Communication

The Jun Kinase/Stress-activated Protein Kinase Pathway Functions to Regulate DNA Repair and Inhibition of the Pathway Sensitizes Tumor Cells to Cisplatin

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We have studied the role of Jun/stress-activated protein kinase (JNK/SAPK) pathway in DNA repair and cisplatin resistance in T98G glioblastoma cells. JUN/SAPK is activated by DNA damage and phosphorylates serines 63 and 73 in the N-terminal domain of c-Jun, which is known to increase its transactivation properties. We show that treatment of T98G glioblastoma cells with cisplatin but not the transplatin isomer activates JNK/SAPK up to 10-fold in a dose-dependent manner. Furthermore, inhibition of this pathway in cells modified by expression of a nonphosphorylatable dominant negative mutant of c-Jun, dnJun, blocks DNA repair as judged by quantitative PCR and markedly decreases viability following treatment with cisplatin but not transplatin. Thus, JNK/SAPK is activated by cisplatin-induced DNA damage and is required for DNA repair and survival following cisplatin treatment.

EXPERIMENTAL PROCEDURES

Cells—Culture conditions and all cell lines and plasmids used here were developed using standard methods as described previously (22, 23). The expression of c-Jun and dnJun was quantitated using the methods (24) and antibodies previously characterized (24).

PCR—The PCR-stop assay (28) was used to quantitate cisplatin-induced DNA adduct formation and subsequent repair. The assay is based on the observation that the efficiency of amplification of cisplatin-treated DNA is inversely proportional to the degree of platination. Genomic DNA was isolated immediately or 6 h after treatment of cells for 1 h 15 min with varying amounts of cisplatin and amplified qualitatively using 32P-end-labeled primers, giving rise to a 2.7-kb and a nested 0.15-kb fragment of the hypoxanthine phosphoribosyl transferase gene. The 5′ and 3′ primers were TGGGATTACACGTGTGAACCAACC and GATCCACAGTCTGCCTGAGTCACT, respectively, with a 5′ nested primer of CCTAGAAAGCACATGGAGAGCTAG. The 0.15-kb segment of genomic DNA sustains undetectable levels of DNA damage under our conditions and serves as an internal PCR control and the basis for normalization of the amount of amplification of the 2.7-kb fragment. The number of lesions/2.7-kb fragment (i.e. Fig. 4) is calculated as 1 – (cpm damaged DNA/cpm undamaged DNA) (8).

JNK Assay—JNK assays were carried out exactly as described previously (29). Viability (29) was assessed by the addition of cisplatin to T98G cells at varying concentrations and determination of surviving cells 5 days later by addition of MTS for 1 h and determination of absorbance at 590 nm of the dissolved formazan product as described by the manufacturer.

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Extended titrations revealed IC50 values of 147 and 154 μM for cisplatin and transplatin for 1 h with a 1-h chase period followed by lysis and assay for JNK activity as described (7). Matching wells of cells were harvested and counted (Coulter counter) and used as the basis for sample loading. PBS, fetal bovine serum. B, positive control. T98G cells were exposed to the indicated doses of UV-C band (Stratalinker® UV cross-linker 1800) radiation and processed as described for A. C, JNK activity of human lung carcinoma M103 cells following treatment with 200 μM cisplatin or transplatin and processing as described for A with the addition of a 1-h chase prior to lysis.

RESULTS

Activation of JNK/SAPK by Cisplatin Requires DNA Adduct Formation—It is known that cisplatin but not transplatin forms covalent covalent cross-links between the N7 position of guanine or adjacent adenine-guanine residues (19, 20). We find that the JNK activity of T98G cells is elevated in a dose-dependent manner up to 10-fold following a 1-h exposure to cisplatin but not to transplatin (Fig. 1A). As a positive control of the effects of a DNA-damaging agent, we examined the response of JNK of T98G cells to UV-C irradiation (Fig. 1B) and observed a similar dose-response relationship. Cisplatin-specific responses have been observed in other cell lines from tumor types that are commonly refractory to cisplatin treatment (20). We find that the JNK activity of T98G cells or lung carcinoma cells M103 remains elevated, suggesting that treatment with cisplatin leads to a prolonged response. These results indicate that only the DNA-damaging cisplatin isomer activates JNK activity.

Dominant Negative c-Jun Sensitizes Tumor Cells to Cisplatin but Not Transplatin—We developed clonal lines of human T98G glioblastoma cells, which stably express a dominant negative inhibitor (1, 2) of the JNK/SAPK pathway, dnJun. Expression of dnJun has no effect on either basal AP-1 activity (1, 2) or on the enzyme activity of JNK (data not shown) but does inhibit phosphorylation-dependent activation of transcription (1, 2, 10, 12). The effect of cisplatin treatment on the viability of representative clonal lines of the dnJun-expressing T98G cells is compared with that of an empty vector control line, T98GLJCX, in Fig. 2A. The viability of empty vector control T98G cells remains largely unaffected by treatment with increasing concentrations of cisplatin even at doses of ≤70 μM. Extended titrations revealed IC50 values of 147 and 154 μM for the parental cells and empty vector control cells, respectively (Table I). In contrast, the dnJun-expressing cells exhibit an IC50 as low as 21 μM (Fig. 2A) or over 7-fold more sensitive to cisplatin than the control cells (Table I). Replicate experiments using additional clones that exhibit varying amounts of steady state dnJun indicate the sensitzation to cisplatin is proportional (rPearson = 0.98) to the amount of dnJun expressed (Fig. 2B). Transplatin has no discernible effect at concentrations where the viability following treatment with cisplatin is less than 25% (Fig. 3B) and in extended titrations no significant effect at 250 μM, indicating that the requirements for sensitization by dnJun depends upon the stereospecific DNA-binding properties of cisplatin, similar to the conditions for the activation of JNK.
Inhibition of JNK/SAPK Sensitizes Tumor Cells to Cisplatin

Sensitization of human tumor lines to cisplatin-induced cytotoxicity

IC₅₀ values were determined by direct titration of viability with cisplatin as described ("Experimental Procedures"). None of the cell lines examined here were made cisplatin-resistant prior to analysis.

| Cell                      | Control IC₅₀ (µM) | dnJun-expressing IC₅₀ (µM) | Cisplatin sensitization⁵ (IC₅₀)Parent/IC₅₀dnJun |
|---------------------------|-------------------|-----------------------------|-----------------------------------------------|
| T98G glioblastoma         | Parental 140 ± 13  | 21 ± 3                      | 7.0                                           |
|                           | Empty vector pLHCX| Empty vector pLHCX          | 140 ± 13                                      |
| U87 glioblastoma          | Parental 130 ± 53 | 50 ± 5                      | 2.6                                           |
|                           | Empty vector pLHCX| Empty vector pLHCX          | ND                                            |
| PC3 prostate carcinoma    | Parental 109 ± 13  | 16 ± 2                      | 6.9                                           |
|                           | Empty vector pMT64AA| Empty vector pMT64AA       | 156 ± 18                                      |
| MCF-7 breast carcinoma    | Parental 145 ± 25  | 38 ± 2                      | 3.8                                           |
|                           | Empty vector pLHCX| Empty vector pLHCX          | 101 ± 9                                       |

⁴ In all cases parental and empty vector cells were analyzed in parallel and with equal concentrations of cisplatin and transplatin in the range 0–250 µM all in quadruplicate. Transplatin had no effect on viability of any cell. ND, not done.

⁵ Sensitization is defined by the ratio of IC₅₀ values for the parental or empty vector control cells to the IC₅₀ value of the dnJun-expressing cells.

**DISCUSSION**

These studies show that the JNK/SAPK pathway is activated by cisplatin-induced DNA damage and is required for DNA repair and viability following cisplatin treatment. T98G glioblastoma cells modified to express a nonphosphorylatable domain of AP-1 owing to a deletion of residues 2–122 (21). As with dnJun, induction of TAM-67 in PC3 cells strongly enhances their sensitivity to cisplatin (Fig. 3A). We have determined that these TAM-67 and dnJun are expressed in approximately equal amounts, suggesting that the comparable degree of sensitization for TAM-67 and dnJun (Fig. 3A) is accounted for by interference in the role of phosphorylation-related function of c-Jun.

Similar results have been observed with an additional human glioblastoma line, U87, and an additional epithelial tumor line, MCF-7 (Table I). Clonal dnJun-expressing lines of these cells exhibit 2.6- and 3.8-fold decreased IC₅₀ values, respectively (Table I). Thus, the sum of results indicate that the JNK/SAPK pathway may have a general role in mediating a functional response to DNA-cisplatin adduct formation. Inhibition of this response sensitizes cells to the cell-killing properties of cisplatin.

Cisplatin Activates and dnJun Inhibits DNA Repair—We assessed the extent of genomic DNA damage and repair following cisplatin treatment using a modified PCR assay (25). For this assay, it has been shown that the degree of inhibition of PCR-amplified and amplified DNA purified from cisplatin-treated cells is a direct measure of the amount of DNA-cisplatin adduct formation as measured by atomic absorption (25). Thus, this assay provides a direct assessment of the extent of cisplatin-induced DNA damage.

DNA isolated from T98G cells immediately after treatment with 0, 100, or 200 µM cisplatin for 1 h exhibit increasing levels of DNA damage (Fig. 4A, circles). However, if a 6-h “recovery” period is introduced prior to the DNA purification, damage is markedly and significantly (p = 0.003) reduced (Fig. 4A, filled circles). As a positive control for the effects of inhibition of genomic DNA repair, an inhibitor of ADP-riboseylation, 2-aminobenzidine, was added at the time of treatment of the cells with cisplatin (Fig. 4A, squares). Following the 6-h recovery period, DNA damage remained unrepaird, and total DNA damage was substantially increased. Next, we compared the level of DNA damage for T98G cells and dnJun-expressing cells following treatment with cisplatin (Fig. 4B). For the dnJun-expressing T98G cells, 6 h after cisplatin treatment DNA damage remains completely unrepaired for cells treated at either 100 or 200 µM cisplatin (p > 0.53). All the results summarized here (Fig. 4, A and B) are the averages of three independent assays, which confirms the reliability of this observation. The sum of results, therefore, strongly indicates that expression of dnJun by T98G cells largely abolishes DNA repair following exposure of the cells to cisplatin.
Inhition of JNK/SAPK Sensitizes Tumor Cells to Cisplatin

Addendum—During the review of this manuscript we became aware that activation of JNK/SAPK by cisplatin has been reported by Liu et al. (Liu, Z.-G., Baskaran, R., Lea-Chou, E. T., Wood, L. D., Chen, Y., Karin, M., and Wang J. Y. J. (1996) Nature 384, 273–276).

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