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Jnk signaling pathway-mediated regulation of Stat3 activation is linked to the development of doxorubicin resistance in cancer cell lines

Ju-Hwa Kim [a], Seok Chul Lee [a], Jungsil Ro [a], Han Sung Kang [a], Hyung Sik Kim [b], Sungpil Yoon [a,*]

[a] Research Institute, National Cancer Center, 809 Madu 1-dong, Ilsan-gu, Goyang-si, Gyeonggi-do 411-764, Republic of Korea
[b] College of Pharmacy, Pusan National University, Busan, Republic of Korea

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ABSTRACT

We sought to identify altered transcription factors (Stat, AP1, and NF-kB) or signal proteins (Erk1/2, p38, Akt, Jnk, Jak, and c-Src) in cancer cell lines whose growth was arrested by doxorubicin (DOX) treatment. Jnk1 was the only signal protein to be activated. DOX increased Stat3 phosphorylation, nuclear localization, and transcriptional activity. Jnk1 activation appeared to be required for Stat3 activity. Stat3 activity via the Jnk pathway was conserved in other cell lines originating from other organs. Transcriptional activity of Stat3 was increased in cells surviving DOX treatment suggesting that Stat3 activation contributed to the resistance to cytotoxicity. To better understand the role of Stat3 in Jnk1 activation, we investigated its effect on the viability of DOX-treated cells. Co-treatment with DOX and Jnk inhibitor negatively correlated with the viability of cancer cells and reduced Stat3 activity. Taken together, these results indicate that Stat3 activation via the Jnk pathway promotes the resistance of cancer cells to DOX.

1. Introduction

Doxorubicin (DOX) is a drug that is widely used for the treatment of a variety of cancers including breast and liver cancers [1,2]. Many cancer cells undergo terminal growth arrest and acquire a senescence-like phenotype when treated with DOX. DOX primarily induces apoptosis, although the exact mechanisms underlying its cytotoxicity are not fully understood [3,4]. DOX is not always efficacious in the treatment of cancer, as development of resistance is common [5].

Identification of proteins whose expression and activity are altered following DOX treatment should yield valuable clues concerning the mechanism of DOX-induced apoptosis and resistance. Since the activation or inactivation of signaling kinases contributes to drug-induced apoptosis or chemo-resistance associated with cancer [6–10], it is important to determine which signaling kinases are altered when cells are exposed to DOX. Signaling kinases relevant to drug-induced apoptosis or resistance include principal signal protein molecules such as Erk1/2, p38, Akt, c-Src, Jak, and c-Jun N-terminal kinase (Jnk) [6–13]. It would also be useful to identify downstream targets such as major transcription factors whose activation or reduction correlates with signaling kinase alterations. Recently, the signal transducers and activators of transcription (Stat), activator protein-1 (AP1), and nuclear factor-kappa B (NF-kB) families of transcription factors have emerged as important participants [14–19].

Here, we investigated changes in the expression and activation of growth signaling kinases and potential downstream transcription factors in DOX-treated cancer cells. We report that DOX activates Stat3 transcription factor via the Jnk signaling pathway, and that Jnk1 and Stat3 activation contributes to the reduction of DOX-induced apoptosis. We conclude that cancer cells activate Stat3 via the Jnk pathway in order to survive or resist the cytotoxic effect of DOX.

2. Materials and methods

2.1. Reagents

DOX (Boryung Pharmacy, Gyeonggi-do, Korea) was obtained from the National Cancer Center in Korea. Paclitaxcel, SP600125, and etoposide were purchased from Sigma–Aldrich (St. Louis, MO). Negative control small interfering RNA (siRNA) and two kinds of validated Stealth siRNA for Jnk1 were obtained from Invitrogen (Carlsbad, CA).

Abbreviations: DOX, doxorubicin; phospho, phosphorylation; Stat, signal transducer and activator of transcription; Jnk, c-Jun N-terminal kinase; AP1, activator protein-1; NF-kB, nuclear factor-kappa B; Erk, RAS/extracellular signal-regulated kinase; Jak, Janus kinase; FBS, fetal bovine serum; siRNA, small interfering RNA; PBS, phosphate buffered saline; S.D., standard deviation.

* Corresponding author. Tel.: +82 31 920 2361; fax: +82 31 920 2002.
E-mail address: yoons@ncc.re.kr (S. Yoon).

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2.2. Antibodies

Antibodies against phosphorylated-serine Stat1, phosphorylated-tyrosine Stat2, phosphorylated-tyrosine Stat3, phosphorylated-serine Stat3, phosphorylated-tyrosine Stat5, phosphorylated-tyrosine Stat6, c-Jun, c-Fos, phosphorylated activating transcription factor-2 (ATF2), ATF2, Akt, phosphorylated Akt, phosphorylated IkappaB kinase (IKK)α/β, phosphorylated p65, phosphorylated Jak2, and phosphorylated Erk1/2 were from Cell Signaling Technology (Danvers, MA). Antibody against β-actin was from Sigma–Aldrich. Antibodies against phosphorylated p38, p38, Stat2, phosphorylated-tyrosine Stat4, Stat5, JunD, p65 (RelB), hSnf2H, Erk1/2, p38, c-Src, Jak1, and Jak2 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Cyclin D1, Jnk1, phosphorylated c-Jun, phosphorylated Jnk1, phosphorylated c-Src, and phosphorylated Jak1 were from Biosource (Camarillo, CA). Antibodies against Stat1, Stat3, and Stat6 were from Stressgen (Victoria, BC, Canada). Antibody against α-tubulin was obtained from Abcam (Cambridge, UK).

2.3. Cell culture

Human cancer cell lines MDA-MB231, MCF7, and HepG2 were obtained from the American Type Culture Collection (ATCC; Manassas, VA). They were cultured in RPMI1640 containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (WelGENE, Daegu, Korea).

2.4. Plasmids and transfection

Cells were transiently transfected using WelFect-Ex™ PLUS transfection reagent (WelGENE) as previously described [20]. Briefly, cells grown in 12-well plates were transiently transfected with pZLuc-TK plasmid [21] and Renilla vector. They were treated with the appropriate drugs at 24 h after transfection. The cells were then washed with 1 ml of phosphate buffered saline (PBS), suspended in passive lysis buffer, and frozen at −20 °C for at least 1 h.

2.5. Luciferase assay

The assay was performed using a dual-luciferase reporter kit (Promega, Madison, WI) according to the manufacturer's instructions. Luciferase activity was analyzed using a VICTOR™ Light Model 1420 luminometer (PerkinElmer, Waltham, MA). Firefly luciferase activity, which was used to determine the transcriptional activity of Stat3, was normalized with Renilla luciferase activity. For transfection of siRNA with lucifersae vectors, HiperFect transfection reagent (Qiagen, Valencia, CA) was used according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were treated with DOX, etoposide, or SP600125 for the indicated times and luciferase activity was determined in each cell lysate. All experiments were performed more than three times with two independent experiments.

2.6. Trypan blue dye exclusion

Cells grown in 60 mm-diameter dishes were washed with 5 ml of PBS, dislodged by trypsin, and pelleted by centrifugation for 1 min at 3000 rpm. The pellet was suspended in 500 μl of culture medium and Trypan blue was added to the cell suspension. The numbers of dye-excluding (viable) cells and stained (dead) cells were counted using a hematocytometer. All experiments were performed more than three times with two independent experiments.

2.7. Cell proliferation assay

Cell proliferation was determined in a colorimetric assay using a 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTS) kit (Promega) according to the manufacturer's instructions. Briefly, cells grown in wells of a 96-well plates were incubated with 20 μl of MTS for 2 h at 37 °C. Absorbance at 490 nm was then immediately determined using a multi-well spectrometer (Bio-Tek Instruments, Winooski, VT). All experiments were performed more than three times with two independent experiments.

2.8. Immunocytochemistry

Cells were plated onto chamber slides, incubated for 24 h, fixed for 10 min with 4% paraformaldehyde, and permeabilized for 20 min at room temperature in PBS containing Triton X-100 and bovine serum albumin (BSA). The permeabilized cells were incubated with anti-phosphorylated Jnk1 or anti-α-tubulin primary antibodies overnight at 4 °C. They were then re-incubated with fluorescein isothiocynate (FITC)-conjugated secondary antibody (Zymed, South San Francisco, CA), fluorescently labeled Alexa Fluor 568 secondary antibody (Molecular Probes, Eugene, OR), and were stained with 4′-6-diamidino-2-phenylindole (DAPI) for 1 h at 37 °C. The stained cells were subsequently examined using an inverted fluorescence microscope [22]. We performed more than two independent tests with each duplicated experiment.

2.9. Fluorescence activated cell sorting (FACS) analysis

Cells grown in 60 mm-diameter dishes were treated with DOX or SP600125 for predetermined times. The cells were dislodged by trypsin and the entire volume was centrifuged to recover the dislodged cells. The pelleted cells were thoroughly washed with PBS, suspended with 75% ethanol for at least for 1 h at 4 °C, washed with PBS, and re-suspended in cold propidium iodide (PI) staining solution (100 μg/ml Rnase A and 10 μg/ml PI in PBS) for 40 min at 4 °C. The stained cells were analyzed for relative DNA content using a FACS-Calibur flow cytometry system (BD, Franklin Lakes, NJ).

2.10. Protein extraction and Western blot analysis

Nuclear and cytoplasmic proteins were extracted using a commercially available kit (Pierce Biotechnology, Rockford, IL) according to a previously described method [20,22]. The proteins were dissolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer (Invitrogen, Carlsbad, CA), and subjected to Western blot analysis. The tricholoracetic acid method was used for Western blot analysis of total proteins [23].

2.11. Statistical analysis

The data were presented as mean ± standard deviation (S.D.). Statistical analysis was conducted using Student's *t*-test and one-way analysis of variance (ANOVA) followed by multiple-comparison test. Results were considered to be statistically significant compared with the control ($P < 0.05$).

3. Results

3.1. DOX influences on activation of the Jnk signaling pathway

We sought to identify important altered proteins in DOX-treated breast and liver cancers. For this purpose, we chose MDA-MB231 and HepG2 cells, which are representative breast and liver cancer cell lines, and which have been well-studied. In addition, we

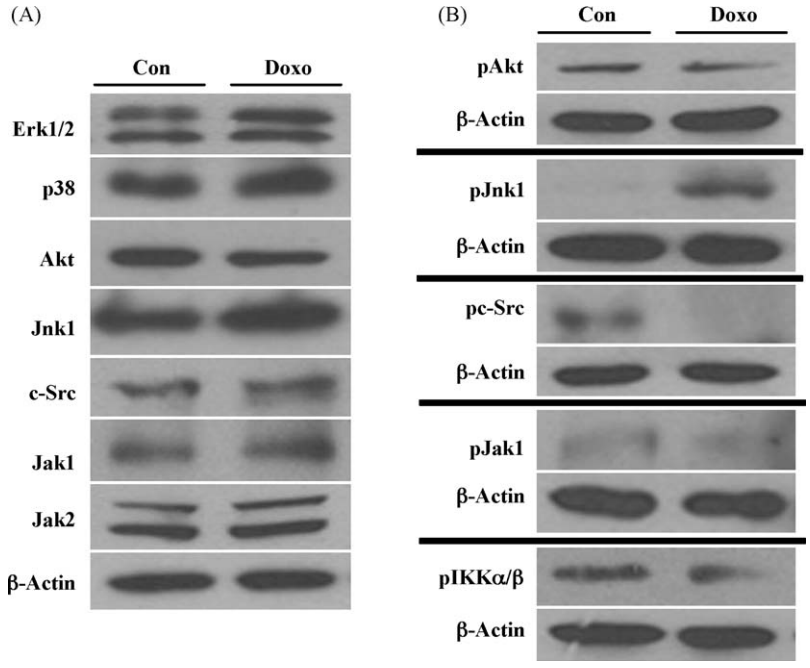

Fig. 1. DOX solely activates the Jnk signaling pathway. MDA-MB231 cell extracts were collected 20 h after treatment with 3.5 μM DOX (Doxo) or from untreated samples (Con). Western blot analyses were performed using antibodies against (A) Erk1/2, p38, Akt, Jnk1, c-Src, Jak1, Jak2, and β-actin; and (B) phospho-Akt, phospho-Jnk1, phospho-c-Src, phospho-Jak1, phospho-IKKα/β, and β-actin.

established in preliminary experiments that these cell lines were more susceptible to the action of DOX than other cell lines. To ensure that these experiments were clinically relevant, we exposed cells to DOX. We assumed that the results would echo the results from in vitro analysis, since DOX was dissolved to a concentration that is used to treat patients at the National Cancer Center in Korea (90 mg dose, administered as 50 mg/25 ml, for a patient with a body mass index of 20 kg/m^2). The DOX concentration of 3.5 μM was selected based on the results of a MTS assay at 20 and 30 h (Supplementary Fig. 1A and B), which showed approximately 50% decreased cellular viability.

After 20 h of growth, the number of MDA-MB231 cells increased by about 1.75-fold. However, cell numbers remained unchanged after 20 h incubation in the presence of 3.5 μM DOX. These results agree with the finding that DOX reduced MTS-determined cell viability by 50% (Supplementary Fig. 2A and B). DOX also reduced the levels of Cyclin D1 (Supplementary Fig. 2C), which are required for proliferation [24]. Next, we assessed the influence of DOX on the activation status or levels of Erk1/2, p38, Akt, Jnk1, Jak1, Jak2, c-Src, and IKKα/β, which are pivotal factors in the major signaling pathways regulating cell growth [6–13]. As shown in Fig. 1A, DOX treatment did not alter the total levels of these proteins. However, DOX did reduce the protein levels of various transcription factors (see Fig. 2A and B). Phosphorylated forms of Erk1/2, p38, and Jak2 were not detected in control or DOX-treated cells (data not shown). Phosphorylated Akt and Jak1 were detected in control cells, but these levels were unaffected by DOX treatment (Fig. 1B). DOX treatment reduced the levels of phosphorylated c-Src and IKKα/β, the upstream kinase of NF-kB [18,25], but it increased Jnk1 phosphorylation (Fig. 1B). After DOX treatment, apoptotic cells appeared as suggested by the circular formation of α-tubulin around nuclear membranes (Supplementary Fig. 3A and B). Also DOX-mediated Jnk1 activation was observed in both MDA-MB231 and HepG2 cells using immunocytochemistry. Collectively, these findings indicate that the inhibition of cellular proliferation by DOX positively correlates with the Jnk pathway.

3.2. DOX activates only the Stat3 transcription factor

We assessed whether DOX-induced growth arrest was associated with changes in the activation status or levels of the Stat, AP1, or NF-kB transcription factors [14–19]. This experiment was grounded on the assumption that the identified transcription factors could be involved in potential DOX-targeted downstream target proteins involved in Jnk1 activation. The total levels of Stat2 and Stat5 were reduced upon DOX treatment, while the levels of Stat1, Stat3, Stat4, and Stat6 remained unchanged (Fig. 2A). DOX treatment decreased the levels of activated Stat1 and Stat4 (Fig. 2A), but increased the levels of activated Stat3. Activated forms of Stat2, Stat5, and Stat6 were not detected in control or DOX-treated cells (data not shown). Analysis of the levels of the major AP1 family members [17] revealed that total c-Jun, ATF2, and JunD were reduced after DOX treatment, while total c-Fos remained unchanged (Fig. 2B). Activated c-Jun and ATF2 were also reduced by DOX (Fig. 2B). DOX had no effect on the total levels of the p65 subunit of NF-kB [18,25] (Fig. 2C). The activated form of p65 was not detected in either control or DOX-treated cells (data not shown). Of the major members of the Stat and AP1 families, only Stat3 was activated by DOX. Analysis of subcellular protein fractions revealed that the activated form of Stat3 accumulated in the nucleus in the presence of DOX, while nuclear levels of activated c-Jun decreased under these conditions (Fig. 2D) suggesting that DOX-activated Stat3 enters the nucleus and stimulates transcription.

3.3. Transcriptional activity of Stat3 increases in cells surviving DOX treatment

To assess the specificity of DOX in Jnk1 and Stat3 activation, the dose-dependent effects of this drug on Jnk1 and Stat3 phosphorylation were tested. Levels of phosphorylated Jnk1, but not of Stat3, increased with increasing DOX concentrations (Fig. 3A). The anticancer drug paclitaxcel [26] activated Jnk1 but not Stat3. These results indicate a DOX specific activation of both Stat3 and Jnk1. To

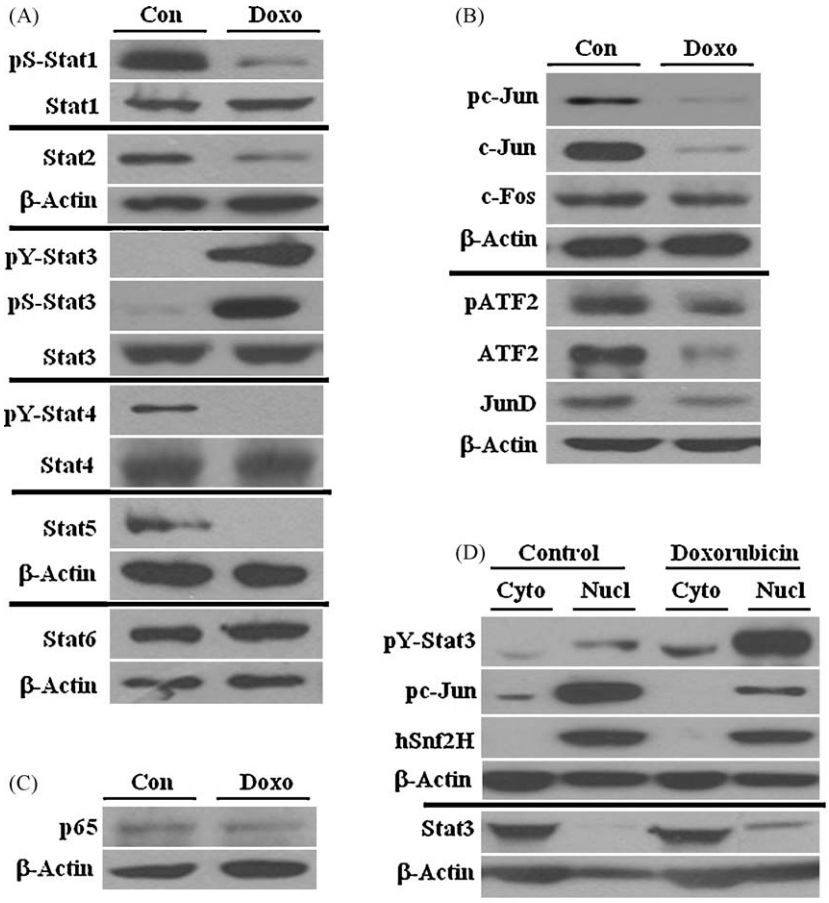

Fig. 2. Stat3 is the only transcription factor activated by DOX. MDA-MB231 cell extracts were collected 20 h after treatment with 3.5 μM DOX (Doxo) or from untreated samples (Con). Western blot analyses were performed using antibodies against (A) phospho-serine (pS) Stat1, Stat1, Stat2, phospho-tyrosine (pY) Stat3, phospho-serine (pS) Stat3, phospho-tyrosine (pY) Stat4, Stat4, Stat5, Stat6, and β-actin; (B) phospho-c-Jun, c-Jun, c-Fos, phospho-ATF2, ATF2, JunD, and β-actin; and (C) p65 and β-actin. (D) Cells were grown and stimulated as described in Fig. 1A. The cytoplasmic (Cyto) and nuclear (Nucl) protein extracts were prepared and Western blot analysis was performed using antibodies for phospho-tyrosine (pY) Stat3, phospho-c-Jun, Stat3, hSnf2H, and β-actin. Probing with β-actin and hSnf2H antibodies provided loading controls.

measure DOX associated Stat3 transcriptional activity, we performed luciferase assays using the pZLuc-TK reporter plasmid [21], which includes three repeats of the Stat3 binding site. Stat3 activity positively correlated with increasing DOX concentration (Fig. 3B). Stat3 activity was decreased by treatment with 15 μM DOX since the majority of the cells were killed at this concentration. Moreover, Stat3 activity also increased with the length of DOX exposure (Fig. 3C). This observation was consistent with Western blot results that revealed DOX-mediated Stat3 activation occurred from 6 h and increased at 20 h (Fig. 3D). The observations are also consistent with the suggestion that Stat3 activity by DOX negatively correlates with cellular apoptosis, and that cell survival following DOX exposure involves Stat3 activation-mediated resistance to DOX cytotoxicity.

3.4. DOX-induced Stat3 activation is Jnk1-dependent

To determine whether the Jnk1 activation is required for Stat3 activation by DOX, cells were co-treated with DOX and SP600125, a specific Jnk inhibitor [27]. DOX-mediated activation of Stat3 was reduced by this co-treatment (Fig. 3E), as was Jnk1 activation (Fig. 3D). Effect of SP600125 treatment on DOX activity seemed to be long lasting, as no Jnk1 or Stat3 activation was detected at 20 h (Fig. 3D). These observations are consistent with the suggestion that the Jnk pathway is required for DOX-mediated Stat3 activation. To assess whether Stat3 transcriptional activity also depended on Jnk1 activation, luciferase activity was determined. Stat3 activation by DOX was reduced after co-treatment with the inhibitor (Fig. 3F), confirming that Stat3 activity depended on Jnk1 activation by DOX.

3.5. The DOX-mediated Jnk pathway is also required for Stat3 activation in other cancer cell lines

Jnk1 activation was increased by DOX in HepG2 liver cancer cells (Supplementary Fig. 3B). Stat3 activity in HepG2 cells depended on exposure-time and concentration of DOX (Fig. 4A and B), as also seen in MDA-MB231 cells. Stat3 activity also depended on Jnk1 activation, since the increased activity by DOX was reduced when cells were co-treated with either SP600125 or Jnk1 siRNA (Fig. 4C and D). Similar results were found using MCF7 breast cancer cells (Fig. 4E and F). Jnk1 activation has been detected in etoposide-treated cells [28] (Supplementary Fig. 3A and B). However, Stat3 activity was not detected in etoposide-treated cells suggesting that these activities are DOX specific (Figs. 3F and 4C and F). Together, the results suggest that the DOX-mediated increased Stat3 activity via the Jnk pathway can be conserved in cancer cells that originate from different organs.

3.6. Co-treatment with Jnk inhibitor increases apoptosis of DOX-treated cells

To better understand the roles of Jnk1 and Stat3 activation in cell growth, we investigated the effect of these activations on the

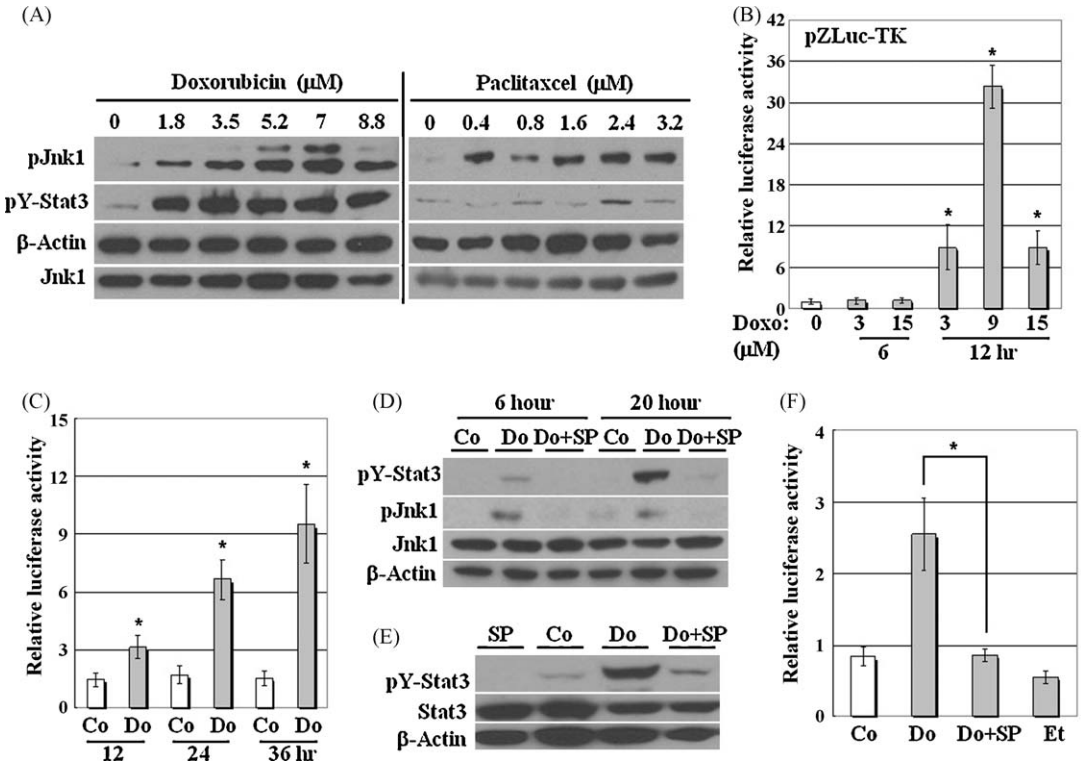

Fig. 3. Jnk1 activation by DOX is required for Stat3 activation. (A) Left panel: MDA-MB231 cell extracts were collected 20 h after treatment with 1.8, 3.5, 5.2, 7, and 8.8 μM DOX, and from untreated cells. Right panel: MDA-MB231 cell extracts were collected 20 h after treatment with 0.4, 0.8, 1.6, 2.4, and 3.2 μM paclitaxcel, and from untreated cells. Western blot analyses were performed using antibodies against phospho-Jnk1, Jnk1, phospho-tyrosine (pY) Stat3, and β-actin. (B and C) MDA-MB231 cells were plated on 22 mm-diameter dishes, grown to 50% confluence, and incubated for 24 h prior to tranfection using pZLuc-TK. Cells were stimulated with indicated concentration or 3.5 μM DOX (Doxo or Do) after 24 h of transfection. The control (Co) group consisted of non-treated transfected samples. Luciferase analysis was performed with all of the extracts at the indicated time after the treatment. (D and E) MDA-MB231 cell extracts were collected after 20 h or at the indicated times after treatment with 3.5 μM DOX (Do) or from untreated cells in the absence (Do) or presence (Do + SP) of 20 μM SP600125 (SP). The cells were used for Western blot analyses using antibodies against phospho-tyrosine(pY)-Stat3, Stat3, phospho-Jnk1, Jnk1, and β-actin. (F) The luciferase assay for MDA-MB231 cells was performed as described in (B) and (C). The cells were stimulated with 3.5 μM DOX in the absence (Do) or presence (Do + SP) of 20 μM SP600125 (SP) after 24 h of transfection. Transfected samples were treated with etoposide (Et). The control (Co) group consisted of untreated transfected samples. Luciferase analysis was performed with all the extracts at 20 h after treatment. The data represented the mean ± S.D. of at least triplicate experiments; $^{*}P < 0.05$ compared to corresponding control.

MTS-determined viability of DOX-treated cells in the presence and absence of Jnk inhibitor. After 24 or 48 h of co-treatment with DOX and the Jnk inhibitor, viability of MDA-MB231 cells was similar to that of cells treated solely with DOX, whereas viability for HepG2 cells was reduced by about 25% after 24 h and 30% after 48 h (ANOVA analysis, $P < 0.05$) (Fig. 5A and B). The results suggest that Jnk1 activation by DOX negatively correlates with DOX cytotoxicity of HepG2 cells, further suggesting that Jnk1 activation contributes to DOX resistance.

Co-treatment of DOX with Jnk inhibitor was largely increased the pre-G1 region at 48 h in MDA-MB231 cells by FACS analysis (Fig. 6B) suggesting that apoptosis increased compared to treatment solely with DOX. We also observed increased apoptosis by co-treatment of DOX with Jnk inhibitor in HepG2 cells (Fig. 6C). Collectively, these observations are consistent with the suggestion that Jnk1 activation by DOX negatively correlates with apoptosis in both MDA-MB231 and HepG2 cells, and suggest that cells with inhibited Jnk1 activation are more sensitive to DOX and that Stat3 activation positively correlates with cell survival after DOX exposure, since co-treatment with Jnk inhibitor resulted in reduced Stat3 activity.

4. Discussion

In the present in vitro study, we identified proteins that either increased or decreased in cancer cells exposed to DOX. Activated Stat3 increased upon DOX treatment but not with either paclitaxcel or etoposide. Therefore, the observed change in Stat3 activity appears to be DOX specific. Stat3 is typically activated by Jak or c-Src [29,30]. However, phosphorylation of these kinases was not observed after DOX treatment. DOX reduced c-Jun activation, which is a downstream target for Jnk1 activation [31]. Our data clearly demonstrate that Jnk1 activation is required for DOX-mediated Stat3 activation. Previous reports showed that Stat3 activation can be stimulated by the Jnk pathway [32,33]. The finding that only Jnk1 was activated following paclitaxcel treatment suggests that signaling molecules other than Jnk1 might be required for Stat3 activation under the condition, and that DOX engages a unique signaling pathway for Stat3 activation via Jnk1. Our observations that DOX increased Stat3 activity via Jnk1 activation in MDA-MB231, MCF7, and HepG2 cancer cells suggest that the Jnk pathway is generally conserved in different cancer cell lines. Since no direct interaction between Stat3 and Jnk1 was detected either in the presence or absence of DOX (data not shown), other molecules must be involved in mediating the connection between the activation of Jnk1 and Stat3. In the future, it would be interesting to determine the exact mechanism by which Jnk1 activation increases Stat3 activity and the mediators for Stat3 activation via Jnk1.

Stat3 and Jnk1 activations may have two possible roles. First, they may contribute to growth-arrest and apoptosis of cells induced by DOX treatment. Second, activation of these proteins may represent an attempt to overcome DOX-induced apoptosis. Our results support the latter possibility as being more likely, since

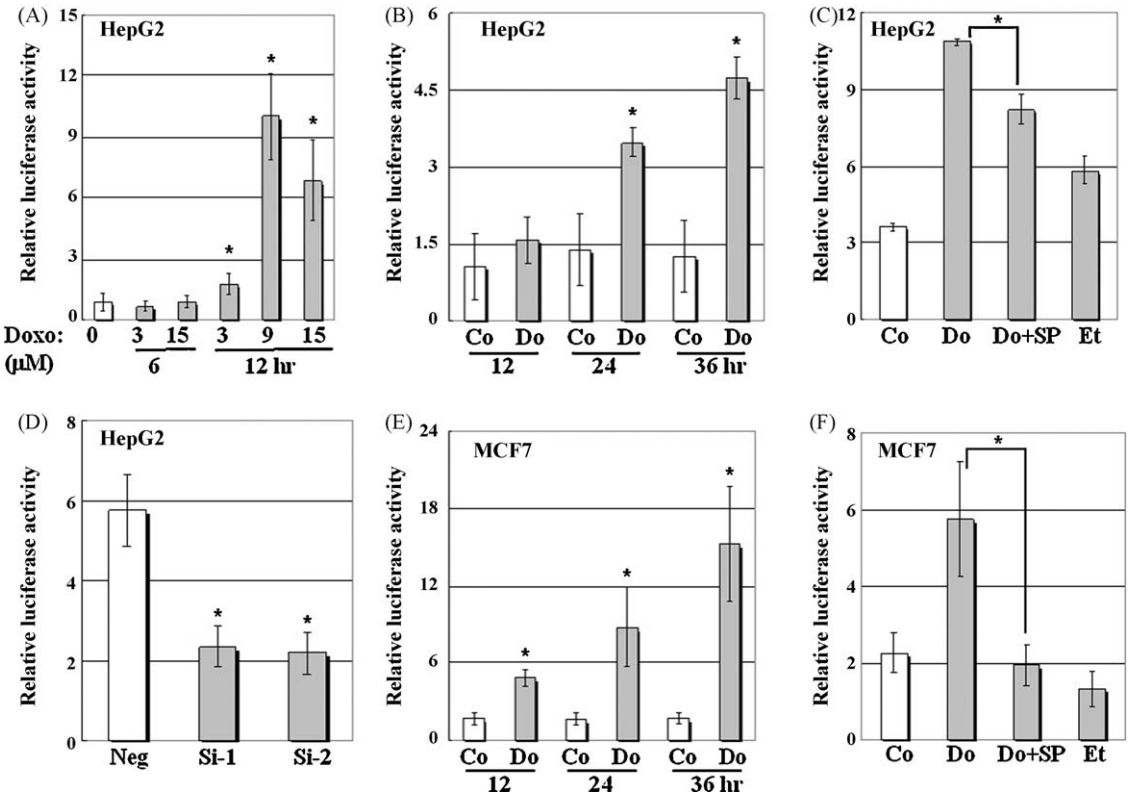

Fig. 4. DOX increases Stat3 activity via the Jnk pathway in HepG2 and MCF7 cells. (A–C) HepG2 cells were plated on 22 mm-diameter dishes, grown to 50% confluence, and incubated for 24 h prior to transfection using pZLuc-TK. The cells were stimulated with the indicated concentrations or 3.5 μM DOX (Doxo or Do) after 24 h of transfection. Transfected samples were treated with etoposide (Et). The control (Co) group consisted of untreated transfected samples. Luciferase analysis was performed with all the extracts at the indicated time or 20 h after treatment. (D) pZLuc-TK was co-transfected with negative siRNA (Neg) or Jnk1 siRNA (Si-1 and Si-2). The luciferase assay for the transfected HepG2 cells was performed as described in (A). (E and F) The luciferase assay for MCF7 cells was performed as described in (A), (B), and Fig. 3F. The data represented the mean ± S.D. of at least triplicate experiments; $^{*}P < 0.05$ compared to corresponding control.

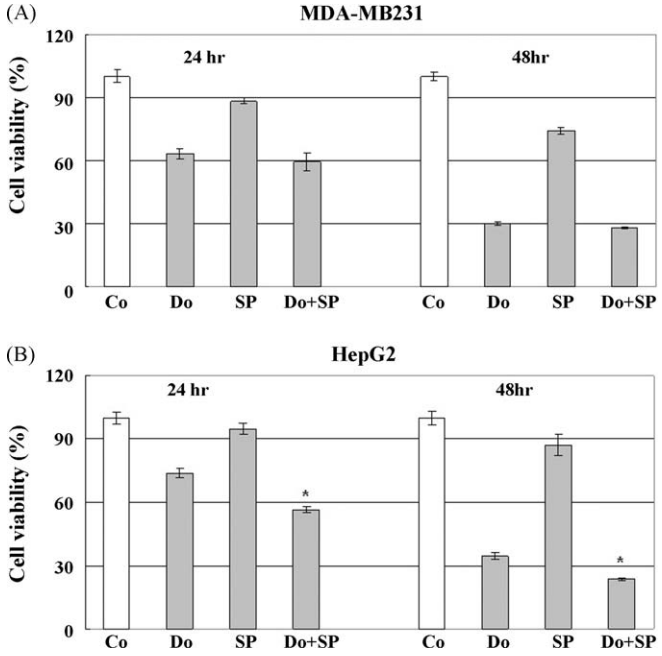

Fig. 5. Co-treatment of Jnk inhibitor with DOX contributes to reduced proliferation. (A) MDA-MB231 and (B) HepG2 cells were collected at 24 or 48 h after treatment with 3.5 μM DOX in the absence (Do) or presence (Do + SP) of 20 μM SP600125 (SP). The control (Co) group consisted of untreated samples. The cells were then used for the MTS assay. The data represented the mean ± S.D. of at least triplicate experiments; $^{*}P < 0.05$ compared to corresponding control.

Jnk1 inhibition reduced cellular viability and increased apoptosis. Considering that DOX-mediated Stat3 activity depended on the Jnk pathway, we can conclude that DOX-mediated Stat3 activity also contributes to protection from the drug-induced cytotoxicity. Our study suggests that cells protect themselves from DOX by Stat3 activation via the Jnk pathway. The observations that Stat3 activity increased with time and concentration of DOX doxorubicin support our conclusion. Recent reports also support our conclusion that Stat3 activation contributes to DOX resistance [34–36]. Although Stat3 activation by DOX or other anti-cancer drugs has recently been shown to contribute to cell survival [34,35], no relationship between Jnk1 activation has hitherto been established. To the best of our knowledge, this is the first report demonstrating that Stat3 activation via Jnk1 by DOX contributes to cellular survival from the cytotoxic drug. We assumed that DNA breakages by DOX increased activation of the Jnk signaling pathway. The activated Jnk pathway then increased Stat3 activation to increase survival from DOX and could have resulted in DOX resistance.

The present study confirms that the major Stat3 target protein, Cyclin D1 is down-regulated by DOX exposure. It will be of interest to identify the target proteins or functions involved in DOX-mediated increased Stat3 activity. It is possible that the activation of Stat3 by Jnk signaling pathway might play a role in inducing genes, whose products can repair DOX-mediated DNA breakage. It will also be important to investigate whether Stat3 and Jnk1 activations are associated with DOX resistance in cancer patients. Such studies may contribute to the improvement of combination-chemotherapeutic treatments for cancer patients who develop resistance to DOX.

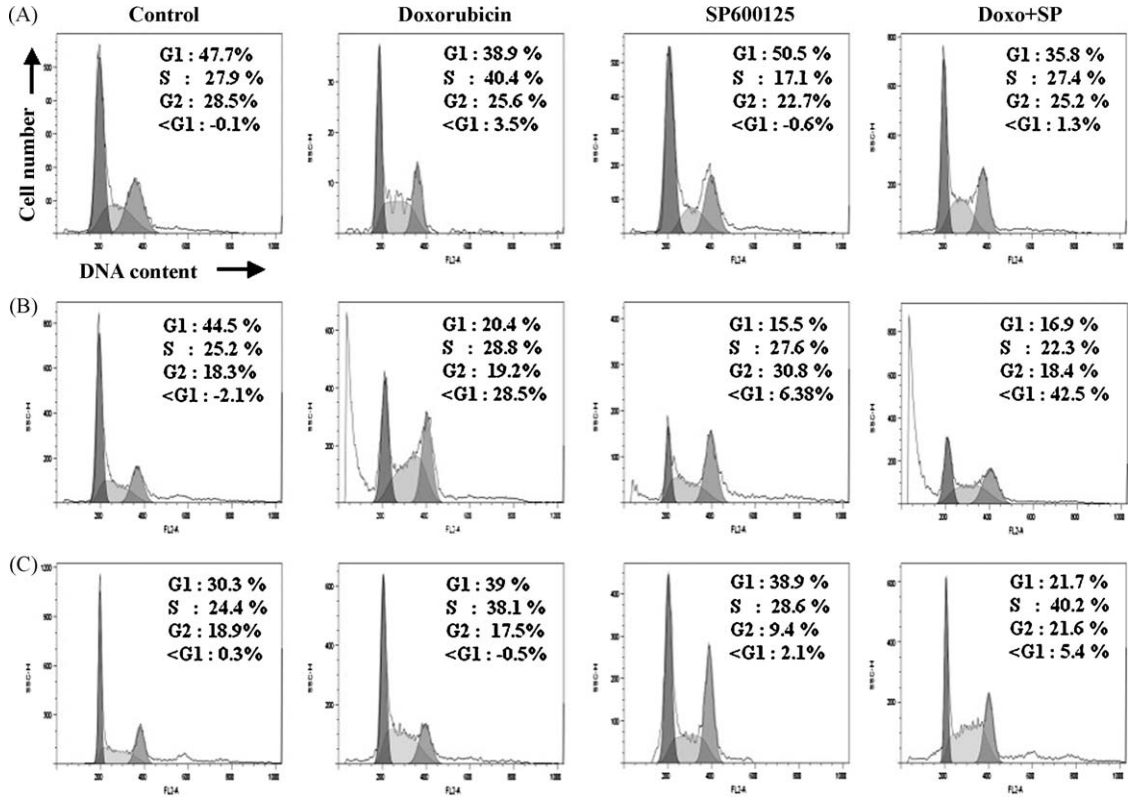

Fig. 6. Co-treatment of Jnk inhibitor with DOX contributes to increased apoptosis. (A) MDA-MB231 cells were plated on 60 mm-diameter dishes, grown to 50% confluence, and incubated for 24 h. The cells were then stimulated with 3.5 μM DOX in the absence or presence (Doxo + SP) of 20 μM SP600125. The control group consisted of untreated samples. FACS analysis was performed with all the extracts 24 h after the treatment. (B) FACS analysis was performed for MDA-MB231 cells with all the extracts 48 h after the treatment as described in (A). (C) FACS analysis was performed for HepG2 cells with all the extracts 24 h after the treatment as described in (A).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.09.008.

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