Original Article

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Regulation of Janus-activated kinase-2 (JAK2) by diindolylmethane in ovarian cancer *in vitro* and *in vivo*

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ABSTRACT: Janus-activated kinase-2 (JAK2) plays an important role in the activation of signal transducer and activation of transcription 3 (STAT3), which is over expressed in majority of ovarian tumors. We have reported previously that diindolylmethane (DIM) induces apoptosis in ovarian cancer cells by inhibiting STAT3. However, the role of JAK2 in our model was not yet understood and hence evaluated in this report. SKOV-3 human ovarian cancer cells were used to evaluate concentration and time dependent effects of DIM. Interleukin 3 (IL-3) and epidermal growth factor (EGF) were used to activate JAK2. Tumor xenograft studies were used to determine modulation of JAK2 *in vivo*. DIM treatment blocked the phosphorylation of JAK2 at Tyr-1007 in a concentration-dependent manner. In a time-dependent study, inhibition of JAK2 by DIM was as early as 1 h, which was followed by the inhibition of STAT3 and survivin. IL-3-induced phosphorylation of JAK2 and STAT3 was significantly blocked by DIM. IL-3 treatment blocked DIM-induced apoptosis. EGF treatment resulted in the activation of JAK2 and STAT3 but suppressed by DIM. These results indicate the involvement of cytokines and growth factors in the activation of JAK2/STAT3 and that DIM suppress their activation. Furthermore, DIM in combination with cisplatin drastically reduced the phosphorylation of JAK2 when compared to cisplatin alone. Western blot analysis of tumors from DIM treated mice showed significant inhibition of JAK2 activation as compared with controls. These findings provide a rationale for further clinical investigation of DIM for its potential use alone or in combination with chemotherapy of ovarian cancer.

Keywords: Diindolylmethane, JAK2, STAT3, apoptosis, EGF, cisplatin

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1. Introduction

Ovarian cancer still remains a leading gynecological malignancy not only in western countries, but all over the world (*1*). It is the fifth leading cause of cancer related deaths, has the highest mortality rate among gynecologic cancers and is the most lethal malignancy of the female reproductive system (*2*). Greater than 90% of ovarian cancers arise from the surface epithelium. Ovarian tumorigenesis is associated with ovulation-associated wound repair and inflammation (*1,3*). Janus activated kinase/signal transducer and activation of transcription (JAK/STAT) is a major pathway that is activated during inflammation (*4*).

JAK/STAT pathway is pivotal in transducing multitude of signals for development and homeostasis in mammals (*5*). JAK activation plays an important role in cell proliferation, differentiation, migration and apoptosis (*5-7*). These cellular events are critical in growth and development of various organs. Mutations such as JAK2V617F that constitutively activates or fails to regulate JAK signaling confer oncogenic properties to a cell (*6,8*). JAK family comprises of four members, JAK1, JAK2, JAK3, and TYK2 (*9*). Activation of JAKs occurs by ligand receptor mediated mutlimerization (*10*). Activated JAKs subsequently auto-phosphorylate their receptors and additionally phosphorylate STATs (*11*). Phosphorylated STATs dimerize and translocate to nucleus, where they bind to specific regulatory sequences to activate or repress transcription of target genes such as *Mcl-1* and *survivin* (*12,13*). Thus, JAK/STAT signaling provides a direct mechanism to translate an extracellular signal into a transcriptional response and hence an important target for therapeutic intervention.

Accumulating epidemiological studies strongly indicate an inverse relation between intake of cruciferous vegetables and ovarian cancer (*14*). 3,3'-Diindolylmethane (DIM, Figure 1) is an important indole compound present in cruciferous vegetables (*15*). DIM was shown to possess anti-cancer properties against several cancers (*15*). Our laboratory previously demonstrated that DIM inhibits proliferation of ovarian cancer by inducing apoptosis in ovarian cancer cells by targeting STAT3 and epidermal growth factor receptor (EGFR) (*16,17*). However, whether these effects are mediated through JAK2 was not clear.

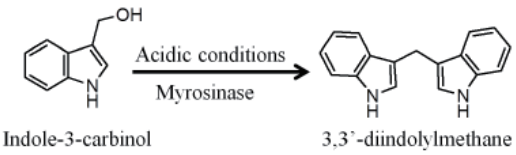

Figure 1. Formation of 3,3'-diindolylmethane (DIM) from indole-3-carbinol (I3C).

In the current study, we show that JAK2 is a mediator of STAT3 activation in our model. We also show that JAK2 is an important downstream signal transducer of EGFR.

2. Materials and Methods

2.1. *Chemicals*

BR-DIM was a kind gift from Dr. Michael Zeligs (Bio Response, Boulder, CO, USA). Antibodies against p-JAK2 (Tyr-1007), p-STAT3 (Tyr-705), survivin, Cl-caspase 3, and Cl-PARP were procured from Cell Signaling Technology (Danvers, MA, USA). Actin antibody, EGF, and IL-3 were procured from Sigma Aldrich (St. Louis, MO, USA). McCoy's 5A medium was purchased from Mediatech (Manassas, VA, USA). Cisplatin was obtained from Novaplus (Bedford, OH, USA).

2.2. *Cell lines and cell culture*

SKOV-3 cells were procured from American Type Culture Collection (ATCC, Manassas, VA, USA). SKOV-3 cells were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic mixture. Cells were maintained at 37°C in a humidified incubator circulated with 5% CO_2/95% air. Normal ovarian surface epithelium (NOSE) cells were kind gift from Dr. Jinsong Liu (M.D. Anderson Cancer Center, TX, USA) were cultured in 1:1 mixture of MCDB105 and medium 199 supplemented with 15% FBS and 1% PSN as described previously (*17*).

2.3. *Western blotting*

SKOV-3 cells were exposed to varying concentrations of DIM alone or in combination with cisplatin. In another experiment cells were exposed to 10 ng/mL IL-3 or 50 ng/mL EGF prior to treating with 75 µM DIM. Cells were collected, lysed, and 20-80 µg of protein was subjected to SDS gel electrophoresis and proteins were blotted onto PVDF membrane. After blocking with 10% nonfat dry milk in Tris buffered saline (TBS), the membrane was incubated overnight with corresponding primary antibodies. Subsequently, the membrane was incubated with appropriate secondary antibody, and the immunoreactive bands were visualized using enhanced chemiluminiscence kit (Thermo Scientific, Rockford, IL, USA) according to manufacturer's instructions as described by us previously (*18*).

2.4. *Annexin V apoptosis assay*

SKOV-3 cells were plated at a density of 0.3×10^6 cells per well in a six-well plate and allowed to attach overnight. Cells were exposed to IL-3 an hour before treatment with DIM. After 24 h, cells were washed, suspended in binding buffer and incubated for 15 min with annexin V-FITC (BD Biosciences, San Jose, CA, USA). Fluorescence was measured using C6 Accuri flow cytometer (Ann Arbor, MI, USA) with a minimum of 10,000 events per sample as described by us previously (*19*).

2.5. *EGF and IL-3 treatment*

SKOV-3 cells were exposed to 50 ng/mL EGF or 10 ng/mL IL-3 for 15 min followed by treatment with 75 µM DIM for 24 h. Cells were then processed for apoptosis assay or Western blotting as described above.

2.6. *Analysis of tumors from control and DIM treated mice*

Four to six week old female athymic nude mice were purchased from Charles River Laboratories (Wilmington, MA, USA). The use of mice and their treatment was approved by Institutional Animal Care and Use Committee (IACUC), Texas Tech University Health Sciences Center, and all experiments were carried out in strict compliance with regulations. Mice were fed with antioxidant-free AIN-76A special diet for a week before starting the experiment. About 5×10^6 SKOV-3 cells were injected subcutaneously into both right and left flanks. Mice were randomly arranged in each group. Control group received PBS whereas mice in the treatment group received 3 mg DIM suspended in PBS by oral gavage every day. Tumors from female athymic nude mice that received PBS or 3 mg DIM suspended in PBS by oral gavage every day were excised, snap frozen and lysed as explained by us previously (*18*). Tumor lysates were subjected to Western blotting for monitoring the activation of JAK2 as explained above.

2.7. *Quantitation and statistical analysis*

Densitometric analysis and quantitation was analyzed using UNSCAN-IT (Orem, UT, USA). All the statistical analysis was performed using Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). The data represents mean ± S.D. Student's *t*-test was used to compare the control and treated groups. All statistical tests were two sided. Differences were considered statistically significant when the *p* value was less than 0.05.

3. Results

3.1. *DIM inhibits activation of JAK2 in ovarian cancer cells*

JAK2/STAT3 is aberrantly expressed in ovarian tumors (*20*). Therefore targeting JAK2-STAT3 may inhibit the

growth of ovarian cancer cells. We previously identified STAT3 as a critical target of DIM in ovarian cancer cells (*17*). However, the role of JAK2 in DIM mediated inhibition of STAT3 was not clear. Hence to determine the effect of DIM on JAK2, SKOV-3 ovarian cancer cells were treated with various concentrations of DIM for 24 h. Our Western blotting results reveal that DIM inhibits the activation of JAK2 by blocking its phosphorylation at Tyrosine 1007 (Figure 2A). The effect of DIM on JAK2 was concentration dependent with about 80% inhibition of p-JAK2 (Tyr-1007) by exposure to 75 µM DIM (Figure 2B). We had previously shown that DIM was least effective on normal ovarian surface epithelial (NOSE) cells (*17*). Interestingly, our current results exhibit that normal ovarian cells do not show any constitutive activation of JAK2 as indicated by the absence of phosphorylation at Tyr-1007 (Figure 2C). These studies clearly suggest that JAK2 is a target of DIM in ovarian cancer cells.

3.2. *Early inhibition of JAK-2 by DIM*

Since we observed remarkable inhibition of JAK2 phosphorylation by DIM, we next determined the kinetics of JAK2 inhibition. SKOV-3 cells were treated with 75 µM DIM for desired time points and the cell lysates were evaluated by Western blotting. Our results indicate that inhibition of JAK2 activation by DIM was as early as 1 h (Figure 3). Since recent literature suggests that STAT3 was down stream of JAK2, we also looked for p-STAT3. DIM treatment substantially blocked the activation of STAT3 by suppressing its phosphorylation at Tyr-705 in SKOV-3 cells, and this inhibition started 1 h after treatment. Inhibition of STAT3 activation correlated with inhibition of JAK2 activation (Figure 3). Similarly, survivin, a downstream target of JAK2-STAT3 was substantially downregulated by 1 h of DIM treatment (Figure 3). These results not only substantiate that JAK2 is a target of DIM, but also indicate that STAT3 and survivin were regulated by JAK2 in our model.

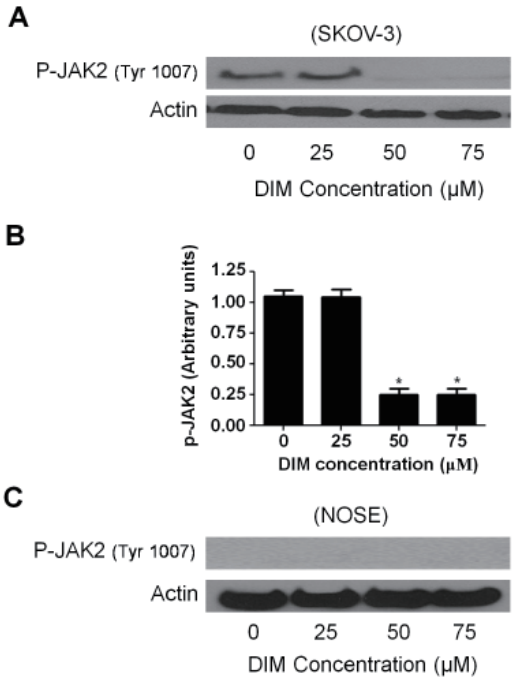

Figure 2. DIM treatment inhibits the activation of JAK2 in ovarian cancer cells but not in normal ovarian surface epithelial cells. SKOV-3 cells or normal ovarian surface epithelial cells (NOSE) were treated with various concentrations of DIM or treated with 75 µM DIM for desired time internals. Cells were collected, lysed and subjected to Western blotting. **(A)** Representative blots of p-JAK2 (Tyr-1007) and its **(B)** quantitation showing the concentration-dependent effect of DIM in SKOV-3 cell. **(C)** Representative blot of p-JAK2 (Tyr-1007) showing that DIM has least effect on NOSE cells. Actin was used as loading control.

3.3. *DIM regulates STAT3 pathway via JAK2 activation*

Our above observations demonstrate a link between JAK2, STAT3, and survivin in our model. To firmly establish the association between these molecules, SKOV-3 cells were stimulated with IL-3. IL-3 is a cytokine that phosphorylates JAK2 at Tyr-1007 and activates it. Our results show that treatment of SKOV-3 cells with 10 ng/mL IL-3 not only increased the activation of JAK2 by 2-fold but also increased the phosphorylation of STAT3 at Tyr-705 and expression of survivin by approximately 2.3- and 2-fold, respectively (Figure 4). To determine the effect of DIM on IL-3 induced activation/expression of these molecules, cells were treated with IL-3 for 15 min followed by treatment with DIM for 24 h. Our interesting results show that DIM treatment significantly blocked IL-3 induced phosphorylation of JAK2 (Tyr-1007), p-STAT3 (Tyr-705) and expression of survivin (Figure 4). These results confirm that STAT3 and survivin were regulated by JAK2 in our model.

3.4. *IL-3 treatment blocks DIM-induced apoptosis in SKOV-3 cells*

We further questioned ourselves, whether activating

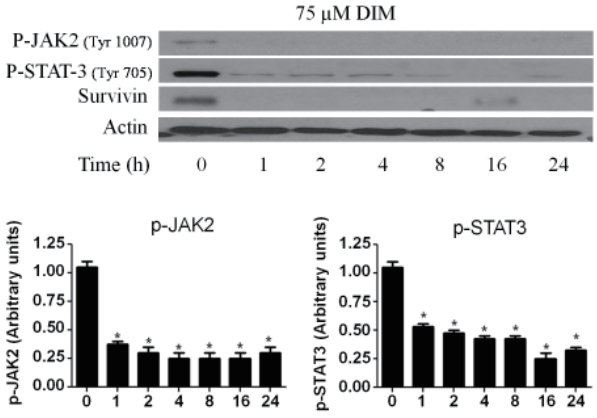

Figure 3. DIM treatment inhibits activation of JAK2 as early as one hour. Representative blots and their quantitation showing the time dependent effect of 75 µM DIM on p-JAK2 (Tyr-1007), p-STAT3 (Tyr-705) and survivin. Actin was used as loading control. * $p < 0.05$ compared to control.

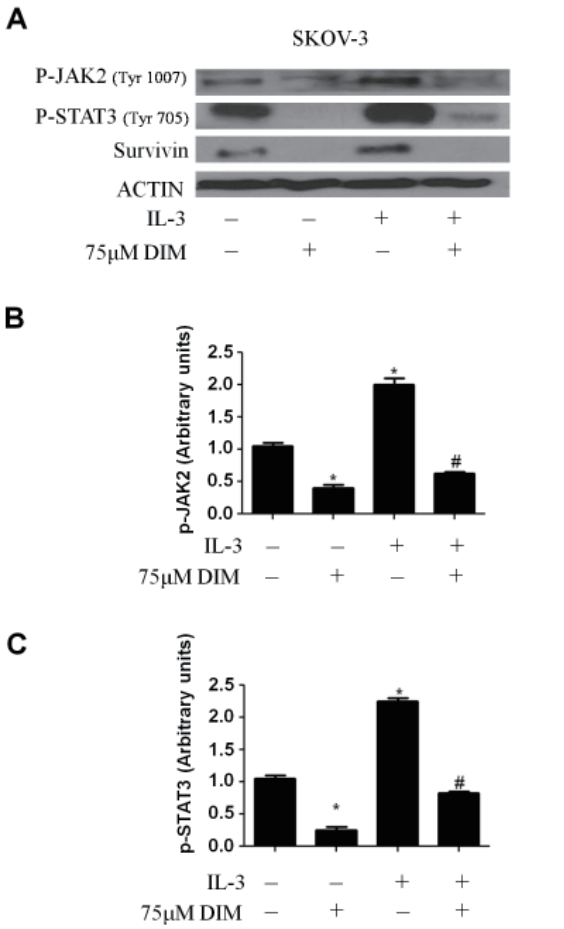

Figure 4. JAK2 is a critical target in DIM mediated apoptosis. SKOV3 cells were stimulated with 10 ng/mL IL-3 for 15 min followed by treatment with 75 μM DIM for 24 h. **(A)** Whole cell lysates were resolved on 10% SDS-PAGE for the analysis of phosphorylation of JAK2 at Tyr-1007, phosphorylation of STAT3 at Tyr-705 and expression of survivin. Actin was used as loading control. Quantitation of **(B)** p-JAK2 and **(C)** p-STAT3 were represented as bar diagram.

JAK2 would abrogate DIM mediated apoptosis. To answer this question, SKOV-3 cells were treated with IL-3 followed by 75 μM DIM for 24 h and apoptosis was determined. As expected, IL-3 treatment reduced the apoptosis induced by DIM in SKOV-3 cells (Figure 5A). For example, apoptosis mediated by 75 μM DIM alone was approximately 2.5-fold over control whereas in presence of IL-3, DIM-induced apoptosis was only 1.2-fold over control suggesting that IL-3 substantially blocked DIM-induced apoptosis (Figure 5B). Accordingly, IL-3 treatment significantly reduced the cleavage of caspase-3 and PARP induced by DIM treatment in SKOV-3 ovarian cancer cells (Figure 5C).

3.5. *EGFR regulates JAK2-STAT3 pathway in our model*

EGFR is activated in around 70% of ovarian tumors. Several studies demonstrated the possibility that EGFR activation in ovarian cancer would lead to phosphorylation of JAK2/STAT3 (*20*). Interestingly, our published studies indicated that EGFR is a target of DIM in ovarian cancer cells both *in vitro* and *in vivo* (*16*). We hypothesized that regulation of JAK2/STAT3 by DIM in ovarian cancer cells was mediated through EGFR. To test our hypothesis, SKOV-3 ovarian cancer cells were treated with EGF before treatment with DIM. EGF is a ligand of EGFR, subsequent binding of which leads to the activation of EGFR. Figure 6A clearly demonstrates that EGF treatment lead to the activation of JAK2 and STAT3 in SKOV-3 cells. Activation of JAK2 and STAT3 was approximately 2.5- and 2-fold, respectively by EGF as compared to constitutive expression in SKOV-3 cells (Figures 6B and 6C). Interestingly, DIM treatment significantly suppressed EGF mediated phosphorylation of JAK2 and STAT3 (Figures 6A-6C), confirming the role of EGF in the activation of JAK2 and STAT3.

3.6. *JAK2 inhibition potentiates the effect of cisplatin*

Cisplatin is a drug used clinically to treat patients with ovarian cancer. However, cisplatin has several side effects including systemic toxicity. DIM was previously shown by us to potentiate the effect of cisplatin in ovarian cancer cells (*17*). To determine the involvement of JAK2 in the effect of DIM with cisplatin combination, SKOV-3 cells were treated with 50 μM DIM for 24 h followed by treatment with 10 μM cisplatin for 24 h. As expected, our results indicate that DIM treatment alone and in combination with cisplatin remarkably reduced the phosphorylation of JAK2 at Tyr-1007 (Figure 7A). Approximately 70% reduction in the expression of p-JAK2 (Tyr-1007) was observed in DIM treated SKOV-3 cells (Figure 7B). The decrease in the phosphorylation of JAK2 in combination treatment (DIM and cisplatin) was further reduced when compared with cisplatin treatment alone (Figure 7B). These results suggest that DIM treatment can potentiate the effect of cisplatin in ovarian cancer cells by targeting JAK2.

3.7. *DIM treatment suppresses the growth of ovarian tumors by inhibiting JAK2 phosphorylation*

In our previously published studies, we have shown that oral administration of 3 mg DIM per day for 48 days significantly suppressed the growth of SKOV-3 ovarian tumor xenografts in athymic nude mice (*16,17*). To further establish whether JAK2 activation play any role in the growth of ovarian tumors, the tumors from control and DIM treated mice were analyzed by Western blotting. Our results reveal that JAK2 phosphorylation in tumors from the mice that received DIM was approximately 50% lesser than in the tumors from mice from control group (Figure 8). Based on these observations, it can be concluded that reduced tumor growth in DIM treated mice was associated with reduced activation of JAK2.

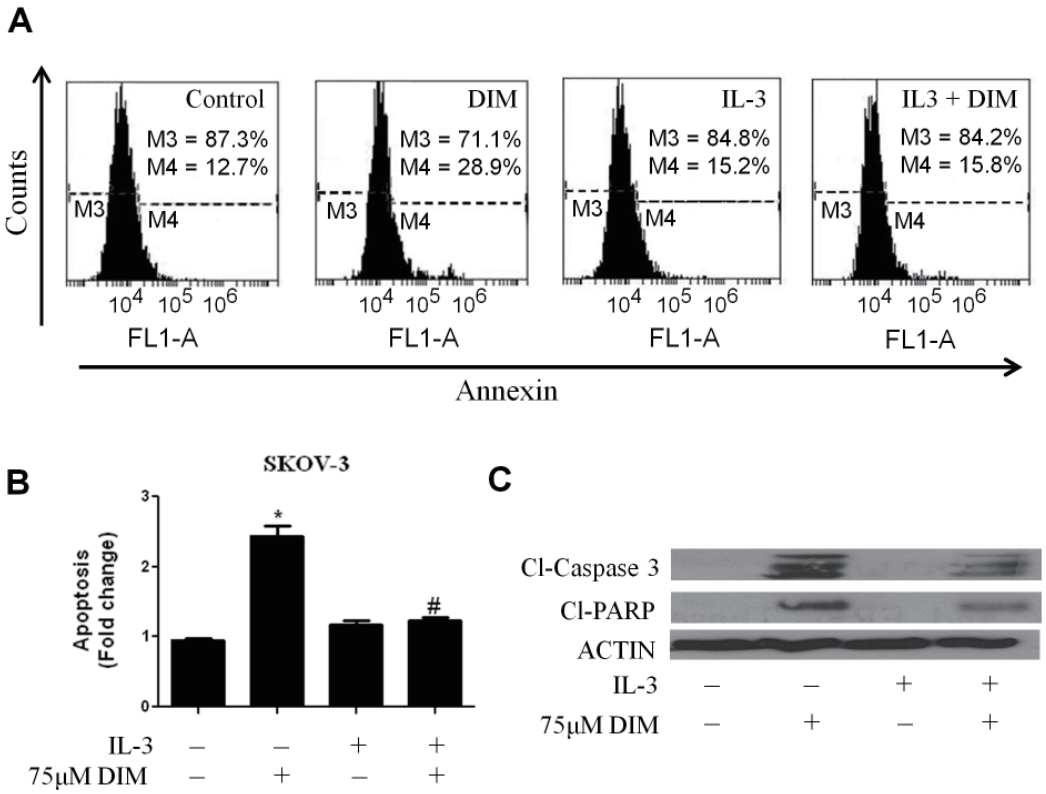

Figure 5. IL-3 treatment abrogates DIM induced apoptosis. (A) Effect of IL-3 on DIM induced apoptosis in SKOV-3 cells as detected by annexin V staining. **(B)** Bar graphs representing the apoptosis as indicated by annexin positive cells. * $p < 0.05$ compared to control. # $p < 0.05$ when compared to IL-3 treatment. **(C)** SKOV3 cells were stimulated with 10 ng/mL IL-3 for 15 min followed by treatment with 75 μM DIM for 24 h. Whole cell lysates were resolved on 10% SDS-PAGE for the analysis for cleavage of caspase-3 and PARP.

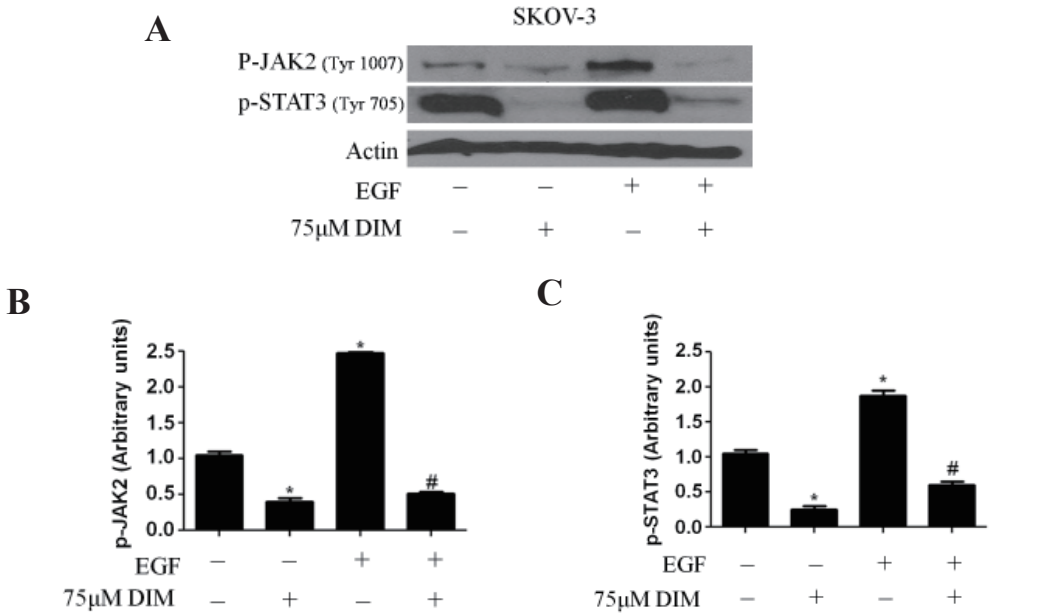

Figure 6. EGFR regulates JAK2/STAT3. SKOV3 cells were stimulated with 50 ng/mL EGF for 15 min and then treated with 75 μM DIM for 24 h. **(A)** Whole cell lysates were resolved on 10% SDS-PAGE to detect the phosphorylation of JAK2 at Tyr-1007 and phosphorylation of STAT3 at Tyr-705. Actin was used as loading control. Quantitation of **(B)** p-JAK2 and **(C)** p-STAT3 blots was also represented in bottom panel.

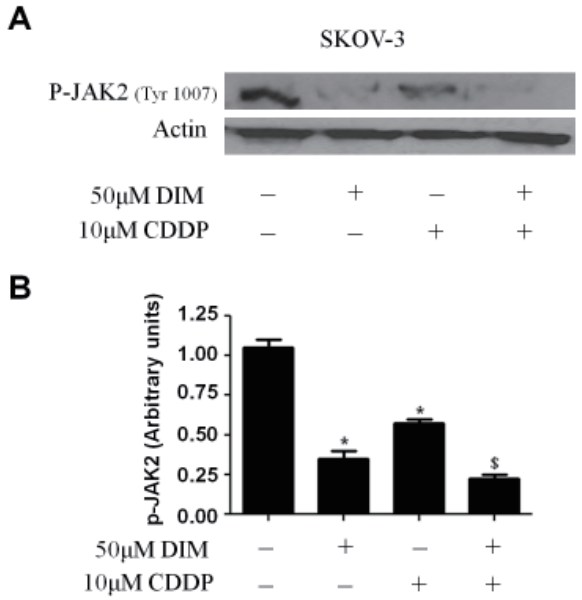

Figure 7. DIM potentiates the effect of cisplatin by inhibiting JAK2 phosphorylation. (A) Representative blots and **(B)** quantitation of p-JAK2 showing the effect of 50 μM DIM, 10 μM cisplatin or combination of both in SKOV-3 ovarian cancer cells. * $p < 0.05$ when compared to control. # $p < 0.05$ when compared to IL-3 treatment. $ $p < 0.05$ when compared to cisplatin treatment.

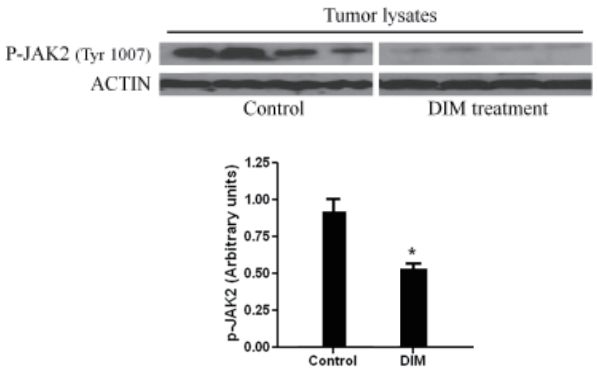

Figure 8. DIM suppresses ovarian tumor growth by inhibiting p-JAK2. Tumors from control and DIM treated mice were excised, lysed and analyzed by Western blot for p-JAK2 (Tyr-1007). Actin was used as loading control. Densitometric quantitation was represented. * $p < 0.05$ compared to control.

4. Discussion

The primary objective of the current study was to test whether the anti-cancer effects of DIM were mediated by inhibition of JAK2. JAK2 is an important therapeutic target in ovarian cancer. Its activation has been observed in a large fraction of human ovarian tumors compared with normal ovarian tissues (*20*). Moreover JAK2 signaling regulates gene expression of various pro-survival and anti-apoptotic molecules (*5*). Inhibition of JAK2 signaling triggers cell death in human cancer cells (*20,21*). Our results show that SKOV-3 cells, which come from high grade ovarian tumors, have higher constitutive phosphorylation of JAK2, whereas normal ovarian surface epithelial (NOSE) cells do not express JAK2 in its activated form. Our findings are in agreement with previously published study which reported that normal and benign ovaries lack activated JAK2 and it is aberrantly expressed only in low and high grade ovarian carcinomas (*6*).

Various cytokines and growth factors can activate JAK2. IL-3, a pleotripic cytokine is well known to activate JAK2 by phosphorylation at Tyr-1007 (*22*). DIM treatment not only eliminated constitutive activation of JAK2, but also blocked IL-3 mediated activation of JAK2. Interestingly, IL-3 treatment abrogated DIM-induced apoptosis as shown by reduced cleavage of caspase 3 and PARP as well as by annexin V staining of treated cells. Interestingly, AG490 a JAK2 inhibitor was shown to induce apoptosis in various cancer cells suggesting that JAK2 is required for cell survival and hence a target in cancer cells (*14,20*).

One of the major downstream effector of JAK2 is STAT3 (*6,14,20*). Activated JAK2 subsequently auto-phosphorylates its receptors and additionally phosphorylates STAT3. Phosphorylated STAT3 dimerizes and translocates to nucleus, where it binds to specific regulatory sequences to activate or repress transcription of target genes (*23*). Hence, JAK2 and thereby STAT3 play an important role in various stages of cancer progression. Our results demonstrate a relationship between JAK2 and STAT3 in ovarian cancer cells. The inhibition of STAT3 activation was consistent with inhibition of JAK2 activation in our time dependent study. Importantly, IL-3 treatment not only activated JAK2, but subsequently increased the phosphorylation of STAT3 but inhibited by DIM. Our results also demonstrate a link between JAK2/STAT3 and survivin. Expression of survivin was increased by IL-3 treatment and reduced by DIM. It is noteworthy that the gene expression profiling of a previous study showed that survivin is a target of DIM in breast cancer (*24*). Our results establish that JAK2 causes STAT3 activation in ovarian cancer cells and the apoptotic effects of DIM were due to inhibition of JAK2/STAT3 activation. These results are in agreement with previous studies which demonstrated that inhibition of JAK2/STAT3 pathway induced apoptosis and inhibited cell growth (*21,25*).

Over expression of EGFR occurs in almost 70% of ovarian cancers (*26*). Activation of EGFR has been associated with poor progression and increase in survival of ovarian cancer cells. Our previous studies showed that EGFR as a target of DIM (*16*). Our current study established a cross-talk between EGFR and JAK2/STAT3 pathway. EGF, a ligand known to activate EGFR, significantly activated JAK2 and STAT3 by phosphorylation. Nevertheless, DIM treatment substantially suppressed the phosphorylation of JAK2 and STAT3 mediated by EGF. Our studies indicate that

perhaps DIM targets EGFR-JAK2-STAT3 signaling axis to inhibit the growth of ovarian cancer. Our results are in agreement with the findings of previous study which indicated a link between EGFR and JAK2/STAT3 in carcinomas (*5*).

Our previously published results showed that administration of 3 mg DIM/day substantially retarded the growth of ovarian tumors *in vivo* (*17*). The tumor suppressive effects were mediated by reduction in JAK2 phosphorylation in the present study. DIM is a major phytochemical present in cruciferous vegetables (*3*). We showed that DIM is minimally toxic to normal cells. For example, 120 μM DIM inhibited 75% proliferation of cancer cells whereas affected only 25% of NOSE cells, showing selectivity towards cancer cells (*17*). A recent clinical study showed that dose of up to 300 mg DIM was well tolerated in humans (*27*). Importantly, DIM is in clinical trials to treat cervical neoplasia and prostate carcinomas (*28,29*). Our current results also established that inhibition of JAK2 activation by DIM potentiates the effects of cisplatin, a clinically used drug with several side effects. Hence, this gives a possibility of using low non-toxic doses of cisplatin if DIM is administered concomitantly with cisplatin. Nevertheless, further clinical evaluations are required to confirm the safety of DIM alone or in combination with cisplatin in patients with ovarian cancer.

Taken together, our current study demonstrates the rationale for further clinical investigation of DIM for its potential use alone or in combination with chemotherapy of ovarian cancer.

Acknowledgements

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