-fold increase in efficacy was observed in the C30 cells. These results have been replicated in more resistant cell lines of this series also,[1] and imply a selective action against resistant cells in this model. These results warrant further testing *in vivo*, and human trials directed to tumors with appropriate expression of c-*jun* are planned.

It was important to perform the complementary experiment in the sensitive cell lines: could overexpression of c-*jun* confer the resistant phenotype? The transfected cells

[1] Pan and O'Dwyer, unpublished results.

exhibited a 9.2-fold increase in c-Jun protein, a 10.7-fold elevation of steady-state GSH levels, and a 2-fold increase in the IC_{50} for cisplatin. This level of resistance is generally regarded as clinically significant as estimated from explants of human tumors. It does not reach the level of resistance in C30 cells (5.2-fold), but in addition to altered regulation of detoxication pathways, C30 differs from A2780 in a number of respects that influence sensitivity to cisplatin: C30 has a deletion of one p53 allele and, based upon p21 expression, is non-functional in the other. C30 cells also have enhanced DNA repair capacity, and greater DNA damage tolerance, i.e. the amount of DNA–platinum adduct accumulation required for cell kill is 8.8-fold higher than that in A2780 cells using the 4-hr IC_{50} value [63]. Thus, the demonstration of a 2-fold increase in resistance supports a role for enhanced detoxication capacity in determining the resistant phenotype.

The underlying hypothesis, that reactive platinum species are quenched by cellular thiols before they reach their target, and that their removal from DNA is facilitated in a reducing environment, supports the development of GSH-depleting agents, such as buthionine sulfoximine, to reverse resistance. However, the resistance-reversing effects of targeting c-Jun, while predictably decreasing thiol synthesis, may have additional effects on unrelated genes. An interaction between c-Jun and p53 has been demonstrated recently to control progression through the cell cycle by an effect on Cdks [64]; an emerging role for c-Jun in the control of apoptotic pathways may contribute equally. A more complete functional genomic picture of the cellular effect of c-Jun disruption may elucidate additional loci relevant to resistance.

Acknowledgments

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References

[1] McGuire WP, Hoskins WJ, Brody MF, Kucera PR, Partridge EE, Look KY, Clarke-Pearson DL, Davidson M. Cyclophosphamide and cisplatin compared with paclitaxel and cisplatin in patients with Stage III and Stage IV ovarian cancer. N Engl J Med 1996;334: 1–6.

[2] O'Dwyer PJ, Johnson SW, Hamilton TC. Platinum analogues. In: DeVita Jr VT, editor. Cancer: principles and practice of oncology, 5th ed. Philadelphia: Lippincott Co., 1997. p. 418–32.

[3] Hamilton TC, Winker MA, Louie KG, Batist G, Behrens BC, Tsuruo T, Grotzinger KR, McKoy WM, Young RC, Ozols RF. Augmentation of adriamycin, melphalan, and cisplatin cytotoxicity in drug-resistant and -sensitive human ovarian cancer cell lines by buthionine sulfoximine mediated glutathione depletion. Biochem Pharmacol 1985;34: 2583–6.

[4] Godwin AK, Meister A, O'Dwyer PJ, Huang CS, Hamilton TC, Anderson ME. High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis. Proc Natl Acad Sci USA 1992;89:3070–4.

[5] Yao K-S, Godwin AK, Johnson SW, Ozol RF, O'Dwyer PJ, Hamilton TC. Evidence for altered regulation of γ-glutamycysteine synthetase gene expression among cisplatin-sensitive and cisplatin-resistant human ovarian cancer cell lines. Cancer Res 1995;55:4367–74.

[6] Henderson CJ, McLaren AW, Moffat GJ, Bacon EJ, Wolf CR. Pi-class glutathione transferase: regulation and function. Chem Biol Interact 1998;111/112:69–82.

[7] Yao K-S, Hageboutros A, Ford P, O'Dwyer PJ. Involvement of activator protein-1 and nuclear factor-κB transcription factors in the control of the DT-diaphorase expression induced by mitomycin C treatment. Mol Pharmacol 1997;51:422–30.

[8] Kharabanda S, Ren R, Pandey P, Shafman TD, Feller SM, Weichselbaum RR, Kufe DW. Activation of the c-Abl tyrosine kinase in the stress response to DNA-damaging agents. Nature 1995;376:785–8.

[9] Potapova O, Haghighi A, Bost F, Liu C, Birrer MJ, Gjerset R, Mercola D. The jun kinase/stress-activated protein kinase pathway functions to regulate DNA repair and inhibition of the pathway sensitizes tumor cells to cisplatin. J Biol Chem 1997;272:14041–4.

[10] Zanke BW, Boudreau K, Rubie E, Winnett E, Tibbles LA, Zon L, Kyriakis J, Liu F-F, Woodgett JR. The stress-activated protein kinase pathway mediates cell death following injury induced by *cis*-platinum, UV irradiation or heat. Curr Biol 1996;6:606–13.

[11] Sanchez-Perez I, Murguia JR, Perona R. Cisplatin induces a persistent activation of JNK that is related to cell death. Oncogene 1998;16:533–40.

[12] Yao K-S, Xanthoudakis S, Curran T, O'Dwyer PJ. Activation of AP-1 and of a nuclear redox factor, Ref-1, in the response of HT29 colon cancer cells to hypoxia. Mol Cell Biol 1994;14:5997–6003.

[13] Crooke ST. Therapeutic application of oligonucleotides. Annu Rev Pharmacol Toxicol 1994;32:329–76.

[14] Flanagan WM. Antisense comes of age. Cancer Metastasis Rev 1998;17:169–76.

[15] Lin SB. Do overexpressed oncoproteins cause malignant growth of cancer cells? Studied by antisense oligonucleotides. Clin Chem Lab Med 1998;36:541–4.

[16] Quartin RS, Brakel CL, Wetmur JG. Nuclear and distribution of methylphosphonate linkages in oligodeoxynucleotides affect exo- and endo-nuclease sensitivity and ability to form RNase H substrates. Nucl Acids Res 1989;17:7253–62.

[17] Cook PD. Antisense medicinal chemistry. In: Crooke ST, editor. Antisense research and applications. New York: Springer, 1998. p. 51–101.

[18] Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, Danielsen M. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc Natl Acad Sci USA 1987;84:7413–7.

[19] Chu G, Sharp PA. SV40 DNA transfection of cells in suspension: analysis of the efficiency of transcription and translation of T-antigen. Gene 1981;13:197–202.

[20] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Meth 1983;65:55–63.

[21] Angel P, Allegretto EA, Okino ST, Hattori K, Boyle WJ, Hunter T, Karin M. Oncogene *jun* encodes a sequence-specific *trans*-activator similar to AP-1. Nature 1988;332:166–71.

[22] Cleveland DW, Lopata MA, MacDonald RJ, Cowan NJ, Rutter WJ, Kirschner MW. Number and evolutionary conservation of α- and β-tublin and cytoplasmic β- and γ-actin genes using specific cloned cDNA probes. Cell 1980;20:95–105.

[23] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.

[24] Towbin H, Staehlin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1970;76:4350–4.

[25] Griffith OW. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal Biochem 1980; 106:207–12.
[26] Duff JL, Monia BP, Berk BC. Mitogen-activated protein (MAP) kinase is regulated by the MAP kinase phosphatase (MKP-1) in vascular smooth muscle cells. Effect of actinomycin D and antisense oligonucleotides. J Biol Chem 1995;270:7161–6.
[27] Curran T, Franza Jr. BR. Fos and Jun: the AP-1 connection. Cell 1988;55:395–7.
[28] Rahmsdorf HJ. Jun: transcription factor and oncoprotein. J Mol Med 1996;74:725–47.
[29] Lamph WW, Wamsley P, Sassone-Corsi P, Verma IM. Induction of proto-oncogene JUN/AP-1 by serum and TPA. Nature 1988;334:629–31.
[30] Devary Y, Gottlieb RA, Lau LF, Karin M. Rapid and preferential activation of the c-*jun* gene during the mammalian UV response. Mol Cell Biol 1999;11:2804–11.
[31] Schonthal A, Alberts AS, Frost JA, Feramisco JR. Differential regulation of *jun* family gene expression by the tumor promoter okadaic acid. New Biol 1991;3:977–86.
[32] Schonthal A, Sugarman J, Brown JH, Hanley MR, Feramisco JR. Regulation of c-*fos* and c-*jun* proto-oncogene expression by the Ca^{2+}-ATPase inhibitor thapsigargin. Proc Natl Acad Sci USA 1991;88:7096–100.
[33] Chiu R, Boyle WJ, Meek J, Smeal T, Hunter T, Karin M. The c-Fos protein interacts with c-Jun/AP-1 to stimulate transcription of AP-1 responsive genes. Cell 1988;54:541–52.
[34] Rauscher III FJ, Cohen DR, Curran T, Bos TJ, Vogt PK, Bohmann D, Tjian R, Franza Jr. BR. Fos-associated protein p39 is the product of the *jun* proto-oncogene. Science 1988;240:1010–6.
[35] Sassone-Corsi P, Ransone LJ, Lamph WW, Verma IM. Direct interaction between fos and jun nuclear oncoproteins: role of the ''leucine zipper'' domain. Nature 1988;336:692–5.
[36] Gass P, Bruehl C, Herdegen T, Kiessling M, Lutzenburg M, Witte OW. Induction of Fos and Jun proteins during focal epilepsy: congruences with and differences to [^{14}C]deoxyglucose metabolism. Brain Res Mol Brain Res 1997;46:177–84.
[37] Angel P, Karin M. The role of Fos, Jun and AP-1 complex in cell-proliferation and transformation. Biochim Biophys Acta 1991;1072: 129–57.
[38] Hai T, Curran T. Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. Proc Natl Acad Sci USA 1991;88:3720–4.
[39] Johnson W, Albanese C, Handwerger S, Williams T, Pestell RG, Jameson JL. Regulation of the human chorionic gonadotropin α- and β-subunit promoters by AP-2. J Biol Chem 1997;272:15405–12.
[40] O'Dwyer PJ, Yao K-S, Ford P, Godwin AK, Clayton M. Effects of hypoxia on detoxicating enzyme activity and expression in HT29 colon adenocarcinoma cells. Cancer Res 1997;54:3082–7.
[41] Vasilevskaya IA, O'Dwyer PJ. Effects of geldanamycin on signaling through activator protein-1 in hypoxic HT29 human colon adenocarcinoma cells. Cancer Res 1999;59:3935–40.
[42] Kim R, Beck WT. Differences between drug-sensitive and -resistant human leukemic CEM cells in c-*jun* expression, AP-1 DNA-binding activity, and formation of Jun/Fos family dimers, and their association with internucleosomal DNA ladders after treatment with VM-26. Cancer Res 1994;54:4958–66.
[43] Ritke MK, Bergoltz VV, Allan WP, Yalowich JC. Increased c-*jun*/AP-1 levels in etoposide-resistant human leukemia K562 cells. Biochem Pharmacol 1994;48:525–33.
[44] Yamazaki H, Schneider E, Myers CE, Sinha BK. Oncogene overexpression and *de novo* drug-resistance in human prostate cancer cells. Biochim Biophys Acta 1994;1226:89–96.
[45] Yang L-M, Kim H-T, Munoz-Medellin D, Reddy P, Brown PH. Induction of retinoid resistance in breast cancer cells by over-expression of c-Jun. Cancer Res 1997;57:4652–61.
[46] Johnston SRD, Lu B, Scott GK, Kushner PJ, Smith IE, Dowsett M, Benz CC. Increased activator protein-1 DNA binding and c-Jun NH_2-terminal kinase activity in human breast tumors with acquired tomoxifen resistance. Clin Cancer Res 1999;5:251–6.
[47] Chen Z, Seimiya H, Naito M, Mashima T, Kizaki A, Dan S, Imaizumi M, Ichijo H, Miyazono K, Tsuruo T. ASK1 mediates apoptotic cell death induced by genotoxic stress. Oncogene 1999;18:173–80.
[48] Santoro R, Wölfl S, Saluz HP. UV-laser induced protein/DNA crosslinking reveals sequence variations of DNA elements bound by c-Jun *in vivo*. Biochem Biophys Res Commun 1999;256:68–74.
[49] Hayakawa J, Ohmichi M, Kurachi H, Ikegami H, Kimura A, Matsuoka T, Jikihara H, Mercola D, Murata Y. Inhibition of extracellular signal-regulated protein kinase or c-Jun-N-terminal protein kinase cascade, differentially activated by cisplatin, sensitizes human ovarian cancer cell line. J Biol Chem 1999;274:31648–54.
[50] Rushmore TH, Pickett CB. The antioxidant response element: activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. J Biol Chem 1993;268: 11475–8.
[51] Jaiswal AK. The antioxidant response element. Biochem Pharmacol 1994;48:438–44.
[52] Mulcahy RT, Wartman MA, Bailey HH, Gipp JJ. Constitutive and β-naphthoflavone-induced expression of the human γ-glutamylcysteine synthetase heavy subunit gene is regulated by a distal antioxidant response element/TRE sequence. J Biol Chem 1997;272:7445–54.
[53] Li Y, Jaiswal AK. Regulation of human NAD(P)H:quinone oxidoreductase gene: role of AP-1 binding site contained within the human antioxidant response element. J Biol Chem 1992;267:15097–150104.
[54] Xie T, Belinsky M, Xu Y, Jaiswal AK. ARE- and TRE-mediated regulation of gene expression: response to xenobiotics and antioxidants. J Biol Chem 1995;270:6894–900.
[55] Prestera T, Holtzclaw WD, Zhang Y, Talalay P. Chemical and molecular regulation of enzymes that detoxify carcinogens. Proc Natl Acad Sci USA 1993;90:2965–9.
[56] Bergelson S, Pinkus R, Daniel V. Induction of AP-1 (Fos/Jun) by chemical agents mediates activation of glutathione S-transferase and quinone reductase gene expression. Oncogene 1994;9:565–71.
[57] Wasserman WW, Fahl WE. Functional antioxidant responsive elements. Proc Natl Acad Sci USA 1997;94:5361–6.
[58] Chen JY, Han X, Kan YW. Cloning of Nrf-1, an NF-E2-related transcription factor by genetic selection in yeast. Proc Natl Acad Sci USA 1993;90:11371–5.
[59] Moi P, Chan K, Asunis I, Cao A, Kan YW. Isolation of NF-E2-related factor 2 (Nrf-2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP-1 repeat of the β-globin locus control region. Proc Natl Acad Sci USA 1994;91:9926–30.
[60] Venugopal R, Jaiswal AK. Nrf-1 and Nrf-2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase gene. Proc Natl Acad Sci USA 1996;93:14960–5.
[61] Moinova HR, Mulcahy RT. An electrophile responsive element (EpRE) regulates β-naphthoflavone induction of the human γ-glutamylcysteine synthetase regulatory subunit gene: constitutive expression is mediated by an adjacent AP-1 site. J Biol Chem 1998;273:14683–9.
[62] Venugopal R, Jaiswal AK. Nrf-2 and Nrf-1 in association with Jun proteins regulate antioxidant response element-mediated expression and coordinated induction of genes encoding detoxifying enzymes. Oncogene 1998;17:3145–56.
[63] Johnson SW, Swiggard PA, Handel LM, Brennan JM, Godwin AK, Ozols RF, Hamilton TC. Relationship between platinum–DNA adduct formation and removal and cisplatin cytotoxicity in cisplatin-sensitive and -resistant human ovarian cancer cells. Cancer Res 1994;54: 5911–6.
[64] Schreiber M, Kolbus A, Piu F, Szabowski A, Möhle-Steinlein U, Tian J, Karin M, Angel P, Wagner EF. Control of cell cycle progression by c-Jun is p53 dependent. Genes Dev 1999;13:607–19.