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Factors Secreted by Human Neuroblastoma Mediate Doxorubicin Resistance by Activating STAT3 and Inhibiting Apoptosis

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Abstract

Background: The transcription factor Stat3 has been reported to play a key role in protecting cells against apoptosis by up-regulating expression of the anti-apoptotic gene *Bcl-x_L*. This investigation analyzes the relationship between the development of resistance to doxorubicin-mediated apoptosis in neuroblastoma cells (SKN-SH) and activation of the Stat3 signaling pathway.

Materials and Methods: A drug-resistant cell line (SKN-SH/Dox6) was generated by continuous exposure to incremental concentrations of doxorubicin. Specific antibodies were utilized for Western blots and confocal microscopy to determine the nuclear localization of activated Stat3.

Results: Doxorubicin-mediated DNA fragmentation was inhibited and caspase-3 activity decreased in SKN-SH/Dox6 cells. Up-regulation of Stat3 phosphorylation and Bcl-x_L expression, increased nuclear translocation of phospho-Stat3, and binding to DNA occurred only in resistant SKN-SH/Dox6 cells. The expression of Bcl-x_L was inhibited by AG490, an inhibitor of the JAK/Stat3 signaling pathway, suggesting that the regulation of Bcl-x_L and Stat3 involved a common mechanism. Activation of Stat3 in SKN-SH/Dox6 cells was contingent upon stimulation evoked by ligands secreted by the drug-resistant cells. Evidence to support this hypothesis was provided by experiments in which doxorubicin-sensitive SKN-SH cells were preincubated with conditioned media obtained from doxorubicin-resistant SKN-SH/Dox6 cells. This treatment increased Stat3 activation. It also rendered SKN-SH cells resistant to doxorubicin as demonstrated by a sharp decrease in doxorubicin-induced DNA degradation and cytotoxic potency.

Conclusions: These findings suggest that the resistance of SKN-SH/Dox6 cells to doxorubicin may be mediated by anti-apoptotic factor(s) that are synthesized and secreted by tumor cells in response to cytotoxic agents.

Introduction

Stats are a family of transcription factors that become activated by phosphorylation in response to extracellular ligands such as cytokines, hormones, or growth factors (1). Upon activation, Stat proteins dimerize and translocate to the nucleus, where they bind to the promoter regions of target genes. The physiologic roles of Stat molecules have been examined by a variety of approaches including the use of knock-out mice (2). Animals lacking Stat3 did not survive beyond early embryogenesis (3), suggesting that this transcription factor is essential for cell survival and organ development.

Recently it has been reported that constitutive activation of Stat3 inhibits fas-mediated apoptosis and, therefore, contributes to the pathogenesis of multiple myeloma (4), squamous cell carcinoma (5), and prostate cancer (6). Inhibition of apoptosis by activated Stat3 was found to be mediated by up-regulation of *Bcl-x_L* gene expression. The direct relationship between Stat3 and *Bcl-x_L* was demonstrated by in vitro experiments showing the ability of this transcription factor to interact with the *Bcl-x_L* gene promoter, causing its up-regulation in myeloma cells (4). Humoral factors that stimulate the gp130/JAK signaling pathway upstream of Stat3, such as interleukin-6, ciliary neurotrophic factor, and leukemia inhibitory factor, can also suppress apoptosis by inducing expression of anti-apoptotic genes of the Bcl-2 family (7–10). Pharmacologic inhibition of this signaling pathway with the JAK2 inhibitor, AG490, inhibited Stat3 activation, decreased expression of the anti-apoptotic gene Bcl-2, and enhanced expression of the pro-apoptotic gene *Bax* (11,12). Additional data implicating Stat3 in the protection of tumor cells against apoptosis have been provided by specific targeting of this signaling pathway, using a dominant negative Stat3. Introduction of the dominant negative

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Stat3 in mice led to the inhibition of tumor progression by enhancement of tumor cell apoptosis (6,13).

Treatment of tumor cells with cytotoxic drugs is often associated with increased apoptosis (14,15). Moreover, recent studies have demonstrated that inhibition of apoptosis was associated with drug resistance in different types of malignant tumors (16–18). Accordingly, the role of Stat3 in promoting tumor cell protection against cytotoxic drug-mediated apoptosis and the development of drug resistance has become of critical interest.

The present investigation addresses the putative association between Stat3 mediated anti-apoptosis and resistance to doxorubicin in human neuroblastoma cells. These findings demonstrate that extracellular secretion of factors synthesized by drug-resistant tumor cells can stimulate Stat3 signaling and modulate doxorubicin-mediated cytotoxicity.

Material and Methods

Chemicals and Reagents

The following items were purchased from the companies cited: Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) (BioWhittaker, Walkersville, MD, USA); doxorubicin, 3-(4, 5-dimethyl-2-thiazolyl)2,5-diphenyl tetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA); antibodies to Stat3 and phospho-Stat3 (New England Biolabs, Beverly, MA, USA); antibodies to Bcl-2, Bcl-$x_{L/S}$, and NF kappa B (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); secondary antibodies conjugated to horseradish peroxidase and enhanced chemiluminescence reagents (ECL) (Amersham, Arlington Heights, IL, USA); immobilon-P transfer membrane for Western blot (Millipore, Bedford, MA, USA); and [γ^{32}P]-ATP (9.25 Mbq) (Dupont NEN, Boston, MA, USA). The oligonucleotide sequence SIE (59-GTG CAT TTC CCG TAA ATC TTG TCT ACA-39) was obtained from Santa Cruz Biotechnology. The T4 polynucleotide kinase was acquired from USB (Cleveland, OH, USA), poly (dI-dC), tyrphostin (AG490) from Pharmacia Biotech Inc., Piscatoway, NJ, USA, and the caspase-3-3 colorimetric assay kit from R&D Systems Inc. (Minneapolis, MN, USA).

Cell Culture, Generation of Doxorubicin Resistant Cells, and Cytotoxicity Assay

Neuroblastoma (SKN-SH) cells were maintained in DMEM supplemented with 10% FBS at 378C in a 5% CO_2 atmosphere. Doxorubicin-resistant cells (SKN-SH/Dox6), were generated by incubating the wild type SKN-SH cells with incremental concentrations of doxorubicin ranging from 10^{29} to 10^{26} M over a period of 6 months. Cytotoxic activity of the drug was quantitatively determined by a colorimetric assay utilizing MTT (19). Cells were seeded at 10^4 cells/well in 96-well plates and maintained in culture for 24 hr at 378C in DMEM supplemented with 10% FBS. Doxorubicin (10^{29} to 10^{26}M) was then added to designated wells and the cells incubated for an additional 72 hr. MTT (10 μl of 5 mg/ml solution) was added to each well and incubated for 4 hr at 378C. The cells were solubilized in 100 μl of HCl 0.5N/isopropanol and incubated for 15 hr at 378C. The optical density of this solution was measured at 570 nm and cell survival estimated by comparison with untreated control cells.

The effect of conditioned media on the cytotoxic potency of doxorubicin was determined by harvesting media from SKN-SH or SKN-SH/Dox6 cells that had been seeded at 5×10^5 cells/ml and incubated for 48 hr. The media was collected under sterile conditions and transferred to flasks containing 5×10^5 cells/ml of the SKN-SH or SKN-SH/Dox6 cell lines. Following incubation for 48 hr, doxorubicin was added at a specific concentration and incubation continued for an additional 48 hr, at which time cell number or other parameters were determined.

Detection of Apoptosis

Apoptosis was assessed either by determination of DNA fragmentation or by measure of caspase-3 activity. Cells were incubated with doxorubicin (1 μM) for 24 hr at 378C and DNA prepared from Triton X-100 lysates for analysis of fragmentation (20). Briefly, cells were lysed in hypotonic solution containing 10 mM Tris HCl (pH 7.4), 1 mM EDTA, and 0.2% Triton X-100, and centrifuged at 11,000 g for 5 min. Supernatants were electrophoresed on a 1% agarose gel and the DNA fragments visualized by UV light after staining with ethidium bromide.

Caspase-3 activity was determined in cell extracts after incubation with doxorubicin (1 μM) for 24 hr. After washing (twice with PBS), the cells were lysed by addition of 100 μl of lysis buffer (caspase-3 colorimetric assay kit, R&D Systems). Caspase-3 activity was assayed by preincubating 50 μl of cell lysate containing 200 μg of protein in a 96-well plate with 50 μl of $2\times$ reaction buffer and 5 μl of caspase-3 substrate (DEVD-pNA) for 2 hr at 378C. Optical density was determined at 405 nm using an ELISA plate reader.

Western Blot Analysis

The cells were seeded in 25 cm^2 flasks containing DMEM supplemented with 10% FBS and incubated at 378C. After 48 hr of incubation, the cells were washed with cold PBS, and the monolayer was solubilized by the addition of 200 μl of lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 100 mM NaF, 1 mM $MgCl_2$, 1.5 mM EGTA, 10% glycerol, 1% Triton X100, 1 μg/ml leupeptin, and 1 mM phenylmethyl sulfonyl fluoride). Insoluble material was removed by centrifugation and the protein concentration of the supernatant determined. The soluble protein fraction (50 μg) was electrophoresed on SDS-PAGE and transferred to an Immobilon-P

membrane. Stat3, Phospho-Stat3, NF kappa B, and Bcl-x proteins were identified by incubation of the membrane with specific antibodies. Complexes were detected by sequential blotting with biotinylated secondary antibodies linked to peroxidase and reactive bands identified by ECL.

Preparation of Nuclear and Cytoplasmic Protein Fractions

Nuclear extracts were prepared from SKN-SH and SKN-SH/Dox6 cells following a previously described procedure (21). Cells grown in 75 cm^2 flasks were scraped into 10 ml tris-buffered saline (TBS) and pelleted by centrifugation (1500 g for 5 min). After a second wash with TBS, the pellet was re-suspended in 400 μl of buffer A (10 mM HEPES pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 0.5 mM PMSF). The mixture was incubated for 30 min on ice, then 25 μl of a 10% solution of Nonidet NP-40 was added and the homogenate centrifuged for 30 sec. The nuclear pellet was resuspended in 50 μl of ice cold buffer B (20 mM HEPES pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM dithiothreitol; 1 mM PMSF). The samples were then rocked at 48C for 30 min. Following centrifugation (12000 g for 5 min), protein concentration was determined in the supernatant and samples stored at 2 708C for Western blot or DNA gel shift assay.

DNA Gel Shift Assay

SIE oligonucleotides were radiolabeled with [^{32}P]-ATP using T4 polynucleotide kinase as described previously (21). The radiolabeled sequence was then purified by passage through a Nuc-Trap Probe column (Stratagene, La Jolla, CA, USA). Radiolabeled SIE (50,000 cpm) was incubated with 5 μg of nuclear protein in the presence of 2 μg poly (dI-dC) for 30 min at room temperature in a total volume of 20 μl, containing 10 mM HEPES pH 7.9, 50 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$, 10% (v/v) glycerol, and 5 mM dithiothreitol. The samples were resolved by electrophoresis using a 5% polyacrylamide gel. The gel was dried and radioactive bands detected by autoradiography.

Confocal Microscopy

Cellular localization of phospho-Stat3 was determined by confocal microscopy. Cells were seeded onto sterile glass slides and incubated for 24 hr in DMEM containing 10% FBS. The media was aspirated, the cells washed with PBS, and then fixed with PBS containing 3% paraformaldehyde for 10 min at 48C. Nonspecific sites were blocked by incubating slides in TBS-0.1% Tween 20 buffer (TBST) containing 5.5% Normal Goat Serum for 1 hr at room temperature. The cells were then incubated with anti-phospho-Stat3 for 24 hr at 4°C. After washing with TBST, the cells were incubated with FITC-labeled anti-rabbit (1/500) in TBST for an additional 45 min and localization of phospho-Stat3 visualized by confocal microscopy.

Statistical Analyses

A two-sided paired *t*-test was used to determine statistical significance. *p* values for all comparisons are presented in the text (significance is set at p , 0.05).

Results

Characterization of Doxorubicin-Induced Cell Death in SKN-SH and SKN-SH/Dox6 Human Neuroblastoma Cells

The data shown in Figure 1A demonstrated that SKN-SH/Dox6 cells were much less sensitive to doxorubicin-mediated cell death (IC_{50} of 6.4 3 10^{25} M) than the wild-type SKN-SH cells (IC_{50} of 4 3 10^{27} M). The MTT assay used to quantify cell death in this cellular model does not, however,

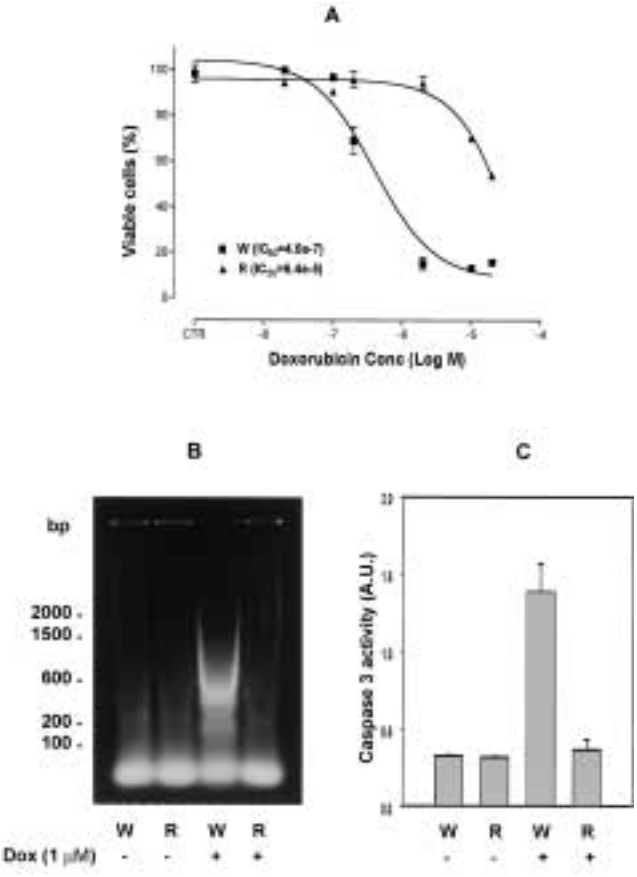

Fig. 1. Differential effects of doxorubicin on SKN-SH wild-type (W) and SKN-SH/Dox6 drug-resistant (R) human neuroblastoma cells. (A) Cytotoxic response of W and R cells to doxorubicin. Cells were incubated with doxorubicin for 72 hr, after which cell viability was determined by the MTT assay. **(B)** DNA degradation following incubation of W and R cells with doxorubicin (1 μM) for 24 hr. Equal amounts of DNA were electrophoresed on 1% agarose gel. DNA fragments were stained with ethidium bromide and visualized under UV light. **(C)** Caspase-3 activity in W and R cells. Cells were treated with doxorubicin (1 μM) for 24 hr and caspase-3 activity assayed in the cell lysate. Note that caspase-3 activity correlates with DNA degradation shown in Panel B. Data are expressed in arbitrary units (AU) and represent the mean 6 SEM of triplicate determinations.

provide information about the cellular mechanism that mediates doxorubicin cytotoxicity.

Because many cytotoxic drugs, including doxorubicin, exert their cytotoxic action by inducing apoptotic cell death (22,23) the role of doxorubicin-mediated apoptosis as a molecular mechanism for drug toxicity was investigated in this model. Doxorubicin (1 μM) induced DNA fragmentation in wild-type SKN-SH cells (Fig. 1B); in contrast, similar treatment did not affect DNA integrity in drug-resistant SKN-SH/Dox6 cells. The activity of caspase-3, a key mediator in the apoptotic signaling cascade (24), was also suppressed in SKN-SH/Dox6 cells when compared to SKN-SH cells, following incubation with doxorubicin (Fig. 1C). These data suggest that alterations in anti-apoptotic signaling pathways may be responsible for the decreased susceptibility of SKN-SH/Dox6 cells to doxorubicin-induced apoptosis.

Stat3 Activation in SKN-SH and SKN-SH/Dox6 Human Neuroblastoma Cells

The expression of Stat3 and its activation were compared in SKN-SH and SKN-SH/Dox6 cells to determine their correlation with the development of drug resistance in these cells.

Western blot analyses demonstrated the presence of Stat3 in both SKN-SH and SKN-SH/Dox6 cell lines. The expression of this transcription factor in both the cytoplasmic and nuclear fractions of SKN-SH/Dox6 cells was slightly greater than that of SKN-SH cells (Fig. 2A). In contrast, phospho-Stat3 was strongly expressed in the nuclei of SKN-SH/Dox6 cells (p , 0.03), and almost absent in SKN-SH cells (Fig. 2A and 2B). Expression of NF-kappa B, which served as an internal control, did not differ between the two cell lines.

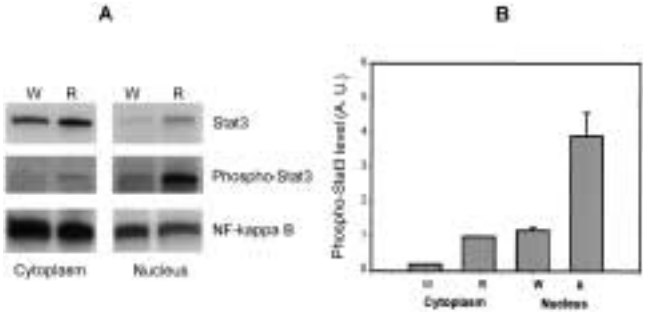

Fig. 2. Expression and phosphorylation of Stat3 in SKN-SH wild-type (W) and SKN-SH/Dox6 drug-resistant (R) human neuroblastoma cells. (**A**) Western blot of Stat3, phospho-Stat3, and NF-kappa B in the cytoplasm and nucleus of W and R cells. Cytoplasmic and nuclear fractions were prepared as described in "Methods." Proteins were electrophoresed on SDS-PAGE and probed with specific antibodies to Stat3, phospho-Stat3, and NF-Kappa B. (**B**) Densitometric analysis of Phospho-Stat3 expression in the cytoplasm and nuclei of W and R cells (Sigma Gel Program). Data represent the mean 6 SEM of three independent experiments expressed in arbitrary units (AU).

It is of interest that phospho-Stat3 was barely detectable in the cytoplasmic fraction of SKN-SH/Dox6 cells, indicating that the activated transcription factor had translocated to the nucleus. Because tyrosine phosphorylation of Stat3 in SKN-SH/Dox6 cells occurred in the absence of any experimentally induced extracellular stimulus, such as the addition of cytokines, hormones, or growth factors, these findings suggest that Stat3 was constitutively activated in SKN-SH/Dox6 cells.

Nuclear Translocation and Binding of Phospho-Stat3 to DNA

The nuclear localization of phospho-Stat3 was determined by confocal microscopy (Fig. 3A). SKN-SH cells had a diffuse staining pattern localized mainly to the cytoplasm with virtually no nuclear localization. In contrast, doxorubicin-resistant cells displayed

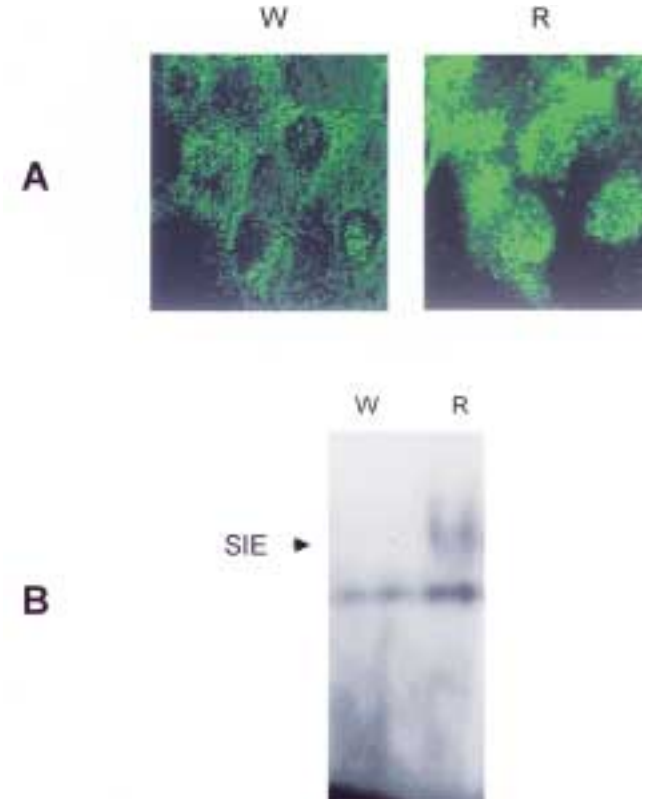

Fig. 3. Cellular localization and binding of phospho-Stat3 to DNA in SKN-SH wild-type (W) and SKN-SH/Dox6 doxorubicin-resistant (R) human neuroblastoma cells. (**A**) Confocal microphotographs showing differences in expression of phospho-Stat3 between W and R cell lines. Note increased nuclear localization of phospho-Stat3 in R cells when compared to W cells. Cells were plated on a sterile slide, cultured for 2 days, and stained with anti-phospho-Stat3 and FITC-conjugated secondary antibody, as described in "Methods." (**B**) DNA mobility gel shift analysis of phospho-Stat3 binding to *sis*-inducible element (SIE). Nuclear proteins were extracted and incubated with radiolabeled DNA. Protein-DNA complexes (SIE) and free SIE were separated on 5% SDS-PAGE and autoradiographs prepared.

intense staining, which was mainly located in the nucleus. These morphologic data confirm the constitutive phosphorylation of Stat3 mentioned above (see Fig. 2), and demonstrate the translocation of phospho-Stat3 from its cytoplasmic site of interaction with a cellular kinase, to the nucleus of SKN-SH/Dox6 cells.

The binding of phospho-Stat3 to DNA was determined by DNA gel shift analysis, using a radiolabeled DNA sequence (*sis*-inducible element [SIE]) known to interact with the phospho-Stat3 homodimer and phospho-Stat3/Stat1 heterodimer (25,26). Binding to SIE was observed only in the SKN-SH/Dox6 cells (Fig. 3B). Western blot analyses with anti-Stat1 and anti-Stat2 antibodies had demonstrated their lack of expression in either SKN-SH or SKN-SH/Dox6 cell lines (data not shown). Collectively, these findings suggest that the Stat3 homodimer may be the principal component of the SIE complex formed in SKN-SH/Dox6 cells. These data also indicate that constitutively phosphorylated Stat3 appears to be functional in terms of its ability to translocate, bind to DNA, and potentially activate specific genes in the nucleus.

Expression of Bcl-2 and Bcl-x_L in SKN-SH and SKN-SH/Dox6 Human Neuroblastoma Cells

Stat3 activation and increased expression of genes of the Bcl-2 family were investigated to determine their relationship to decreased apoptosis in drug-resistant cells. As previously described, this relationship has been demonstrated in the case of resistance to fas-mediated apoptosis (4,11,27). Western blot analyses performed to detect Bcl-2 revealed that this molecule was not expressed in either the SKN-SH or SKN-SH/Dox6 cell lines (Fig. 4A). Although Bcl-x_L was present in both cell lines, it was significantly increased (p , 0.01) in SKN-SH/Dox6 cells when compared to SKN-SH cells (Fig. 4A,B). These data provide further support for a causal relationship between Stat3 activation and up-regulation of Bcl-x_L expression as events associated with the inhibition of apoptosis.

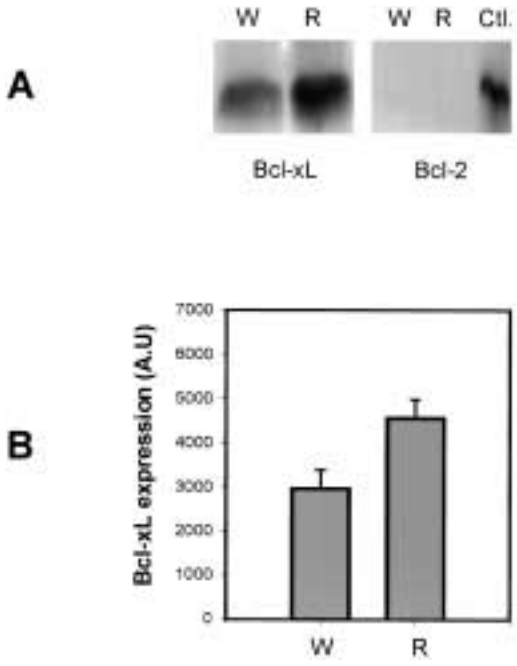

Fig. 4. Bcl-2 and Bcl-x_L expression in SKN-SH wild-type (W) and SKN-SH/Dox6 doxorubicin-resistant (R) cells. (A) Bcl-x_L and Bcl-2 expression in W and R cells. **(B)** Densitometric analysis of Bcl-x_L expression in W and R cell lines. R significantly greater than W (p , 0.01). Data represent mean 6 SEM of four independent experiments.

Inhibition of the JAK/Stat Signaling by Tyrphostin (AG490): Effects on Stat3 Activation and Bcl-x_L Expression

Further analysis of the putative association between activation of Stat3 and expression of Bcl-x_L was investigated using the JAK antagonist, tyrophostin AG490 (Fig. 5). Incubation of SKN-SH and SKN-SH/Dox6 cells with AG490 (10 μM) decreased the nuclear concentration of Stat3 (Fig. 5A) and of phospho-Stat3 (Fig. 5B) to a greater extent in the wild-type SKN-SH than drug-resistant SKN-SH/Dox6 cells. Bcl-x_L expression was also inhibited and expression of the pro-apoptotic gene *Bcl-x_s* slightly enhanced, particularly in the wild-type SKN-SH cells (Fig. 5C). These findings provide additional support for the interrelationship between *Bcl-x_L* gene expression and Stat3 phosphorylation. They also suggest that activation of this pathway is JAK dependent.

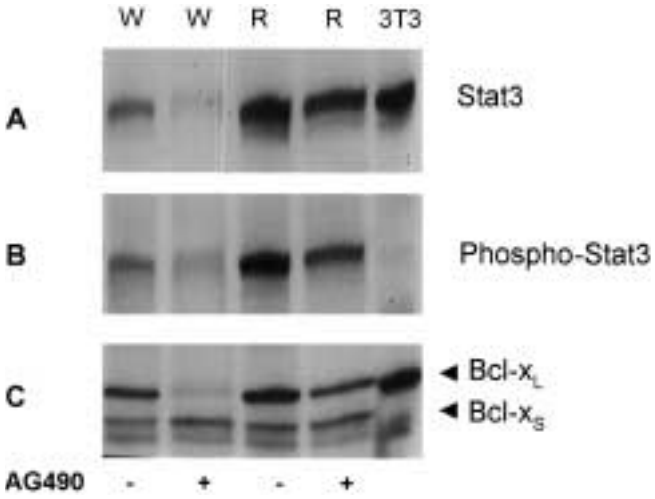

Fig. 5. Effect of tyrphostin (AG490) on Stat3 activation and Bcl-x_L expression in SKN-SH wild-type (W) and SKN-SH/Dox6 doxorubicin-resistant (R) human neuroblastoma cells. W and R cells were treated with AG490 (10 μM) for 15 hr. The nuclear localization of Stat3 **(A)** and phospho-Stat3 **(B)** was determined by Western blot. Expression of Bcl-x_L, and Bcl-x_S **(C)** was determined in whole cell lysates using an antibody that recognized the same epitope on both molecules. 3T3 represents protein cell extract from 3T3 cells that served as control for expression of Stat3 and Bcl-x_L.

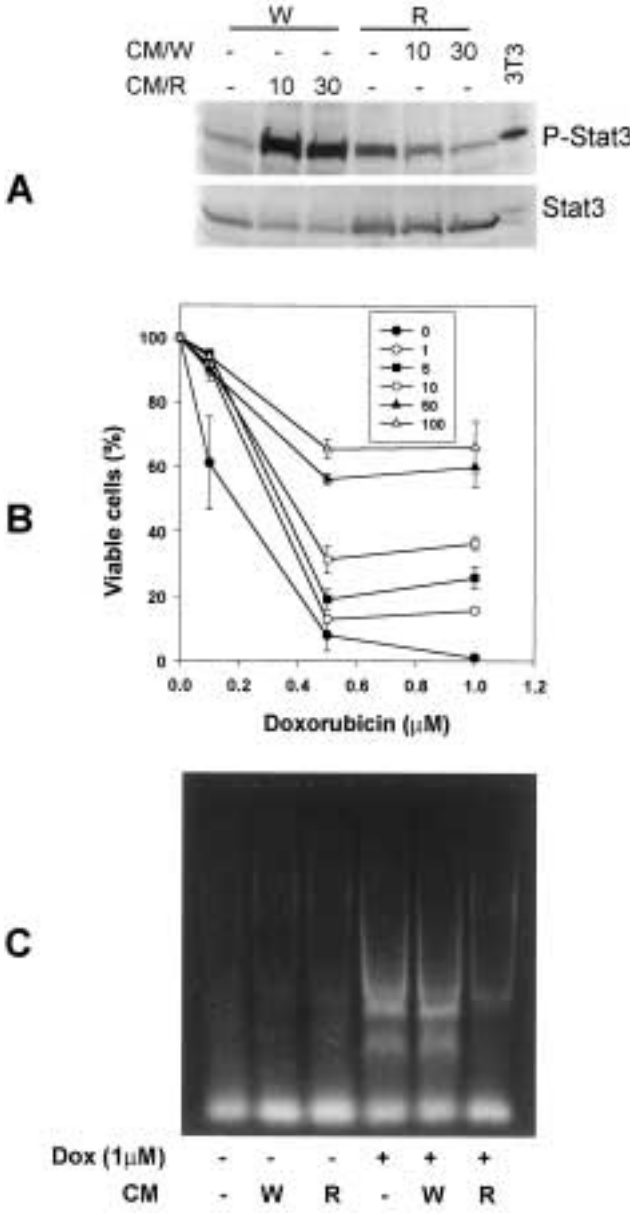

Fig. 6. Response of SKN-SH wild-type cells (W) to incubation with conditioned media (CM) from doxorubicin-resistant cells (R): Effect on Stat3 phosphorylation, cytotoxic response to doxorubicin, and susceptibility to doxorubicin-induced apoptosis. (**A**) Effect on Stat3 phosphorylation: W and R cells were incubated with conditioned media obtained from R and W cells, respectively, for 10 or 30 min. Phosphorylation of Stat3 was determined by Western blot using anti-phosphoStat3 antibody. (**B**) Effect on cell response to doxorubicin: W cells were preincubated for 48 hr with increasing volumes (0–100 μl) of conditioned media from R cells. The W cells were then incubated with varying concentrations of doxorubicin for 72 hr, and viable cells determined by the MTT assay. (**C**) Effect of conditioned media on doxorubicin-induced DNA degradation in W cells: W cells were incubated with conditioned media from W or R cells for 48 hr prior to an additional 24 hr of incubation with doxorubicin (1 μM). DNA was extracted and electrophoresed on a 1% agarose gel.

Modulation of Stat3 Phosphorylation, Doxorubicin Resistance, and Apoptosis by Cellular Secretion of Humoral Factors

Demonstration that Stat3 was constitutively activated in SKN-SH/Dox6 cells led to the concept that molecules synthesized and secreted by drug-resistant cells were putative determinants of this process. This hypothesis was tested by adding conditioned media from drug-resistant SKN-SH/Dox6 cells to wild-type SKN-SH cells for 10 and 30 min at room temperature to determine its effect on Stat3 phosphorylation. At both time periods, phospho-Stat3 was markedly up-regulated in wild-type SKN-SH cells (Fig. 6A). In contrast, conditioned media from SKN-SH cells did not increase Stat3 phosphorylation in drug-resistant SKN-SH/Dox6 cells (Fig. 6A), but was associated with a time-dependent decrease. This probably reflects the action of phosphatases on phosphorylated Stat3. A 48-hr incubation of wild-type SKN-SH cells with varying volumes of conditioned media from SKN-SH/Dox6 cells caused a concentration-dependent decrease in the cytotoxic effect of doxorubicin (Fig. 6B) and inhibition of doxorubicin-induced DNA fragmentation (Fig. 6C). These data suggest that ligand(s) secreted into the media by SKN-SH/Dox6 cells can initiate Stat3 activation, suppression of doxorubicin-induced toxicity and inhibition of apoptosis.

Discussion

The development of resistance to cytotoxic agents represents an adaptive biological response by neuroectodermal tumors that leads to complex and vexing therapeutic outcomes. Many cellular mechanisms causing drug resistance have been described and these involve alterations in membrane transport, drug metabolism, and substrate and enzyme amplification, to cite but a few (28). In addition, tumor cells may also express alterations in the processes that regulate apoptotic cell death (16,29). Therefore, identification of the molecules and mechanisms that modulate apoptosis and define their role in tumor cell adaptation to cytotoxic drugs may be potentially useful for the development of innovative therapeutic strategies.

In this study, we investigated alterations in the Stat3/Bcl-x_L anti-apoptotic pathway in relation to doxorubicin-mediated apoptosis and expression of drug resistance in SKN-SH human neuroblastoma cells. The doxorubicin-resistant cell line (SKN-SH/Dox6) generated for this purpose displayed a sharp decrease in doxorubicin-induced cytotoxicity that was almost 100 times lesser than the parental cells (Fig. 1A). Susceptibility of SKN-SH/Dox6 cells to drug-mediated apoptosis was also reduced as evidenced by inhibition of doxorubicin-mediated DNA degradation (Fig. 1B) and caspase-3 activity (Fig. 1C).

The pattern of Stat3 signaling differed significantly between wild-type and drug-resistant cells.

Although the overall expression of Stat3 was slightly increased in the cytoplasm of SKN-SH/Dox6 cells (Fig. 2), phospho-Stat3 expression was markedly elevated, particularly in the nuclei of these cells. Confocal microscopy and DNA gel shift assays confirmed the nuclear localization of phospho-Stat3 (Fig. 3) and provided additional evidence for the activation of this transcription factor in drug-resistant cells. Activation of Stat3 was constitutively regulated in SKN-SH/Dox6 cells, because no exogenous stimulus (e.g., addition of ligands) was required to elicit this response. Therefore, the possibility that molecular factors responsible for Stat3 activation are constitutively expressed in drug-resistant cells cannot be excluded.

To further define the putative relationship between constitutive activation of Stat3 and resistance to doxorubicin in human neuroblastoma cells, expression of downstream gene targets of the Bcl-2 family were investigated. Western blot analyses revealed that Bcl-2 was absent in both SKN-SH and SKN-SH/Dox6 cells (Fig. 4); however, the expression of Bcl-x_L was significantly elevated in the resistant cell line, SKN-SH/Dox6. This finding, in addition to the inhibition of both Stat3 activation and expression of Bcl-x_L by the JAK2 inhibitor AG490 (Fig. 5), indicates that these two signaling molecules are components of a common anti-apoptotic pathway activated in doxorubicin-resistant human neuroblastoma cells.

The potential mechanism(s) by which Stat3 became constitutively activated in drug-resistant human neuroblastoma cells was also investigated. We hypothesized that this cascade might be initiated by extracellular ligands (cytokines, hormones, or growth factors) secreted by drug-resistant cells into the culture media. Incubation of wild-type SKN-SH cells with conditioned medium obtained from doxorubicin-resistant SKN-SH/Dox6 cells strongly activated Stat3 and inhibited doxorubicin-stimulated toxicity in these cells (Fig. 6A,B). Doxorubicin-mediated DNA degradation in SKN-SH cells was also prevented by this treatment (Fig. 6C). We propose that constitutive activation of Stat3, observed in SKN-SH/Dox6 cells, may be modulated by an autocrine loop initiated by the secretion of stimulatory ligands from drug-resistant cells. The nature of these anti-apoptotic stimuli has not yet been determined and is currently under active investigation in our laboratory.

The role of Stat3 in preventing doxorubicin-induced toxicity has been recently investigated in myocardial tissue (30). Targeted overexpression of Stat3 in the mouse heart provided protection against doxorubicin-mediated cardiomyopathy, and improved survival rate by preventing the progression of cardiac failure. Our data are in agreement with these findings and indicate that in addition to its protective role against short-term treatment with doxorubicin, constitutive activation of Stat3 may be considered as a mediator of cellular adaptation to the sustained administration of doxorubicin. In terms of therapeutic strategies, these findings collectively suggest that targeted anti-Stat3 therapy at the tumor site or Stat3 overexpression in drug sensitive organs, such as the heart, may constitute a valuable adjuvant in the treatment of drug-resistant tumors and attenuation of organ-specific drug toxicity.

In conclusion, the major findings of our investigation suggest that resistance to doxorubicin in the human neuroblastoma cell line, SKN-SH, is associated with decreased apoptosis and constitutive activation of the Stat3/Bcl-x_L signaling pathway. Stimulation of this anti-apoptotic pathway appeared to be elicited by factor(s) secreted only from drug-resistant cells. These data also introduce the concept that production of anti-apoptotic molecules by drug-resistant cells may represent an alternative mechanism underlying tumor cell adaptation to cytotoxic drugs and the development of drug resistance.

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