Leukemia (2006) 20, 1602–1609
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ORIGINAL ARTICLE

Restoration of shp1 expression by 5-AZA-2′-deoxycytidine is associated with downregulation of JAK3/STAT3 signaling in ALK-positive anaplastic large cell lymphoma

Y Han[1], HM Amin[2], C Frantz[1], B Franko[1], J Lee[1], Q Lin[2] and R Lai[1]

[1]Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, Alberta, Canada and [2]Department of Hematopathology, the University of Texas MD Anderson Cancer Center, Houston, TX, USA

Anaplastic lymphoma kinase (ALK)-positive anaplastic large cell lymphoma (ALK+ ALCL) is characterized by constitutive activation of the Janus kinase (JAK)3/signal transducers and activators of transcription 3 (STAT3) signaling pathway. SHP1, a tyrosine phosphatase that negatively regulates JAK/STAT, is frequently absent in ALK+ ALCL owing to gene methylation. To test the hypothesis that loss of SHP1 contributes to JAK3/STAT3 activation in ALK+ ALCL cells, we induced SHP1 expression using 5-aza-2′-deoxycytidine (5-AZA), an inhibitor of DNA methyltransferase, in ALK+ ALCL cell lines, and correlated with changes in the JAK3/STAT3 pathway. 5-AZA gradually restored SHP1 expression in Karpas 299 and SU-DHL-1 cells over 5 days. The initially low level of SHP1 expression did not result in significant changes to the expression or tyrosine phosphorylation of JAK3 and STAT3. However, higher levels of SHP1 seen subsequently correlated with substantial decreases in JAK3 and pJAK3, followed by pSTAT3 (but not STAT3). Importantly, the decrease in JAK3 was abrogated by MG132, a proteasome inhibitor. 5-AZA induced no significant increase in apoptosis but it sensitized ALCL cells to doxorubicin-induced apoptosis. Our findings support the concept that loss of SHP1 contributes to the constitutive activation of JAK3/STAT3 in ALK+ ALCL cells. SHP1 appears to downregulate JAK3 by two mechanisms: tyrosine dephosphorylation and increased degradation via the proteasome pathway.
Leukemia (2006) **20,** 1602–1609. doi:10.1038/sj.leu.2404323; published online 27 July 2006
Keywords: anaplastic large cell lymphoma; SHP1; 5-aza-2′-deoxycytidine; JAK3/STAT3 signaling; DNA methylation

Introduction

Anaplastic large cell lymphoma (ALCL) is a distinct type of aggressive non-Hodgkin's lymphoma characterized by CD30 expression, a sinusoidal infiltrative pattern and a T/null-cell immunophenotype.[1] Approximately 60% of these tumors have the chromosomal translocation, t(2;5)(p23;q35), that juxtaposes the nucleophosmin (*NPM*) gene at 5q35 with the anaplastic lymphoma kinase (*ALK*) gene at 2p23, leading to the expression of the aberrant fusion gene protein, NPM-ALK.[2] NPM-ALK is oncogenic; previous data suggest that it directly contributes to lymphomagenesis through deregulating multiple signaling pathways by virtue of its tyrosine kinase activity.[3–7] More recent studies have also implicated the signal transducer and activator of transcription 3 (STAT3) signaling pathway in the pathogenesis of ALK+ ALCL.[8–10] STAT3 itself is oncogenic,[11] and many types of human cancer demonstrate constitutive activation of STAT3.[12–15] It is believed that STAT3 promotes oncogenesis by upregulating a number of proteins that promote anti-apoptotic effects or cell-cycle progression, such as Bcl-X_L, Mcl-1, survivin and cyclin D3. It has been previously shown that ALK+ ALCL tumors consistently show relatively high levels of STAT3 activation,[8,9,16] and we have previously reported that specific blockade of STAT3 activation in ALK+ ALCL cell lines using a dominant-negative construct effectively triggers apoptosis and cell-cycle arrest, which correlates with downregulation of these STAT3 downstream targets.[10] More recently, Chiarile *et al.*[17] have provided further evidence to support the role of STAT3 in NPM-ALK-mediated tumorigenesis.

Although it is clear that NPM-ALK activates STAT3 in ALK+ ALCL cells, accumulating evidence suggests that other biochemical abnormalities also contribute to the high level of STAT3 activation seen in this cell type. Tyrosine kinases other than NPM-ALK have been shown to activate STAT3, such as src[18] and JAK3,[9,19] with the latter being one of the normal physiologic activators of STAT3. Another group of defects is related to the loss of the negative regulators for the JAK/STAT signaling pathway such as SHP1.[20] SHP1, a non-transmembrane protein tyrosine phosphatase, is expressed primarily in hematopoietic cells.[21–23] It is known to function as an important negative regulator in various signal-transduction pathways including those of cytokine receptors (e.g. Epo-R, IL-3R, IL-2R), growth factors with an intrinsic tyrosine kinase activity (e.g. CSF-1, EGF, c-Kit) and the antigen receptors of B and T cells.[24,25] SHP1 has been shown to silence the JAK/STAT pathway by dephosphorylating and inactivating JAK.[26–29] SHP1 has been reported to have tumor suppressor function.[30] In a previous study, we demonstrated that loss of SHP1 expression related to gene methylation is found in ALK+ ALCL cell lines and is detectable in the majority of ALK+ ALCL tumors.[31] Nevertheless, the role of SHP1 in ALK+ ALCL cells has not been extensively studied. The possible relationship between loss of SHP1 and the high levels of JAK3/STAT3 activation in this tumor type has been largely speculative.

We hypothesized that loss of SHP1 contributes to the constitutive activation of JAK3 and STAT3 in ALK+ ALCL cells. To test this hypothesis, we treated two ALK+ ALCL cell lines with 5-aza-2′-deoxycytidine (5-AZA), a DNA methyltransferase inhibitor, and correlated changes in the JAK3/STAT3 signaling pathway.

Materials and methods

Cell lines and tissue culture

ALK-positive ALCL cell lines, Karpas 299 and SU-DHL1, have been previously characterized and described.[32,33] These two

Correspondence: Dr R Lai, Department of Laboratory Medicine and Pathology, Cross Cancer Institute and University of Alberta, 11560 University Avenue, Alberta T6G 1Z2, Canada.
E-mail: Raymondmail_65@yahoo.com
Received 29 July 2005; revised 1 June 2006; accepted 19 June 2006; published online 27 July 2006

cell lines were maintained in RPMI 1640 (Life Technologies, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), penicillin (10 000 U/ml; Sigma, St Louis, MO, USA), streptomycin (10 mg/ml; Sigma), and L-glutamine (200 mM, Life Technologies). U266, a myeloma cell line, was also maintained in RPMI similar to that described for ALCL cell lines. GP293, an embryonic kidney cell line, was maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% FBS and antibiotics (10 mg/ml streptomycin and 10 000 U/ml penicillin). All cell cultures were maintained under an atmosphere of 95% O_2 and 5% CO_2 in 98% humidity at 37°C.

Pharmacologic agents

5-AZA was purchased from Sigma-Aldrich (Toronto, Ontario, Canada). 5-AZA was initially dissolved in acetic acid and diluted to 10 μg/μl. The aliquoted 5-AZA was frozen at −20°C until being used. The final concentrations of 5-AZA used throughout this study were 0, 5 and 10 μM, and the use of these concentrations was based on the findings of two previous studies[34,35] as well as those of our preliminary studies. In all experiments, cells were seeded in six-well culture plates at a density of 10^6/ml, and 5-AZA in fresh medium was added to the cell culture daily in order to maintain the constant concentration. Cells were harvested daily for 5 days for analysis. MG132, purchased from Calbiochem (EMD Biosciences, San Diego, CA, USA), was initially dissolved in dimethyl sulfoxide (DMSO) and further diluted with sterile, de-ionized water to a final concentration of 1 mM. The aliquoted MG132 was then stored at −20°C. At the time of the experiment, MG132 was thawed and diluted with tissue culture media to a final concentration of 10 μM. AG490 was purchased from Calbiochem (San Diego, CA, USA) and diluted with DMSO (final concentration, 1 mM).

Western blot analysis and antibodies

Western blot analysis was performed using standard techniques and details have been described previously.[19] All antibodies used in this study were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) unless otherwise specified: Bcl-2 (Dako, Carpinteria, CA, USA), Bcl-xL (Zymed, San Francisco, CA, USA), survivin (Novus Biologicals, Littleton, CO, USA), and actin (Sigma, St. Louis, MO, USA). All primary antibodies were used in a 1:1000 dilution.

Cell cycle analysis using propidium iodide and flow cytometry

Karpas 299 and SU-DHL-1 cells treated with 5-AZA were incubated with propidium iodide (Molecular Probes, Eugene, OR, USA) following the manufacturer's protocol. Briefly, the cells were washed and fixed in cold ethanol for 15 min and incubated with propodium iodide for 30 min. Thereafter, cells were analyzed with a flow cytometer (Becton Dickinson, San Jose, CA, USA).

Reverse transcription-polymerase chain reaction

Total cellular RNA was extracted by the RNeasy MINI Handbook Kit (Qiagen, Valencia, CA, USA). Reverse transcriptase-PCR (RT-PCR) was performed using the one-step kit purchased from Qiagen following the manufacturer's protocol. Briefly, the reaction mixture, which was 50 μl in the final volume, consisted of 2 μg RNA template and 0.6 μM of forward and reverse primers for *SHP1*. The RT reaction consisted of one cycle of incubation for 30 min at 50°C, and the PCR consisted of 40 cycles of the following conditions: 95°C for 1 min, then 60°C for 1 min, followed by 72°C for 2 min. The final extension time was 10 min at 72°C. The *SHP1* PCR primer was described previously: forward primer, 5′-CTCTCCGGAAGCCCCCAGGATG-3′, and reverse primer, 5′-CCACCTGAGGACAGCACCGCT-3′.[36] The expected product size of the *SHP1* amplicon is of 1749 bp The sequences of the β-globin primers are as follows: (sense): 5′-CCTGGCTCACCTGGACAACCTCAA-3′ and (antisense): 5′-TAGCCACACCAGCCACCACTTTCT-3′. The expected product size of the *β-globin* amplicon is of 190 bp. The amplified PCR products were then separated on 1% agarose gel and visualized with ethidium bromide stain.

Measurement of ALK tyrosine kinase activity

The activity of NPM-ALK tyrosine kinase was measured using a commercially available kit (Sigma, St Louis, MO, USA) and details of this method have been described previously.[19]

Gene transfection of NPM-ALK

The NPM-ALK cDNA was initially amplified using an *NPM-ALK* plasmid (a kind gift by Dr S Morris, St Jude' Children Research Hospital, Memphis, TN, USA) using a primer set: *ALK* (forward) 5′-ATGGAAGATTCGATGGACATG-3′ and *NPM* (reverse) 5′-TCAGGGCCCAGGCTGGTT-3′. The *NPM-ALK* cDNA was blunt ligated with pCruz His™, which was initially digested with *Eco*RV. The resulted vector was transformed into *Escherichia coli* on ampicillin-resistant plates, and plasmids were isolated by the Mini-prep kit (Qiagen). The sequence and orientation of the insert were confirmed by DNA sequencing. Transfection experiments were carried out in six-well plates using LipofectAMINE (Invitrogen Life Technologies) according to the manufacturer's protocol.

Statistical analysis

Statistical analysis was performed by Student's *t*-test. A *P*-value of <0.05 was considered to be statistically significant.

Results

5-AZA induced SHP1 expression in ALK+ ALCL cells

SHP1 was not detectable in either Karpas 299 or SU-DHL-1 at the steady state, as shown by Western blots and RT-PCR. As shown in Figure 1, SHP1 mRNA became detectable in Karpas 299 with addition of 5-AZA to the cell culture 24 h after the initiation of the experiment, with 10 μM of 5-AZA inducing a

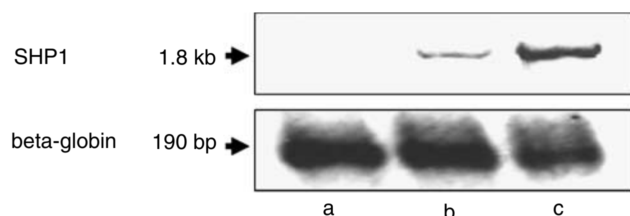

Figure 1 RT-PCR assay to measure the *SHP1* mRNA. Whereas no SHP1 was detectable in Karpas 299 cells at the steady state (lane a), 5-AZA treatment induced expression of SHP1 24 h after initiation of the experiment, and 5 μM of 5 AZA (lane b) induced a lower level than 10 μM did (lane c).

higher level than 5 μM. Western blot analysis showed that SHP1 protein was expressed in Karpas 299, first detectable 2 days after the initiation of the experiment (Figure 2a). The protein level of SHP1 increased gradually with time, reaching the highest level on day 5. On day 2, 10 μM of 5-AZA induced a higher level of SHP1 than 5 μM, but there were no appreciable differences in the SHP1 level between these two different dosages of 5-AZA from day 3 to day 5. Induction of SHP1 was similarly observed for SU-DHL-1, except that a higher level of SHP1 was induced with 10 μM compared to 5 μM, as illustrated in Figure 2b (day 5 results).

SHP1 expression correlated with decreased expression of JAK3, pJAK3 and pSTAT3

We then evaluated if the restoration of SHP1 expression is associated with any changes in the JAK3 tyrosine phosphorylation in both ALK+ ALCL cell lines. As illustrated in Figure 2a and c, no substantial changes in the expression of JAK3 and pJAK3 were identified shortly after treating Karpas 299 cells with 5-AZA. Nevertheless, both JAK3 and pJAK3 markedly decreased on day 3 when compared to cells treated with diluent only (negative controls). When the pJAK3 levels were assessed after correction of the total JAK3 was made, we identified that pJAK3 was downregulated more than that of the total JAK3 (Figure 2c). The decreased levels of JAK3 and pJAK3 inversely correlated with the marked increase in the SHP1 protein level on day 3. Whereas there were no appreciable changes in the total STAT3 protein level throughout the experiment, pSTAT3 decreased with 5-AZA. Compared to the decreases in JAK3 and pJAK3, the decrease in pSTAT3 was delayed, with >50% reduction identifiable on day 5 of treatment with 10 μM of 5-AZA (Figure 2a and c). Similar results were obtained from SU-DHL-1 cells treated with 5-AZA (illustrated in Figure 2b). To ensure these

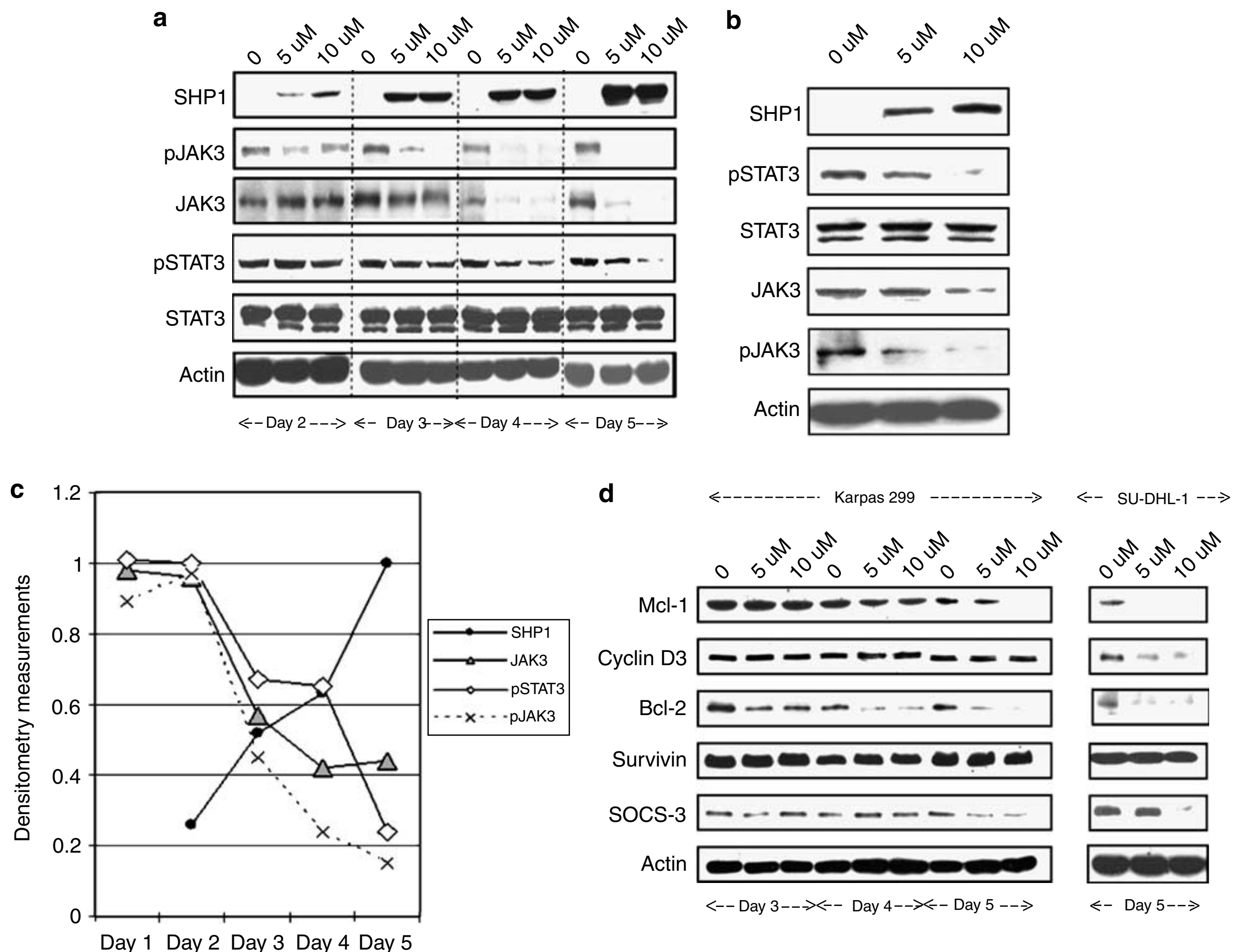

Figure 2 Upregulation of SHP1 by 5-AZA correlates with downregulation of the JAK3/STAT3 signaling in ALK+ ALCL cells. (**a**) Western blot analysis showed that 5-AZA induced time-dependent increase in SHP1 in Karpas 299 cells. In the same experiment, decreases in JAK3, pJAK3 and STAT3 were identified, although the decrease in pSTAT3 was relatively delayed. (**b**) Western blot analysis revealed that SU-DHL-1 cells treated with 5-AZA in 5 days showed restoration of SHP1 and downregulation of pJAK3, JAK3 and pSTAT3, similar to the changes seen for Karpas 299 cells. (**c**) Densitometric measurement of the band intensity for pJAK3, JAK3 and pSTAT3 shown in (**a**). For each day, the band intensity of SHP1, JAK3, pSTAT3 and pJAK3 in cells treated with 10 μM of 5-AZA was normalized to that from cells treated with solvent only (negative controls). pJAK3 was also normalized to the level of JAK3 for each drug dosage and each day. Compared to the untreated samples, pJAK3 decreased more than JAK3, and substantial decrease (i.e. >50% reduction) of pSTAT3 was identified later than that of JAK3 and pJAK3. (**d**) Western blot analysis showed that 5-AZA induced decreases of some but not all of the STAT3 downstream targets in Karpas 299 cells. Mcl-1, Bcl-2 and SOCS-3 were downregulated, whereas cyclin D3 and survivin showed no detectable changes. A similar pattern of changes was identified in SU-DHL-1 cells, except that downregulation in cyclin D3 was also detected in this cell line.

changes were not owing to a nonspecific drug effect of 5-AZA, we performed similar experiments using U266 cells, a myeloma cell line that we have confirmed to have constitutive activation of STAT3 and SHP1 expression at the steady state. No detectable changes in SHP1, pSTAT3 and STAT3 were identified in U266 cells treated with 5-AZA for 5 days. These findings support a link between the restoration of SHP1 expression and changes in the JAK3/STAT3 pathway.

Decreased STAT3 activation was associated with modulation of some but not all STAT3 downstream targets

We have previously shown that specific blockade of STAT3 in ALK+ ALCL cells using a dominant-negative construct induced significant alterations in known downstream targets of STAT3 signaling, including Bcl-2, Mcl-1, SOCS3, survivin and cyclin D3.[10] Thus, we assessed if the decrease in pSTAT3 associated with SHP1 expression is sufficient to induce similar changes in these STAT3 downstream targets. As illustrated in Figure 2d, the expression levels of cyclin D3 and survivin showed no detectable changes throughout the experiment, but Mcl-1, SOCS-3 and Bcl-2 showed concentration-dependent and time-dependent decreases in their protein levels after treatment with 5-AZA. The decrease in the protein level of Bcl-2 was first noted on day 3, whereas the decreases in SOCS-3 and Mcl-1 were first detectable on day 5. Changes in the STAT3 downstream targets for SU-DHL-1 cells are also illustrated in Figure 2d (right panel). The overall pattern was similar although more marked downregulation in cyclin D3 and Mcl-1 was noted. Similar experiments were performed in U266; no detectable changes were noted in these targets.

Proteasome inhibitor, MG132, inhibited 5-AZA-induced downregulation of JAK3, pJAK3 and STAT3

SHP1 has been reported to inhibit JAKs not only by tyrosine dephosphorylation, but also by decreasing the total protein level via the proteasome pathway.[37] Thus, we tested if proteasome inhibitor, MG132, can inhibit the 5-AZA-induced downregulation of the total JAK3 protein level. Both Karpas 299 and SU-DHL-1 cells were incubated in the presence of 5 μM of 5-AZA for 4 days, treated with MG132 (10 μM) and harvested at 0, 2, 6 and 10 h after the addition of MG132. As illustrated in Figure 3, JAK3, which was present at a low level after 4 days of 5-AZA treatments, increased in expression in a time-dependent manner. SHP1, pJAK3 and pSTAT3 all increased in their levels with the addition of MG132.

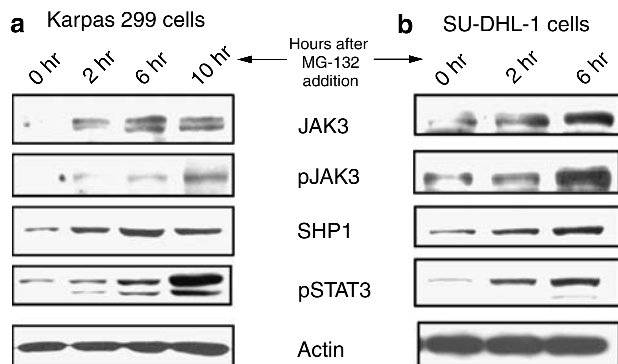

Figure 3 MG132 blocked downregulation of JAK3 induced by 5-AZA. Karpas 299 cells and SU-DHL-1 cells incubated with 5-AZA for 4 days were treated with MG132, a proteasome inhibitor. MG132 induced time-dependent upregulation of JAK3, pJAK3 as well as pSTAT3, in spite of the concomitant increase of SHP1.

5-AZA did not induce significant changes to the ALK tyrosine kinase activity

As we previously showed that JAK3 might potentiate the tyrosine kinase activity of NPM-ALK in ALK+ ALCL cell lines,[19] we tested whether there are changes in the tyrosine kinase of NPM-ALK on day 4, during which both JAK3 and pJAK3 had been downregulated. As shown in Figure 4, *in vitro* tyrosine kinase assay showed that Karpas 299 cells treated with 5 μM of 5-AZA for 4 days induced a slight increase in ALK enzymatic activity to 120% of its baseline level, although this difference is not statistically significant ($P > 0.05$). In contrast, a selective JAK inhibitor, AG490, significantly reduced the ALK enzymatic activity to approximately 42% of the negative control cells (acetic acid only).

5-AZA induced cell-cycle arrest and sensitized ALCL cells to doxorubicin-induced apoptosis

Compared to untreated Karpas 299 and SU-DHL-1 cells, treatment with 5-AZA induced a significant decrease in the cell viability as assessed by Trypan blue staining. As shown in Figure 5a, 5-AZA induced a significant decrease in the number of viable cells in both cell lines compared to the untreated cells. As illustrated in Figure 5b, cell cycle analysis revealed that there was a decrease in the proportion of Karpas 299 cells in the S phase as well as the G_2/M phase. The S-phase fraction decreased from 31 (untreated) to 22 and 21%, at 5 and 10 μM of 5-AZA, respectively. There was no detectable increase in the sub-G_0/G_1 fraction. The lack of an increase in apoptosis was further supported by the absence of a detectable increase in the cleaved caspase-3 level shown on Western blots (not shown).

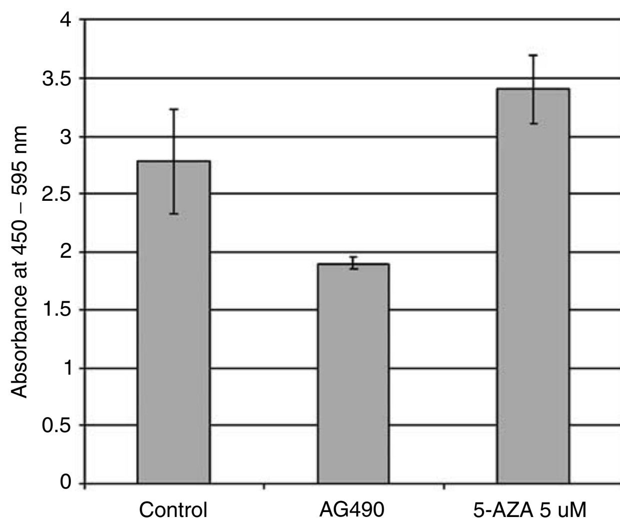

Figure 4 *In vitro* measurement of the NPM-ALK tyrosine kinase activity after 5-AZA treatment. 5-AZA did not significantly decrease the absorbance in Karpas 299 compared to the negative controls (treated with acetic acid only). AG490, which was previously shown to decrease the tyrosine kinase activity of NPM-ALK, served as a control.

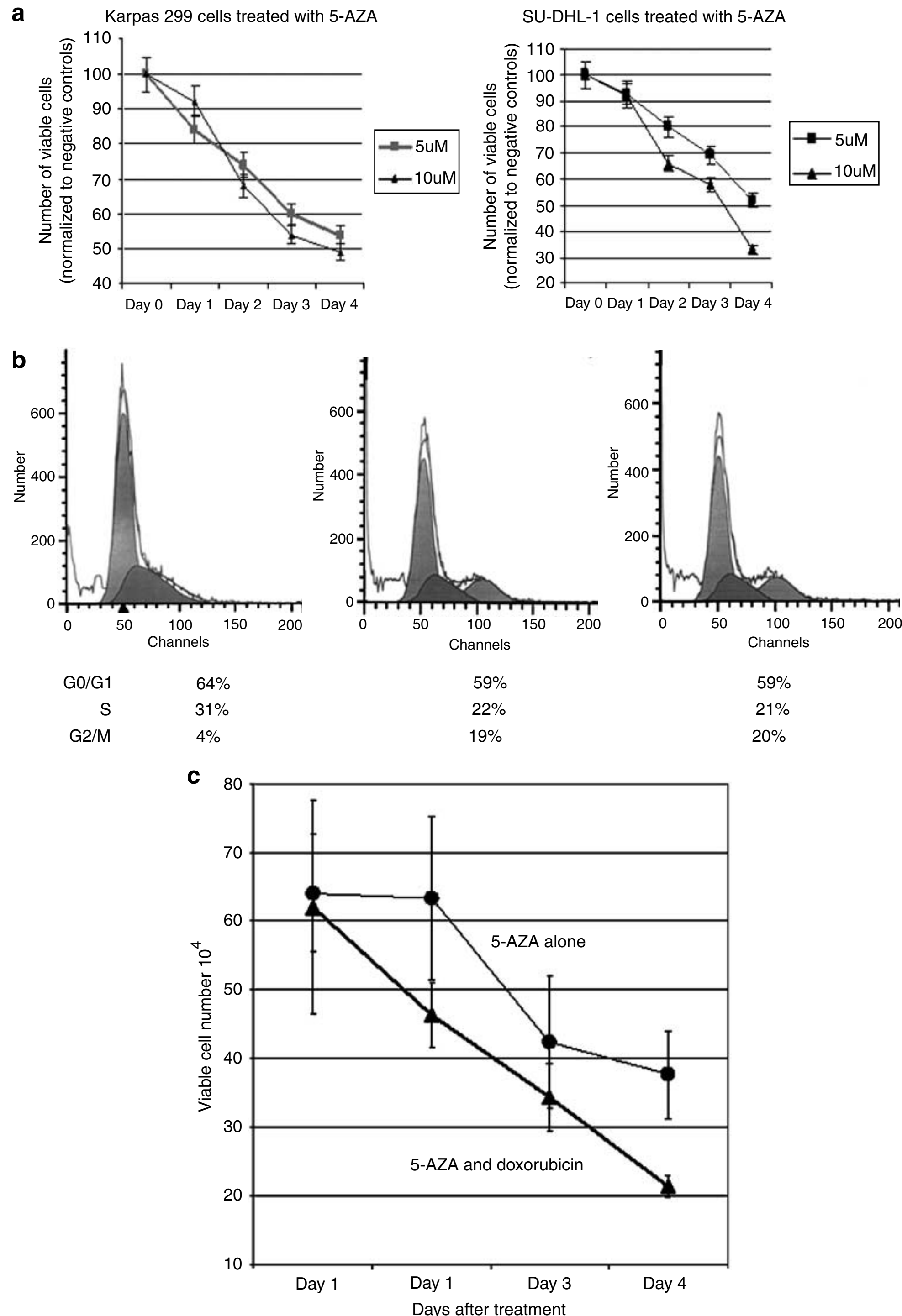

Figure 5 Biological effects of 5-AZA in ALK+ ALCL cells. (**a**) Trypan blue exclusion test in Karpas 299 cells treated with 5-AZA: Karpas 299 cells were treated with solvent only (negative controls), 5 μM of 5-AZA or 10 μM of 5-AZA. The cell numbers of 5-AZA-treated cells were normalized to that of the negative controls. 5-AZA significantly decreased the number of viable cells. No significant difference was seen between the two dosages of 5-AZA. Triplicate experiments were performed. (**b**) Cell cycle analysis was performed using Karpas 299 cells treated with diluent (left) or 5-AZA (5 μM, middle; 10 μM, right) for 4 days. Cells were analyzed by flow cytometry after staining with propidium iodide. Compared to the negative controls, treated cells showed increased proportion of cells in the G2/M phase. (**c**) 5-AZA potentiated the apoptosis induced by doxorubicin: karpas 299 cells treated with a combination of low-dose doxorubicin (0.1 μM) and 5-AZA induced more cell death than low-dose doxorubicin alone. The number of viable cells was assessed by Trypan blue exclusion assay. Triplicate experiments were performed.

Despite the absence of increased apoptosis, 5-AZA potentiated apoptosis induced by doxorubicin, a chemotherapeutic drug used commonly to treat ALCL patients. As shown in Figure 5c, Karpas 299 cells were treated with a relatively low dose (0.1 μM) of doxorubicin. Compared with the use of doxorubicin alone, the combination of doxorubicin and 5-AZA was more effective

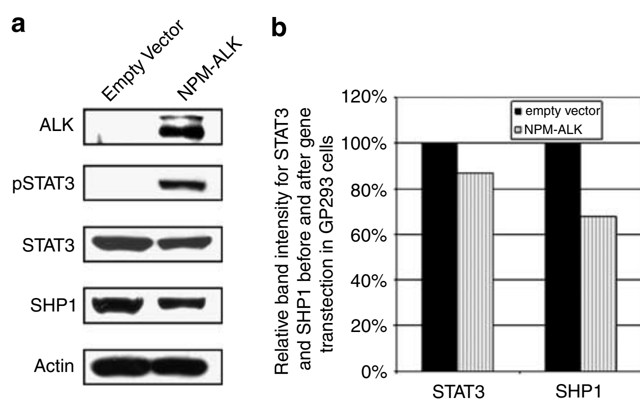

Figure 6 Transfection of NPM-ALK induced a partial downregulation of SHP1. GP293 cells were transfected with an empty vector or an NPM-ALK-expressing vector. Cells transfected with NPM-ALK showed evidence of STAT3 activation (i.e. expression of pSTAT3) and a partial decrease in SHP1 expression, which was shown to be a 32% reduction by densitometry analysis. A relatively slight decrease in the total STAT3 band was also detectable after NPM-ALK transfection.

in reducing the number of viable cells, although the difference between the two experimental conditions is statistically significant only on day 4.

In view of a recent publication that active STAT3 binds to DNA methyltransferase 1 (DNMT1) and promotes methylation/silencing of *SHP1*,[38] we tested if transfection of NPM-ALK, which activates STAT3 in ALK+ ALCL cells, leads to downregulation of SHP1. After gene transfection into GP293 cells, STAT3 became tyrosine phosphorylated and SHP1 was downregulated (Figure 6), and the decrease was estimated to be 32% using densitometry analysis (after correction of a slight difference between the actin bands). Interestingly, the total STAT3 band was consistently decreased slightly (averaging 88% of the negative control).

Discussion

Loss of SHP1 associated with gene hypermethylation is a relatively common abnormality in hematologic cancers;[19,39,40] the exact biological significance of this abnormality has not been extensively studied. The main objective of this study is to correlate the restoration of SHP1 expression using 5-AZA and changes in the JAK3/STAT3 signaling pathway in ALK+ ALCL cells, which are often characterized by the absence of SHP1 and constitutive activation of the JAK3/STAT3 pathway.

We found that 5-AZA induced a time-dependent increase in the SHP1 mRNA and protein level in both ALK+ ALCL cell lines, and this change coincided with the downregulation of pJAK3 and JAK3, followed by a decrease in pSTAT3. Substantial (i.e. >50% of the negative controls) downregulation of both JAK3 and pJAK3 was detectable. The finding that pJAK3 did not decrease in their levels until day 3 of the experiment is rather unexpected, as the current concept is that SHP1 is a rapid acting tyrosine phosphatase. This unexpected result suggests that the initially low level of SHP1 is not sufficient in balancing the overactivated JAK3. We also found that reduction in the total JAK3 protein level was dependent on the proteasome degradation pathway, as a proteasome inhibitor, MG132, efficiently blocked the decrease of JAK3. A parallel observation was made by Wu *et al.*,[37] who found that expression of SHP1 in HTB26 cells negatively regulates JAK1 by dephosphorylation and promotion of proteasome degradation. Thus, SHP1 negatively regulates the JAK/STAT pathway through at least two different mechanisms. Additional studies are required to delineate how SHP1 promotes proteasome degradation of JAK3. Interestingly, as shown in Figure 3, both pSTAT3 and SHP1 increased in their levels after addition of MG132, and these findings support the concept that STAT3 activation is more related to the expression/activation level of JAK3 than the SHP1 expression. Another interesting observation is that a marked reduction of the pSTAT3 level was relatively delayed compared to that of JAK3 or pJAK3. This finding suggests that SHP1 downregulated STAT3 activation indirectly, likely via decreasing JAK3 expression and activation. This is in keeping with our previous finding that JAK3 contributes to STAT3 activation in ALK+ ALCL cells.[18]

Zhang *et al.*[38] recently reported that active STAT3 forms a complex with DNMT1, which binds to the *SHP1* promoter and facilitates gene methylation of *SHP1*. As NPM-ALK is an important activator of STAT3 in ALK+ ALCL, we tested if ectopic NPM-ALK expression in GP293 cells results in downregulation of SHP1. Results from our gene transfection experiments (Figure 6) are entirely in keeping with this concept. However, our findings suggest that STAT3 activation alone is not sufficient to induce complete *SHP1* silencing; additional mechanisms likely exist to achieve complete *SHP1* gene silencing. A mild decrease in the total STAT3 level was identified after NPM-ALK; the mechanism underlying this finding is unclear.

The partial but incomplete downregulation of pSTAT3 after 5-AZA treatment is expected, as the other activators of STAT3 such as NPM-ALK and src are probably functional. With the partial decrease in the pSTAT3 level, a number of STAT3 downstream targets showed detectable decreases in their expression (such as Mcl-1, SOCS3 and Bcl-2). The relative lack of changes in survivin and cyclin D3 in this study is probably owing to the persistence of STAT3 activation, albeit at a relatively low level. These findings suggest that the expression of various STAT3 downstream targets may be triggered at different levels of STAT3 activation.

Despite the decrease in Bcl-2 and Mcl-1 levels, we did not detect significant apoptotic cell death with 5-AZA. Lack of apoptotic cell death in our experiments can at least be partially explained by the persistent expression of survivin. Survivin is an important anti-apoptotic protein.[41] Previous studies have shown that STAT3 induces its anti-apoptoic effects via induction of survivin,[42] and enforced expression of survivin can suppress cell death induced by STAT3 inhibition.[43] Nevertheless, we found that 5-AZA sensitized Karpas 299 cells to doxorubicin-induced apoptosis, and this may be due to decreases in other anti-apoptotic proteins such as Mcl-1 and Bcl-2. In support of this concept, the difference in apoptosis between Karpas 299 cells treated with combined 5-AZA and low-dose doxorubicin and cells treated with low-dose doxorubicin alone was not statistically significant until day 4 of the experiment, and this correlates with the findings that Bcl-2 and Mcl-1 did not show substantial decreases in their expression level until day 4 or 5, respectively.

We found that 5-AZA induced cell-cycle arrest in Karpas 299 cells, predominantly in the G_2/M phase. This is almost certainly not related to cyclin D3, a G_1 promoter that did not show significant changes in its expression level after 5-AZA. From the literature, it has been reported that the p38/MAPK pathway is modulated by 5-AZA,[44] and downregulation of this pathway may be responsible for the G_2/M arrest observed in this study. As 5-AZA can modulate the expression of many genes, depending

on the gene methylation pattern of specific cell types, it is highly likely that other genes that may have been upregulated by this agent in ALK+ ALCL cells are responsible for these biological effects.

Lastly, after we submitted this manuscript for publication, Honorat et al.[45] have published that NPM-ALK is a substrate for SHP1, and SHP1 negatively regulates NPM-ALK and decreases tumorigenecity. Thus, these findings are in keeping with the concept that loss of SHP1 contributes to the pathogenesis of ALK+ ALCL. As mentioned in discussion of this paper, there is a discrepancy related to the SHP1 expression in Karpas 299 cells between the two studies. We believe that this discrepancy is likely owing to the existence of different subclones of this cell line.

Conclusions

We found that restoration of SHP1 expression induced by 5-AZA correlates with a significant downregulation of the JAK3/STAT3 signaling in both ALK+ ALCL cells. Our data support the concept that loss of SHP1 contributes to the pathogenesis of ALK+ ALCL. In addition to its role as a tyrosine phosphatase, SHP1 silences JAK3 by virtue of promoting JAK3 degradation via the proteasome pathway.

Acknowledgements

This study is supported by research grants from the Alberta Cancer Board and the University of Alberta Hospital Research Foundation awarded to RL.

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