[CANCER RESEARCH 61, 3486–3492, April 15, 2001]

# Increased Activator Protein 1 Activity as Well as Resistance to Heat-induced Radiosensitization, Hydrogen Peroxide, and Cisplatin Are Inhibited by Indomethacin in Oxidative Stress-resistant Cells[1]

**C. Matthew Bradbury,[2] Jacob E. Locke,[2] S. Jack Wei, Luis M. Rene, Shervin Karimpour, Clayton Hunt, Douglas R. Spitz, and David Gius[3]**

*Section of Cancer Biology, Radiation Oncology Center, Mallinckrodt Institute of Radiology, Washington University School of Medicine, St. Louis, Missouri 63108 [C. M. B., J. E. L., S. J. W., L. M. R., S. K., D. G.], and Free Radical and Radiation Biology Program, Department of Radiology, B180 Medical Laboratories, University of Iowa, Iowa City, Iowa 52242 [D. R. S.]*

## ABSTRACT

**It has been established that tumor cells develop resistance to a variety of therapeutic agents after multiple exposures to these agents/drugs. Many of these therapeutic agents also appear to increase the activity of transcription factors, such as activator protein 1 (AP-1), believed to be involved in cellular responses to oxidative stress. Therefore, we hypothesized that cellular resistance to cancer therapeutic agents may involve the increased activity of transcription factors that govern resistance to oxidative stress, such as AP-1. To investigate this hypothesis, a previously characterized cisplatin, hyperthermia, and oxidative stress-resistant Chinese hamster fibroblast cell line, OC-14, was compared to the parental HA-1 cell line. Electrophoretic mobility shift and Western blot assays performed on extracts isolated from OC-14 cells demonstrated a 10-fold increase in constitutive AP-1 DNA-binding activity as well as increased constitutive c-Fos and c-Jun immunoreactive protein relative to HA-1 cells. Treatment of OC-14 cells with indomethacin inhibited constitutive increases in AP-1 DNA-binding activity and c-Fos/c-Jun-immunoreactive protein levels. Clonogenic survival assays demonstrated that pretreatment with indomethacin, at concentrations that inhibited AP-1 activity, significantly reduced the resistance of OC-14 cells to heat-induced radiosensitization, hydrogen peroxide, and cisplatin. These results demonstrate a relationship between increases in AP-1 DNA-binding activity and increased cellular resistance to cancer therapeutic agents and oxidative stress that is inhibited by indomethacin. These results support the hypothesis that inhibition of AP-1 activity with nonsteroidal anti-inflammatory drugs, such as indomethacin, may represent a useful adjuvant to cancer therapy.**

## INTRODUCTION

A common dilemma in modern cancer treatment is the tendency of tumors to gain resistance to therapeutic agents following multiple exposures to the treatment agent (1). Radiotherapy, hyperthermia, and chemotherapy are used with differing degrees of regularity in the oncology clinic, partially attributable to differences in therapeutic efficacy between tumor types (2). To further confound their consistent use in cancer therapy, each of these treatments can lead to the development of resistance (3–5). When these treatments are combined, treatment effectiveness improves, and the enhanced responses (*e.g.*, heat-induced radiosensitization) that result can serve to treat malignancies that have developed resistance to single-modality therapies.

Previous investigations into the mechanisms by which IR[4], heat, and other presumptive oxidative stresses affect cells *in vitro* have yielded multiple complex molecular pathways to explain the cellular response to these insults (2, 5–8). The AP-1 transcription factor complex responds to heat shock (8) as well as to a variety of chemical stressors (9–11). The c-Fos and c-Jun immediate early gene transcriptional regulatory families, along with other proteins, comprise the mammalian transcription factor AP-1 (11, 12). These proteins form an array of heterodimeric protein complexes that bind to specific *cis*-acting DNA regulatory elements, known as AP-1 sites, to activate the expression of downstream target genes (12–15). Further work into the mechanism by which this pathway is triggered has shown that AP-1 is induced by the physical interaction of thioredoxin with the redox-sensitive signaling protein Ref-1 (16). Analysis of the effects of hyperthermia on the AP-1 complex has demonstrated that cellular stress from heat shock activates the AP-1 complex via redox-sensitive changes in Ref-1 (8), suggesting that the AP-1 complex may be inducing the expression of downstream genes that are responding to oxidative stress (8, 17). In addition, exposure of cells to $H_2O_2$, a generalized oxidative stress, has also been shown to activate AP-1 DNA binding and protein expression (18–20). Taken together, this suggests that the AP-1 transcription complex has been consistently linked to cellular responses to oxidative stress.

To further evaluate the relationship of AP-1 activity to the phenotype demonstrated by oxidative stress-resistant cells, the previously characterized OC-14 $H_2O_2$-resistant Chinese hamster fibroblast cell line was chosen (6, 17, 21, 22). The OC-14 cell line was selected from the parental HA-1 cell line by continuous exposure to progressively increasing concentrations of $H_2O_2$. In addition to a marked resistance to $H_2O_2$, OC-14 cells are known to have significantly augmented cellular antioxidant defenses, including increased glutathione content as well as enhanced catalase, superoxide dismutase, and glutathione peroxidase activities (22). In addition, these cells have been reported to demonstrate increases in the expression of c-jun mRNA, but the significance of this to AP-1 DNA-binding activity and the oxidative stress-resistant phenotype has not been explored (22).

NSAIDs comprise a class of medication used to alleviate cases of pain and inflammation, often through the inhibition cyclooxygenase activity (23, 24). Although frequently used as anti-inflammatory agents, NSAIDs are being used with greater regularity for additional, and often novel, uses (23, 25, 26). In addition to their cyclooxygenase-inhibitory properties, investigations relating NSAIDs to cellular responses to environmental stress have conclusively demonstrated that drug pretreatment has observable effects on cellular sensitivity to certain cytotoxic insults (23, 26–29). For example, NSAIDs have been shown to increase the responsiveness of murine tumors to IR

Received 9/21/00; accepted 2/19/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by grants from the NIH (1 K08 CA72602-01 and PO1 CA75556) and the American Cancer Society (ACS-IRG-58-010-43 and ACS RPG-00-292-01-TBE), to D. G. D. R. S. was supported by a grant from the NIH (R01 HL51469).

[2] C. M. B. and J. E. L. contributed equally to this manuscript.

[3] To whom requests for reprints should be addressed, at Section of Cancer Biology, Radiation Oncology Center, Mallinckrodt Institute of Radiology, Washington University School of Medicine, 4511 Forest Park Boulevard, Suite 402A, St. Louis, MO 63108. Phone: (314) 362-9781; Fax: (314) 362-9790; E-mail. davidg@radonc.wustl.edu.

[4] Thre abbreviations used are: IR, ionizing radiation; AP-1, activator protein 1; $H_2O_2$, hydrogen peroxide; Ref-1, redox factor 1; NSAID, nonsteroidal anti-inflammatory drug; DMF, dose modification factor; TER, thermal enhancement ratio; EMSA, electrophoretic mobility shift assay; PBS-T, PBS with 0.05% Tween 20; HSF, heat shock factor.

(30) and to potentiate heat-induced radiosensitization in mouse fibrosarcomas *in vivo* (26). Nuclear transcription factors and other biochemical events are also affected, as NSAIDs have been shown to inhibit immediate early response genes, such as nuclear factor-κB (24, 25, 27). Additionally, Dong *et al.* (24) have shown inhibition of AP-1 activity after treatment with the NSAID, sodium salicylate. Indomethacin is another NSAID which will often relieve symptoms at lower relative doses than many other such drugs, suggesting that further study into its role in the cellular response to stress is warranted.

In the current study, resistance of OC-14 cells to $H_2O_2$, cisplatin, and heat-induced radiosensitization, relative to the parental HA-1 cells was found to correlate with elevated constitutive AP-1 DNA-binding activity and overexpression of the affiliated proteins comprising the AP-1 transcription factor complex. Indomethacin administered at anticarcinogenic doses was found to down-regulate AP-1 activity in both HA-1 and OC-14 cells, as is evidenced by the inhibition of both basal and inducible AP-1 DNA binding. Concomitant with this down-regulation was a deceased resistance of OC-14 cells to the cytotoxic effects of hyperthermia, $H_2O_2$, and cisplatin. These results suggest that NSAIDs could be used to reverse cellular resistance to cancer therapeutic agents via the inhibition of AP-1 transcription factor activity and, as such, may represent a useful adjuvant to cancer therapy protocols.

## MATERIALS AND METHODS

**Cell Culture, Drug Treatments, Heat Shock, and IR Exposure.** Parental HA-1 and $H_2O_2$-resistant OC-14 cells (6, 17) were grown in Eagle's MEM supplemented with Earle's basic salt solution, 10% heat-inactivated (56°C, 30 min) FCS, penicillin (100 units/ml), and streptomycin (100 μg/ml) in a humidified 5% $CO_2$/95% air atmosphere at 37°C. Cells were seeded at $2 \times 10^5$ cells/100-mm culture dish and grown to 75–85% confluence ($4–5 \times 10^6$ cells/100-mm dish) prior to experimental treatment unless otherwise stated. Plating efficiencies of both cell lines were 60–70%.

Stock solutions of the NSAID indomethacin (50 mM; Sigma, St. Louis, MO) were prepared in 100% ethanol and added to the growth medium at 100–400 μM. Sham controls treated with ethanol alone were included in all experiments to control for the vehicle used to deliver the indomethacin. Cells were heated by submersing parafilm-sealed plates in a prewarmed, circulating water bath at 43 ± 0.1°C for intervals of 30 or 60 min, returned to 37°C for 1 h, and exposed to IR using a Pantak (Branford, CT) PMC1000 X-ray generator operated at 220 kV and 10 mA that includes an exposure chamber at 37°C in 5% $CO_2$ atmosphere. Control nonirradiated cells were placed into a similar environmentally controlled chamber next to the X-ray machine.

$H_2O_2$ stock solutions were made in sterile PBS, and their molar concentrations were determined by a spectrophotometric method previously described (6). Doses of $H_2O_2$ were delivered directly to the growth medium. Likewise, cisplatin (Sigma) stock solutions (0.25% w/v) were made in sterile $H_2O$ immediately prior to treatment of cells to prevent chemical hydration and potency degradation. Cells were treated with cisplatin added directly to the growth media. After addition of indomethacin, $H_2O_2$, and/or cisplatin to the growth media, dishes of treated cells were returned to a 37°C incubator for 1 h. Doses of $H_2O_2$ and cisplatin were chosen to allow for the calculation of a DMF at 10% or 50% isosurvival (6, 21), where $DMF_{(10, 50)}$ = (Dose to reach 10% (or 50%) survival in OC-14)/(Dose to reach 10% (or 50%) survival in HA-1).

**Clonogenic Cell Survival Assays.** HA-1 and OC-14 cells were plated at densities of $3.0 \times 10^5$ and $3.5 \times 10^5$ cells, respectively, per 100-mm tissue culture dish, grown exponentially for 2 days, pretreated with indomethacin (100–400 μM), incubated at 37°C for 1 h, and then treated with chemical stressors (*e.g.*, $H_2O_2$ or cisplatin) or other environmental stressing conditions (*e.g.*, heat shock or IR). At 1 h after exposure, cells were trypsinized and counted using a Coulter Counter (Beckman Coulter, Fullerton, CA). Dilutions of the treated cells were prepared, and duplicate 60-mm tissue culture dishes were seeded with 200–20,000 cells each, depending on the severity of the challenge treatment. Colonies were allowed to form in an undisturbed, humidified, 37°C/5% $CO_2$ environment for 7–10 days, fixed with 70% ethanol, stained with Coomassie Blue, and counted under a dissection microscope.

Only those plates containing 25–250 colonies were computed as statistically relevant; only those colonies containing at least 50 cells were considered to be viable survivors. Surviving fractions from the treated test cultures were normalized to sham-treated controls and plotted as a function of dose on a log/linear plot. TERs were calculated as previously described (31, 32) to determine the degree of heat-induced radiosensitization demonstrated by OC-14 cells relative to HA-1.

**Preparation of Whole-Cell Protein Extracts and Subcellular Fractions.** Protein extracts from whole cells and nuclear and cytoplasmic subcellular fractions were prepared for analysis via a method slightly modified from Dignam (33) and described by Curry *et al.* (2). Subcellular protein extracts were obtained from freshly trypsinized cells used for clonogenic cell survival by pipetting the remaining volume of the most concentrated dilution of treated cells (after plating dilutions had been made) into 15-ml centrifuge tubes and pelleting at 800 rpm for 5 min at 4°C. Following centrifugation, the pellet was resuspended and washed in 10 ml of 4°C PBS, followed by another 4°C centrifugation at 800 rpm for 5 min. This washing process was performed a second time, followed by resuspension of the pellet in PBS/2.5 mM EDTA and transfer of the cells into 1.5-ml microfuge tubes. From this point, the protocol for obtaining subcellular protein fractions, as previously described, was followed (2).

After preparation of whole-cell extracts or subcellular fractions, protein quantifications within each sample were performed via Bradford analysis (Bio-Rad, Hercules, CA) on a Beckman (Fullerton, CA) DU-640 spectrophotometer to ensure equal protein loading. All samples were thawed on ice and stored at −80°C to minimize protein denaturation.

**EMSAs.** Gel EMSAs were performed as described elsewhere (2, 8). Equal protein amounts (10–20 μg/sample) from whole-cell or subcellular extracts were incubated with a 10% poly(dI:dC) solution on ice for 15 min. After this incubation, a $^{32}$P-labeled oligonucleotide containing either an nuclear factor-κB or AP-1-specific binding domain (Promega, Madison, WI) was added to each sample and they were incubated at 25°C for 30 min. A loading dye consisting of 5× Tris-boric acid-EDTA solution (Bio-Rad), glycerol, and bromphenol blue was added, and the samples were loaded and electrophoresed on native 4.5% polyacrylamide gel. Gels were dried, exposed to a phosphorimaging screen overnight and analyzed on a Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using Image Quant software for quantification.

**Polyacrylamide-SDS Gel Electrophoresis and Western Blot Assays.** Equal amounts of protein, ranging from 10 to 30 μg/sample, were mixed with Laemmli lysis buffer and boiled for 5 min. Protein samples were then separated on a denaturing polyacrylamide-SDS gel and transferred to a nitrocellulose membrane using a semidry transfer apparatus (Owl Scientific, Inc., Portsmouth, NH). The membrane was blocked for 1 h in a 5% milk/PBS-T and was hybridized overnight at room temperature with a polyclonal antibody against c-Fos or c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA), each diluted 1:1000 in 2.5% milk in PBS-T. The membrane was washed three times for 15 min each in PBS-T, and then incubated for 1 h at room temperature with an antirabbit IgG-horseradish peroxidase secondary antibody (Santa Cruz Biotechnology) diluted 1:2000 in 2.5% milk in PBS-T. The membrane was again washed three times for 15 min each in PBS-T and then analyzed via an enhanced chemiluminescence method (Amersham Pharmacia Biotech, Newark, NJ) per the manufacturer's instructions. The assay was resolved on radiographic film (Eastman Kodak, Rochester, NY).

## Results

**OC-14 Cells Exhibit Resistance to Heat-induced Radiosensitization.** Parental HA-1 cells and $H_2O_2$-resistant OC-14 cells have been previously characterized as differing in their responses to a variety of environmental stresses, including survival responses to heat shock, cisplatin, and $H_2O_2$ (6, 17, 21). Experiments were performed with these cell lines to determine whether differences in heat-induced radiosensitization also exist. Both cell lines were treated with heat shock at 43°C for 60 min, followed by exposure to IR, or were treated with IR alone. As shown (Fig. 1*A*), there is no substantial difference in overall cell survival when the cell lines were exposed to IR alone, but an observable difference was seen when heat and radiation were

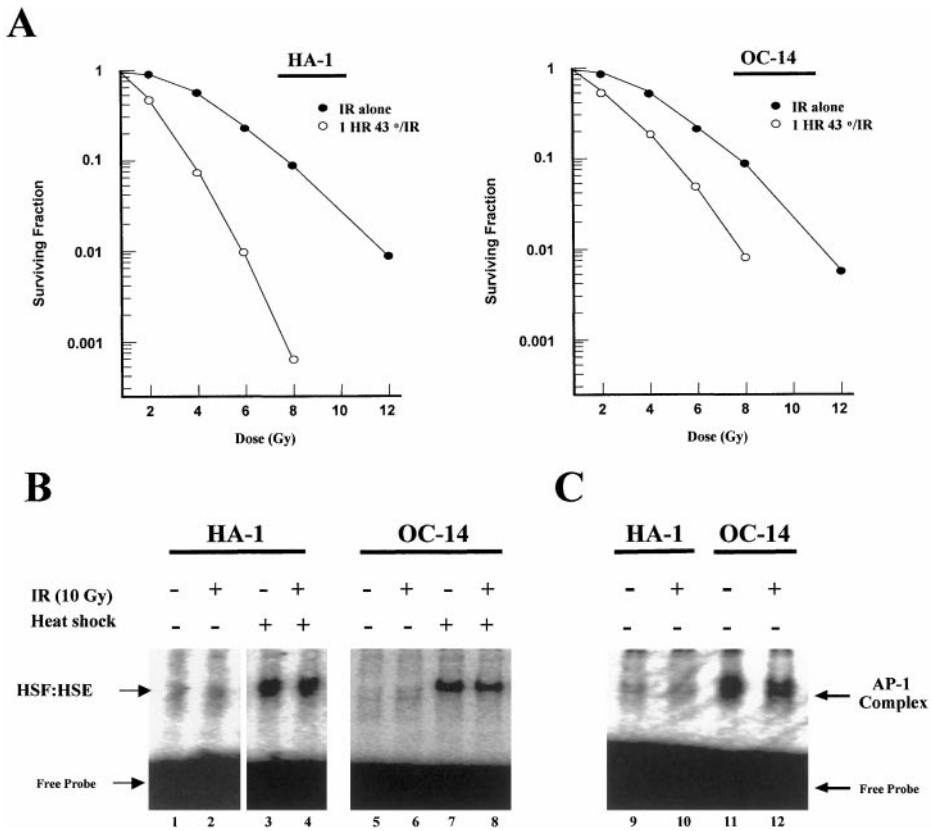


Fig. 1. $H_2O_2$-resistant OC-14 cells demonstrate resistance to heat-induced radiosensitization and increased AP-1 DNA-binding activity relative to the parental HA-1 cell line. *A*, clonogenic cell survival curves with HA-1 (*left panel*) and OC-14 cells (*right panel*) treated with heat shock (43°C for 60 min), followed by IR at 2, 4, 6, 8, and 12 Gy. After exposure, cells were trypsinized and plated at various densities from 200 to 20,000 cells per 60-mm dish. After roughly 7–10 days, colonies were fixed, stained, and counted, and the surviving fraction was plotted *versus* IR dose (Gy). The TERs calculated for HA-1 and OC-14 cells are 2.1 and 1.7, respectively. HSF (*B*) and AP-1 (*C*) DNA-binding activity after heat shock (*Lanes 3*, *4*, *7*, and *8*), IR (*Lanes 2*, *6*, *10*, and *12*), or both (*Lanes 4* and *8*) in parental HA-1 and OC-14 cells. HA-1 and OC-14 cells were heated at 43°C for 60 min, exposed to 10 Gy IR, or heated and then irradiated. Cells were then incubated at 37°C for 1 h before being harvested via subcellular fractionation. EMSA for HSF and AP-1 were accomplished as described in "Materials and Methods." *HSF: HSE* and *AP-1*, the protein-DNA complexes. *Free probe*, unbound HSF or AP-1 radioactive oligonucleotide.

combined. The oxidative stress-resistant OC-14 cell line demonstrated significant resistance to hyperthermic radiosensitization, with a TER at 10% isosurvival ($TER_{10}$) of 1.7 as compared to a $TER_{10}$ of 2.1 in HA-1 cells. The results of these experiments show that the $H_2O_2$-resistant OC-14 cells are also resistant to heat-induced radiosensitization.

**Elevated Constitutive AP-1 DNA Binding in OC-14 Cells.** Since OC-14 cells are resistant to the cytotoxicity of heat shock (17), one possible mechanism accounting for resistance may involve elevated basal and/or inducible HSF levels. Initial investigations determined whether OC-14 cells demonstrated increased HSF DNA-binding activity, which is a nuclear transcription factor that is inducible by heat in eukaryotic cells. HA-1 and OC-14 cells were heated at 43°C for 60 min, without and with IR, and EMSAs were performed using an oligomer containing the HSF DNA-binding element (Fig. 1*B*). These experiments demonstrated that constitutive levels of HSF were nearly identical in HA-1 and OC-14 cells (Fig. 1*B*, *Lanes 1 versus 5*). Following heat shock with or without IR, HA-1 and OC-14 cells demonstrated similar increases in HSF activation (Fig. 1*B*, *Lanes 3* and *4* and *7* and *8*). Likewise, both the constitutive and inducible levels of heat shock protein 70 were found to be identical in these cell lines (data not shown). These data suggest that resistance to environmental stresses in OC-14 cells does not involve the classical elements of the heat shock response.

An investigation into the possible role of the AP-1 transcription factor was initiated based on previous observations that heat shock activates the AP-1 complex via a redox-sensitive signaling protein (8), suggesting that the AP-1 complex may induce the expression of downstream genes that modify thermal cytotoxicity. The hypothesis that resistance to heat-induced radiosensitization demonstrated by OC-14 cells could be attributable to constitutive activation or superinduction of the AP-1 complex was tested.

The cellular extracts shown in Fig. 1*B* were used to determine the constitutive and inducible levels of AP-1 DNA-binding activity in HA-1 and OC-14 cells (Fig. 1*C*). EMSAs demonstrated a 10-fold increase in constitutive AP-1 DNA-binding activity in untreated OC-14 cells when compared to untreated HA-1 cells (Fig. 1*C*, *Lane 9 versus 11*). No change in AP-1 DNA-binding activity was noted in either cell line at this time point when the cells were irradiated (Fig. 1*C*, *Lane 9 versus 10* and *11 versus 12*). In addition, heat shock failed to further induce AP-1 DNA-binding activity in OC-14 cells, whereas a 3-fold increase in AP-1 binding was observed in HA-1 cells following heating (data not shown). These experiments demonstrate that heat- and $H_2O_2$-resistant OC-14 cells have markedly elevated constitutive AP-1 DNA-binding activity relative to the parental HA-1 cell line. These results suggest that increased AP-1 activity may contribute to the oxidative stress-resistant phenotype demonstrated by OC-14 cells.

**Indomethacin Inhibits Increased AP-1 DNA Binding in OC-14 Cells.** As previously discussed, indomethacin and other NSAIDs have been shown to have a variety of molecular consequences in multiple cell types, including inhibition of AP-1 and other transcription factor activity (24, 27). For this reason, pretreatment of OC-14 cells with indomethacin was hypothesized to inhibit the activation of AP-1 DNA-binding activity. To test this concept, OC-14 cells were exposed to increasing concentrations of indomethacin and harvested at 1 or 2 h after exposure. As previously demonstrated (Fig. 1*C*), AP-1 DNA-binding activity was constitutively increased in OC-14 cells (Fig. 2*A*, *Lane 1*) as compared to HA-1 cells (*Lane 2*). In OC-14 cells treated with 100, 200, or 300 $\mu$M indomethacin, a dose-dependent decrease in basal AP-1 DNA binding was observed at 1 h (Fig. 2*A*, *Lanes 3–5*) and at 2 h (*Lanes 6* and *7*). Specifically, at 200 $\mu$M the AP-1 DNA-binding activity is 2-fold greater than HA-1 cells (*lanes 4* and 6 *versus* 2) and at 300 $\mu$M the AP-1 DNA-binding activity is identical to HA-1 cells (*Lanes 5* and 7 *versus* 2). These results

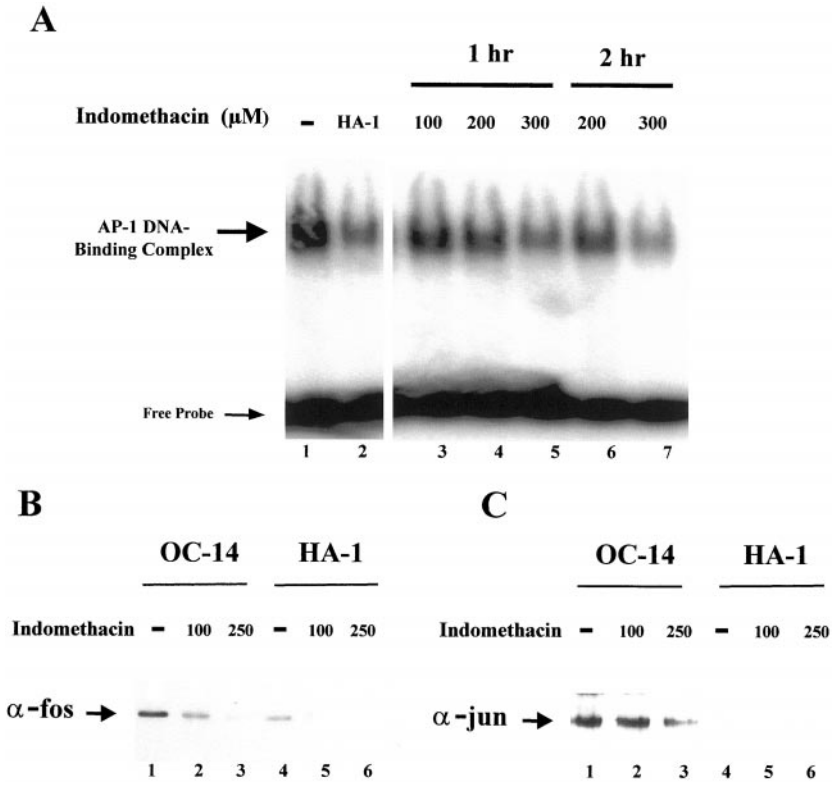


Fig. 2. Indomethacin inhibits constitutive AP-1 DNA binding and c-Fos and c-Jun protein levels in $H_2O_2$-resistant OC-14 cells. *A*, OC-14 cells were treated with indomethacin (via addition to the growth media), and cells were incubated at 37°C for 1 h or 2 h before being harvested for subcellular fractionation. Control OC-14 (*Lane 1*) and HA-1 cells (*Lane 2*) as well as OC-14 cells treated with 100, 200, or 300 $\mu$M indomethacin are shown. Samples were prepared for EMSA analysis by labeling with an AP-1-specific $^{32}$P-labeled oligonucleotide as previously described. The $H_2O_2$-resistant OC-14 cells constitutively expressed elevated levels of c-Fos (*B*)- and c-Jun (*C*)-immunoreactive protein, which was decreased by indomethacin treatment. Control (*Lanes 1* and *4*) OC-14 and HA-1 cells and cells treated with 100 $\mu$M (*Lanes 2* and *5*) or 250 $\mu$M (*Lanes 3* and *6*) indomethacin and harvested after 1 h. Levels of c-Fos- and c-Jun-immunoreactive protein levels were determined using anti-c-Fos or anti-c-Jun antibodies (Santa Cruz Biotechnology). Equal protein loading was determined using the Bradford assay.

demonstrate that exposure of OC-14 cells to indomethacin inhibits constitutive AP-1 DNA binding activity in a dose-dependent manner.

To determine whether the constitutively increased AP-1 DNA-binding activity was accompanied by increased accumulation of immunoreactive c-Fos and c-Jun proteins, total cellular protein was isolated from HA-1 and OC-14 cells and analyzed via Western blotting (Fig. 2, *B* and *C*, *Lanes 1 versus 4*). These results demonstrated that the increases in the constitutive levels of c-Fos- and c-Jun-immunoreactive protein in OC-14 cells (relative to HA-1) correlated with the relative increases seen in AP-1 DNA-binding activity (Fig. 1*C*). Finally, cells were treated with indomethacin to determine whether a similar decrease in c-Fos/c-Jun proteins paralleled the decrease in AP-1 DNA-binding activity seen in Fig. 2*A*. Indomethacin treatment resulted in a decrease in c-Fos and c-Jun protein (Fig. 2, *B* and *C*, *Lane 1 versus 2* and *3* and *Lane 4 versus 5* and *6*), similar to that observed with AP-1 DNA binding (see Fig. 2*A*). These experiments demonstrated that constitutive increases in AP-1 DNA-binding activity and c-Fos/c-Jun-immunoreactive protein levels observed in OC-14 cells, relative to HA-1, were suppressed by treatment with indomethacin.

**Indomethacin Inhibits the Resistance of OC-14 Cells to Heat-induced Radiosensitization.** The results presented above demonstrate that $H_2O_2$-resistant OC-14 cells exhibit resistance to a variety of cytotoxic agents and exhibit a marked increase in AP-1 DNA binding activity that can be inhibited by treatment with indomethacin. Interestingly, these cytotoxic agents ($H_2O_2$, cisplatin, and heat-induced radiosensitization) have all been suggested to cause oxidative stress (6, 17, 21). Furthermore, it is well documented that agents that induce oxidative stress also induce AP-1 DNA binding and transcriptional activity (8–10, 15, 16). Thus, experiments using indomethacin were performed to determine whether resistance of OC-14 cells to these agents was in part mediated by increased AP-1 activity.

OC-14 cells were treated with indomethacin (100 or 250 $\mu$M) for 1 h before and during heat shock and subsequent IR (Fig. 3*B*). These concentrations were chosen because 100 $\mu$M resulted in a roughly 50% decrease in AP-1 DNA-binding activity (Fig. 2*A*, *Lane 1 versus 3*) and 250 $\mu$M was the lowest concentration of indomethacin that appeared to completely inhibit the constitutive increase in OC-14 AP-1 DNA-binding activity, relative to HA-1 (data not shown). Clonogenic survival experiments demonstrated that pretreatment of OC-14 cells with indomethacin for 1 h before and during heat shock and IR resulted in a dose-dependent decrease in survival at 100 and 250 $\mu$M indomethacin (Fig. 3*B*). Similar results were obtained with HA-1 cells (Fig. 3*A*), although the effect of indomethacin was very modest relative to that seen in OC-14 cells (Fig. 3*B*). No change in survival was observed with indomethacin alone or with indomethacin and IR (data not shown). The results of these experiments show that indomethacin inhibits resistance of OC-14 cells to the cytotoxicity of heat-induced radiosensitization (relative to HA-1) and suggests that the mechanism may involve the inhibition of constitutive increases in AP-1 DNA-binding activity demonstrated by these cells.

**Indomethacin Inhibits AP-1 DNA-binding Activity and Resistance of OC-14 Cells to Cisplatin-induced Cytotoxicity.** It has previously been suggested that tumor cells respond to the cytotoxic stress of cisplatin via activation of the AP-1 transcription factor (34–37). OC-14 cells are also resistant to the cytotoxicity of cisplatin relative to HA-1 (21). Therefore, the effect of indomethacin on AP-1 DNA-binding activity and cellular resistance to cisplatin-induced cytotoxicity was tested in OC-14 cells. OC-14 cells were pretreated with indomethacin 1 h before and during a 1-h treatment with 0, 2.5, or 5.0 $\mu$g/ml of cisplatin (Fig. 4*A*, *Lanes 4–6*) and compared with OC-14 cells treated with 0, 2.5, or 5.0 $\mu$g/ml of cisplatin in the absence of indomethacin (Fig. 4*A*, *Lanes 1–3*). A small (1.5- to 2-fold), but reproducible increase in AP-1 DNA-binding activity was observed in OC-14 cells treated with cisplatin alone (Fig. 4*A*, *Lane 1 versus 2* and *3*). Constitutive AP-1 DNA-binding activity (Fig. 4*A*, *Lane 1 versus 4*) as well as the cisplatin-inducible AP-1 activity (Fig. 4*A*, *Lanes 2* and *3 versus 5* and *6*) were inhibited by pretreatment with 400 $\mu$M

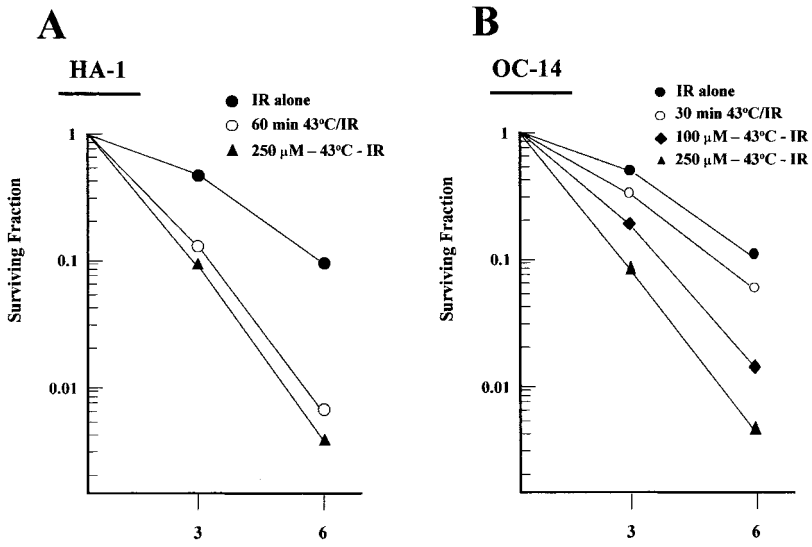


Fig. 3. Indomethacin inhibits resistance to heat-induced radiosensitization in $H_2O_2$-resistant OC-14 cells. Clonogenic cell survival curves with HA-1 (*left panel*) and OC-14 (*right panel*) cells after exposure to IR alone (●), pretreatment with heat shock (43°C) after IR (○), or treatment with 100 $\mu$M (♦) or 250 $\mu$M (▲) indomethacin 1 h before and during heating and IR. After exposure, cells were trypsinized and plated, and survival results were analyzed and plotted as described in the legend to Fig. 1.

indomethacin. Indomethacin had no apparent effect on the cytotoxic response to cisplatin in HA-1 cells (Fig. 4*B*). However, in OC-14 cells, the resistance to cisplatin, relative to HA-1, was nearly abolished by pretreatment with indomethacin (Fig. 4, *B* and *C*, *solid circles versus open circles*). These results show that indomethacin inhibits both AP-1 DNA-binding activity and cisplatin resistance in OC-14 cells, relative to HA-1.

**Indomethacin Inhibits $H_2O_2$-induced AP-1 Activity and Decreases Resistance of OC-14 Cells to $H_2O_2$-induced Cell Death.** To determine whether AP-1 DNA binding activity contributed to the $H_2O_2$-resistant phenotype demonstrated by OC-14, cells were pretreated with indomethacin (400 $\mu$M) 1 h before and during exposure to $H_2O_2$ (30 or 60 $\times$ $10^{-13}$ mol/cell). Similar to cisplatin, a small (1.5- to 2-fold) but reproducible increase in AP-1 DNA-binding activity

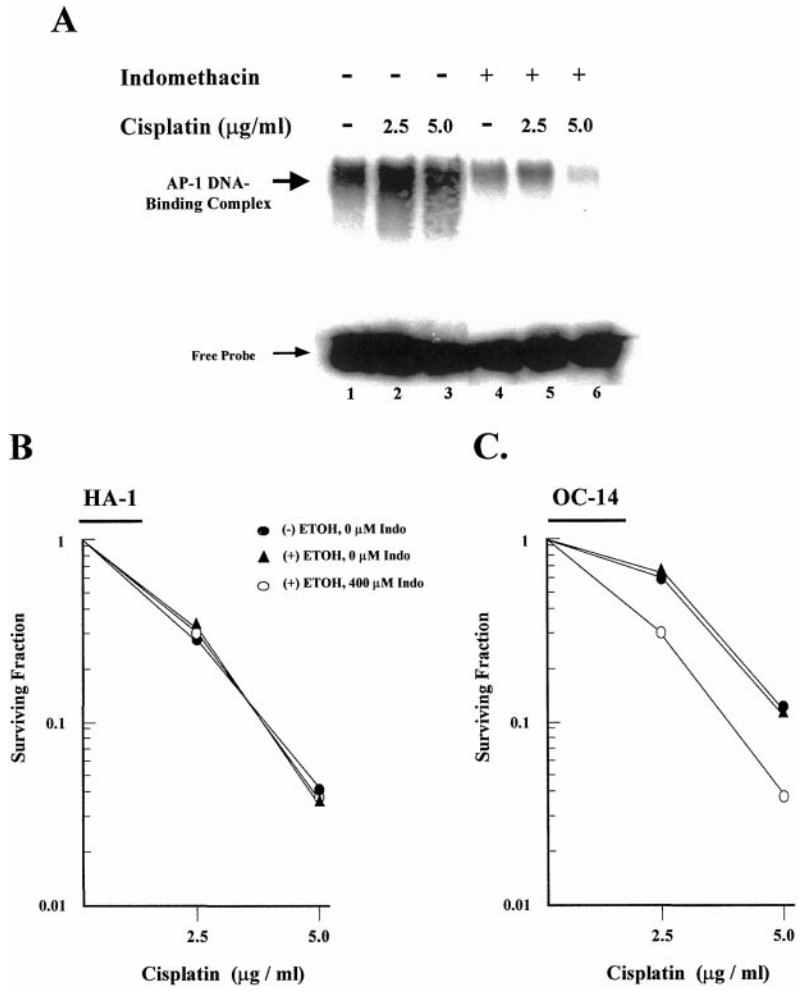


Fig. 4. Indomethacin (*Indo*) inhibits AP-1 DNA-binding activity and resistance to cisplatin in OC-14 cells. *A*, OC-14 cells were treated with or without 400 $\mu$M indomethacin for 1 h before and during exposure to cisplatin for 1 h at doses of 0 (*Lanes 1* and *4*), 2.5 $\mu$g/ml (*Lanes 2* and *5*), or 5.0 $\mu$g/ml (*Lanes 3* and *6*). Samples were prepared for EMSA analysis and probed with an AP-1-specific $^{32}$P-labeled oligonucleotide as previously described. *B* and *C*, indomethacin inhibits the resistance of OC-14 cells to the cytotoxicity of cisplatin, relative to HA-1. Clonogenic cell survival curves from HA-1 (*B*) and OC-14 (*C*) cells exposed as described in *A* to cisplatin (●), ethanol (*EtOH*) and cisplatin (▲), or 400 $\mu$M indomethacin and cisplatin (○). Survival data were analyzed and plotted as previously described.

was observed in cells treated with $H_2O_2$ (Fig. 5*A*, *Lanes 1–3*). ethanol had no effect on AP-1 DNA binding in the presence or absence of $H_2O_2$ (Fig. 5*A*, *Lanes 4–6*). Pretreatment of OC-14 cells with indomethacin substantially decreased AP-1 DNA-binding activity both in non-$H_2O_2$ challenged (Fig. 5*A*, *Lane 7*) as well as in $H_2O_2$-challenged OC-14 cells (Fig. 5*A*, *Lanes 8* and *9*). These results, along with those shown in Fig. 4, suggest that manipulation of AP-1 DNA-binding activity by indomethacin in OC-14 cells follows a similar pattern whether the cells are treated with cisplatin or $H_2O_2$. Finally, the effect of indomethacin on the resistance of OC-14 cells to $H_2O_2$ was tested. As shown in Fig. 5*B*, the survival of $H_2O_2$-treated OC-14 cells was reduced by pretreatment with indomethacin. The results of these experiments are similar to those observed for heat-induced radiosensitization (Fig. 3) and cisplatin (Fig. 4*C*), suggesting a possible common pathway involving AP-1 activity that could partially account for resistance to these cytotoxic agents demonstrated by OC-14 cells.

## DISCUSSION

The resistance of tumor cells to anticancer agents remains a major cause of failure in the treatment of cancer patients. The classic mechanism for the acquisition of a multidrug-resistant phenotype by cancer cells was believed to involve a single molecular mechanism, such as overexpression of P-glycoprotein (38, 39). However, it now appears that the multidrug-resistant phenotype represents a complex multifactorial process, with at least two or more resistance mechanisms (38). This may include resistance associated with decreased drug accumulation, altered intracellular drug distribution, increased detoxification, diminished drug-target interaction, increased DNA repair, altered cell-cycle regulation, and, most recently, changes in the levels of proteins and molecules that regulate the cellular oxidation/reduction status (6, 17, 21, 37–39).

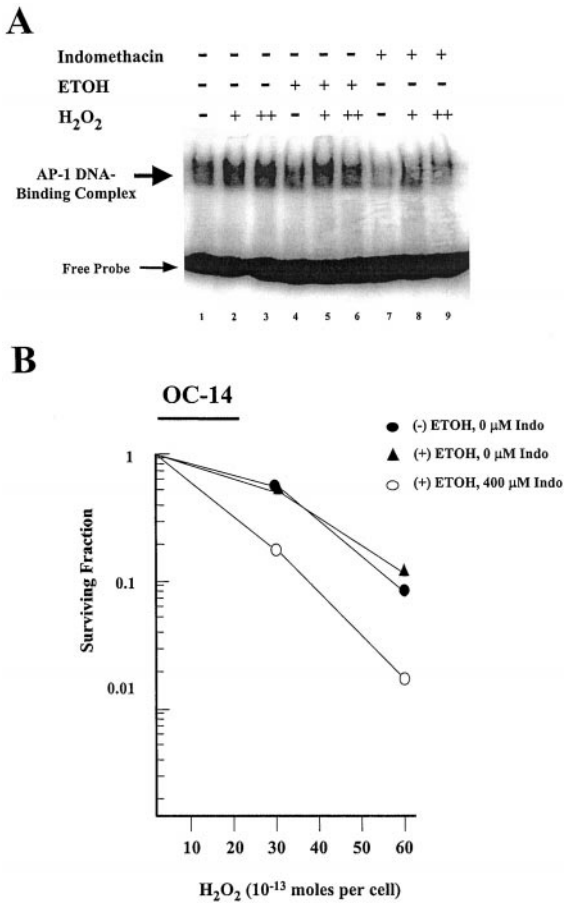


Fig. 5. Indomethacin (*Indo*) inhibits AP-1 DNA-binding activity and resistance to $H_2O_2$-induced cytotoxicity in OC-14 cells. *A*, OC-14 cells were exposed to $H_2O_2$ for 1 h at 37°C (30 or 60 $\times$ $10^{-13}$ mol/cell) without (*Lanes 2*, *3*, *5*, and *6*) or with 400 $\mu$M indomethacin (*Lanes 8* and *9*) 1 h before and during $H_2O_2$ exposure. Cells treated with ethanol (*EtOH*) served as a control for the solvent used to deliver the indomethacin (*Lanes 4–6*). Samples were prepared for EMSA analysis and probed with an AP-1-specific $^{32}$P-labeled oligonucleotide as previously described. *B*, indomethacin inhibits the resistance of OC-14 cells to the cytotoxicity of $H_2O_2$. Clonogenic cell survival curves obtained from OC-14 cells exposed as described in *A* to $H_2O_2$ (●), ethanol plus $H_2O_2$ (▲), or 400 $\mu$M indomethacin plus $H_2O_2$ (○). Survival data were analyzed and plotted as previously described.

Oxidative stress-resistant OC-14 cells (relative to parental HA-1 cells) exhibit remarkable constitutive increases in AP-1 DNA-binding activity and overexpression of the affiliated proteins of the AP-1 transcription factor complex (Figs. 1 and 2) as well as resistance to hyperthermia (17), cisplatin (21), heat-induced radiosensitization (Fig. 1), and $H_2O_2$ (6). Indomethacin has now been shown to inhibit both basal and inducible AP-1 DNA-binding activity in OC-14 cells as well as inhibit the resistance of OC-14 cells to the cytotoxic effects of heat-induced radiosensitization, cisplatin, and $H_2O_2$ (Figs. 2–5), relative to HA-1. The results of these experiments strongly suggest that increased AP-1 DNA-binding activity and the resultant downstream target genes activated by this transcription complex play a significant role in the multimodality-resistant phenotype demonstrated by the oxidative stress-resistant OC-14 cell line.

Relative to HA-1, $H_2O_2$-resistant OC-14 cells contain increased levels of several cellular antioxidants, including catalase, CuZn superoxide dismutase, heme oxygenase, total and reduced glutathione, glutathione *S*-transferase activity, and glutathione peroxidase activity (6, 17, 21, 22, 40, 41). These cellular antioxidants represent some of the major detoxification pathways that regulate intracellular oxidation/reduction status and cellular responses to oxidative stress. The precise mechanism(s) responsible for the broad spectrum of cellular antioxidant overexpression seen in OC-14 cells is currently not well understood, but thought to involve some type of coordinate regulation. Interestingly, the upstream regulatory elements for several of the aforementioned genes that regulate intracellular oxidation/reduction status contain AP-1 enhancer elements that are thought to play central roles in modulating the gene expression of cellular antioxidants (8, 9, 15, 22, 42). These previous results combined with the results presented in the current report support the hypothesis that the AP-1 complex contributes to the coordinate regulation of cellular oxidative stress responses that may contribute to the multimodality resistance demonstrated by tumors treated with systemic cytotoxic agents thought to induce oxidative stress. This hypothesis also fits well with the growing idea that transcription factors play a central role in the cellular response to cytotoxic agents, and, in selected tumors, immediate early gene overexpression may be predictive of clinical outcome (43, 44).

The mechanism of the effect of indomethacin on the regulation of the AP-1 complex in this work remains unknown. In this regard, it is interesting to speculate that indomethacin may increase the intracellular oxidative stress level above the threshold that the OC-14 antioxidant pathways can detoxify. Thus, when combined with a cytotoxic agent that also induces oxidative stress, there may be an increase in cell killing. Although this idea is speculative, recently it has been suggested that indomethacin increases intracellular oxidative stress as shown by others (45, 46) and our group.[5]

In summary, we have used indomethacin to inhibit the increased AP-1 DNA binding activity demonstrated by oxidative stress-resistant OC-14 cells exposed to heat-induced radiosensitization, $H_2O_2$, and

[5] C. M. Bradbury and D. Gius, manuscript in preparation.

cisplatin. In addition, the doses of indomethacin required to inhibit AP-1 DNA-binding activity also inhibited the resistance of OC-14 cells to the cytotoxicity of heat-induced radiosensitization, $H_2O_2$, and cisplatin. The results of these experiments suggest that indomethacin, and possibly other NSAIDs at intermediate concentrations, may represent novel agent(s) that can sensitize resistant tumor cells to therapeutic modalities via the inhibition of AP-1 activity. Taken together with previous investigations (8, 47, 48), these results also support the concept that a common central pathway mediating cellular responses to heat shock or other types of environmental or metabolic oxidative stress may involve redox-sensitive signaling pathways leading to AP-1 activation.

## REFERENCES


1. Kaye, S. B. Multidrug resistance: clinical relevance in solid tumours and strategies for circumvention. Curr. Opin. Oncol., *10:* S15–S19, 1998.
2. Curry, H. A., Clemens, R. A., Shah, S., Bradbury, C. M., Botero, A., Goswami, P., and Gius, D. Heat shock inhibits radiation-induced nuclear localization and activation of NF-κB DNA-binding. Possible role in radiation sensitization of tumor cells. J. Biol. Chem., *274:* 23061–23067, 1999.
3. Yu, L., Cohen, D., Piekarz, R. L., and Horwitz, S. B. Three distinct nuclear protein binding sites in the promoter of the murine multidrug resistance *mdr1b* gene. J. Biol. Chem., *268:* 7520–7526, 1993.
4. Dicato, M., Duhem, C., Pauly, M., and Ries, F. Multidrug resistance: molecular and clinical aspects. Cytokines. Cell. Mol. Ther., *3:* 91–99, 1997.
5. Hunt, C. R., Sim, J. E., Sullivan, S. J., Featherstone, T., Golden, W., Von Kapp-Herr, C., Hock, R. A., Gomez, R. A., Parsian, A. J., and Spitz, D. R. Genomic instability and catalase gene amplification induced by chronic exposure to oxidative stress. Cancer Res., *58:* 3986–3992, 1998.
6. Spitz, D. R., Kinter, M. T., and Roberts, R. J. Contribution of increased glutathione content to mechanisms of oxidative stress resistance in hydrogen peroxide resistant hamster fibroblasts. J. Cell. Physiol., *165:* 600–609, 1995.
7. Dewey, W. C. Arrhenius relationships from the molecule and cell to the clinic. Int. J. Hyperthermia, *10:* 457–463, 1994.
8. Diamond, D. A., Hunt, C. R., Spitz, D. R., Albee, L. D., II, Parsian, A., Goswami, P., and Gius, D. Redox factor-1 (Ref-1) mediates the activation of AP-1 in HeLa and NIH 3T3 cells in response to heat. J. Biol. Chem., *274:* 16959–16964, 1998.
9. Bergelson, S., Pinkus, R., and Daniel, V. Intracellular glutathione levels regulate Fos/Jun induction and activation of glutathione *S*-transferase gene expression. Cancer Res., *54:* 36–40, 1994.
10. Friling, R. S., Bergelson, S., and Daniel, V. Two adjacent AP-1-like binding sites form the electrophile-responsive element of the murine glutathione *S*-transferase Ya subunit gene. Proc. Natl. Acad. Sci. USA, *89:* 668–672, 1992.
11. Franza, B. R., Jr., Rauscher, F. J., III, Josephs, S. F., and Curran, T. The Fos complex and Fos-related antigens recognize sequence elements that contain AP-1 binding sites. Science (Wash DC), *249:* 1150–1153, 1988.
12. Karin, M., and Smeal, T. Control of transcription factors by signal transduction pathways: the beginning of the end. Trends Biochem. Sci., *17:* 418–422, 1992.
13. Angel, P., and Karin, M. The role of Jun, Fos, and the AP-1 complex in cell-proliferation and transformation. Biochim. Biophys. Acta, *1072:* 129–157, 1991.
14. Kerppola, T. K., and Curran, T. Transcription. Zen and the art of Fos and Jun. Nature (Lond.), *373:* 199–200, 1995.
15. Abate, C., Patel, L., Rauscher, F. J., III, and Curran, T. Redox regulation of Fos and Jun DNA-binding activity *in vitro*. Science (Wash DC), *249:* 1157–1161, 1990.
16. Hirota, K., Matsui, M., Iwata, S., Nishiyama, A., Mori, K., and Yodoi, J. AP-1 transcriptional activity is regulated by a direct association between thioredoxin and Ref-1. Proc. Natl. Acad. Sci., *94:* 3633–3639, 1997.
17. Spitz, D. R., and Li, G. C. Heat-induced cytotoxicity in $H_2O_2$-resistant Chinese hamster fibroblasts. J. Cell. Physiol., *142:* 255–260, 1990.
18. Li, W. C., Wang, G. M., Wang, R. R., and Spector, A.,. The redox active components $H_2O_2$ and *N*-acetyl-L-cysteine regulate expression of c-Jun and c-Fos in lens systems. Exp. Eye Res., *59:* 179–190, 1994.
19. Meyer, M., Schreck, R., and Baeuerle, P. A. $H_2O_2$ and antioxidants have opposite effects on activation of NF-κB and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. EMBO J., *12:* 2005–2015, 1993.
20. Meyer, M., Pahl, H. L., and Baeuerle, P. A. Regulation of the transcription factors NF-κB and AP-1 by redox changes. Chem. Biol. Interact., *91:* 91–100, 1994.
21. Spitz, D. R., Phillips, J. W., Adams, D. T., Sherman, C. M., Deen, D. F., and Li, G. C. Cellular resistance to oxidative stress is accompanied by resistance to cisplatin: the significance of increased catalase activity and total glutathione in hydrogen peroxide-resistant fibroblasts. J. Cell. Physiol., *156:* 72–79, 1993.
22. Guyton, K. Z., Spitz, D. R., and Holbrook, N. J. Expression of stress-response genes *GADD153, c-Jun*, and *heme oxygenase-1* in $H_2O_2$- and $O_2$-resistant fibroblasts. Free Radical Biol. Med., *20:* 735–741, 1996.
23. Shenoy, M. A., and Singh, B. B. Studies on indomethacin as a potentiator for hyperthermic and radiation responses in a mouse fibrosarcoma. Cancer Lett., *45:* 227–232, 1989.
24. Dong, Z., Huang, C., Brown, R. E., and Ma, W. Y. Inhibition of activator protein 1 activity and neoplastic transformation by aspirin. J. Biol. Chem., *272:* 9962–9970, 1997.
25. Scheuren, N., Bang, H., Munster, T., Brune, K., and Pahl, A. Modulation of transcription factor NF-κB by enantiomers of the nonsteroidal drug ibuprofen. Br. J. Pharmacol., *123:* 645–652, 1998.
26. Floersheim, G. L. Allopurinal, indomethacin, and riboflavin enhance radiation lethality in mice. Radiat. Res., *139:* 240–247, 1994.
27. Yamamoto, Y., Yin, M. J., Lin, K. M., and Gaynor, R. B. Sulindac inhibits activation of the NF-κB pathway. J. Biol. Chem., *274:* 27307–27314, 1999.
28. Jurivich, D. A., Sistonen, L., Sarge, K. D., and Morimoto, R. I. Effect of sodium salicylate on the human heat shock response. Science (Wash DC), *255:* 1243–1245, 1992.
29. Jurivich, D. A., Pachetti, C., Qiu, L., and Welk, J. F. Salicylate triggers heat shock factor differently than heat. J. Biol. Chem., *270:* 24489–24495, 1995.
30. Furuta, Y., Hunter, N., Barkley, T., Hall, E., and Milas, L. Increase in radioresponse of murine tumors by treatment with indomethacin. Cancer Res., *48:* 3008–3013, 1988.
31. Iliakis, G., Seaner, R., and Okayasu, R. Effects of hyperthermia on the repair of radiation-induced DNA single- and double-strand breaks in DNA double-strand break repair-deficient and repair-proficient cell lines. Int. J. Hyperthermia, *6:* 813–833, 1990.
32. Maity, A., Kao, G. D., Muschel, R. J., and McKenna, W. G. Potential molecular targets for manipulating the radiation response. Int. J. Radiat. Oncol. Biol. Phys., *37:* 639–653, 1997.
33. Dignam, J. D. Preparation of extracts from higher eukaryotes. Methods Enzymol., *182:* 194–203, 1990.
34. Bhushan, A., Abramson, R., Chiu, J. F., and Tritton, T. R. Expression of c-fos in human and murine multidrug-resistant cells. Mol. Pharmacol., *42:* 69–74, 1992.
35. Yao, K. S., Godwin, A. K., Johnson, S. W., Ozols, R. F., O'Dwyer, P. J., and Hamilton, T. C. Evidence for altered regulation of γ-glutamylcysteine synthetase gene expression among cisplatin-sensitive and cisplatin-resistant human ovarian cancer cell lines. Cancer Res., *55:* 4367–4274, 1995.
36. Rabo, Y. B., Shoshan, M. C., Linder, S., and Hansson, J. Different mechanisms are responsible for c-jun mRNA induction by cisplatin and ultraviolet light. Int. J. Cancer, *65:* 821–826, 1996.
37. Kern, M. A., Helmbach, H., Artuc, M., Karmann, D., Jurgovsky, K., and Schadendorf, D. Human melanoma cell lines selected *in vitro* displaying various levels of drug resistance against cisplatin, fotemustine, vindesine, or etoposide: modulation of proto-oncogene expression. Anticancer Res., *17:* 4359–4370, 1997.
38. Larsen, A. K., Escargueila, A. E., and Skladanowski, A. Resistance mechanisms associated with altered intracellular distribution of anticancer agents. Pharmacol. Ther., *85:* 217–229, 2000.
39. Larsen, A. K., and Skladanowski, A. Cellular resistance to topoisomerase-targeted drugs: from drug uptake to cell death. Biochim. Biophys. Acta, *1400:* 257–274, 1998.
40. Winters, R., Zukowski, J., Ercal, N., Matthews, R., and Spitz, D. R. Analysis of glutathione, glutathione disulfide, cysteine, homocysteine, and other biological thiols by high-performance liquid chromatography following derivatization by *N*-(1-pyrenyl)maleimide. Anal Biochem., *227:* 14–21, 1995.
41. Dennery, P. A., Wong, H. E., Sridhar, K. J., Rodgers, P., Sim, J. E., and Spitz, D. R. Differences in basal and hyperoxia associated heme oxygenase expression in oxidant resistant hamster fibroblasts. Am. J. Physiol., *15:* L672–L679, 1996.
42. Sekhar, K. R., Meredith, M. J., Kerr, L. D., Soltaninassab, S. R., Spitz, D. R., Xu, Z. Q., and Freeman, M. L. Expression of glutathione and γ-glutamylcysteine synthetase mRNA is JUN dependent. Biochem. Biophys. Res. Commun., *234:* 588–593, 1997.
43. Russo, A., Mitchell, J. B., DeGraff, W., Friedman, N., and Gamson, J. Depletion of cellular glutathione by exogenous spermine in V79 cells: implications for spermine-induced hyperthermic sensitization. Cancer Res., *45:* 4910–4914, 1985.
44. Weichselbaum, R. R., Hallahan, D. E., Fuks, Z., and Kufe, D. Radiation induction of immediate early genes: effectors of the radiation-stress response. Int. J. Radiat. Oncol. Biol. Phys., *30:* 229–234, 1994.
45. Barton, A. E., Bayley, D. L., Mikami, M., Llewellyn-Jones, C. G., and Stockley, R. A. Phenotypic changes in neutrophils related to anti-inflammatory therapy. Biochim. Biophys. Acta, *1500:* 108–118, 2000.
46. Giardina, C., and Inan, M. S. Nonsteroidal anti-inflammatory drugs, short-chain fatty acids, and reactive oxygen metabolism in human colorectal cancer cells. Biochim. Biophys. Acta, *1401:* 277–288, 1998.
47. Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Llamazares, A., Zamanillo, D., Hunt, T., and Nebreda, A. A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. Cell, *78:* 1027–1037, 1994.
48. Stevenson, M. A., Zhao, M. J., Asea, A., Coleman, C. N., and Calderwood, S. K. Salicylic acid and aspirin inhibit the activity of RSK2 kinase and repress RSK2-dependent transcription of cyclic AMP response element binding protein- and NF-κB-responsive genes. J. Immunol., *163:* 5608–5616, 1999.