STAT3 Inhibition Is a Therapeutic Strategy for ABC-like Diffuse Large B-Cell Lymphoma

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Abstract

Persistent STAT3 signaling contributes to malignant progression in many diverse types of human cancer. STAT3 is constitutively active in activated B-cell (ABC)–like diffuse large B-cell lymphomas (DLBCL), a class of nongerminatal center derived DLBCL cells for which existing therapy is weakly effective. In this report, we provide a preclinical proof of concept that STAT3 is an effective molecular target for ABC-like DLBCL therapy. Direct inhibition of STAT3 with short hairpin RNA suppressed the growth of human ABC-like DLBCL in mouse models in a manner associated with apoptosis, repression of STAT3 target genes, and inhibition of a tumor-promoting microenvironment. Together, these results suggest that STAT3 is essential to maintain the pathophysiology of ABC-like DLBCL and therefore that STAT3 inhibition may offer a promising approach in its therapy. Cancer Res; 71(9); 3182–8. © 2011 AACR.

Introduction

Among the non-Hodgkin lymphomas, the diffuse large B-cell lymphoma (DLBCL) represents the most frequent (30%) of the aggressive lymphomas and is, pathophysiologically and clinically, a very heterogeneous disease (1). In contrast to normal cells, STAT3 is persistently activated in many diverse human tumors, where it dysregulates the transcription of genes involved in fundamental functions such as proliferation, survival, angiogenesis, and immune evasion (2).

High levels of STAT3 expression and activation have been found in activated B-cell (ABC)–like DLBCL (3), which is a subtype of DLBCL associated with a worse prognosis and molecularly characterized by constitutively activated NF-xB, and differing from germinal center (GC)–like subtype, which is characterized by high levels of CD10 and Bcl-6 (4). Recently, it has been shown that Bcl-6 is a negative regulator of STAT3 expression in GC-like DLBCL (3) and also there is cooperation between NF-xB and STAT3 in the ABC-like subtype (5). However, a more detailed study on the role of STAT3 in these tumor cells has not been conducted. We analyzed several specimens from ABC-like DLBCL patients by immunofluorescence staining, and in all of them we observed STAT3 activation (Supplementary Fig. S1), confirming previous observations (3, 5). We therefore hypothesized that STAT3 inhibition may have antilymphoma effects.

The main purpose of this study was to evaluate whether STAT3 could be a good target for treating ABC-like DLBCL. The importance of this study is that treatment with anti-STAT3 drugs directed against this specific target, which is aberrantly activated only in tumors, may be more effective and have fewer side effects than conventional therapy. Our findings show that inhibition of STAT3 with short hairpin RNA (shRNA) suppresses DLBCL tumorigenesis.

Materials and Methods

Establishment of stable transduced cell lines and cell culture conditions

Human ABC-like DLBCL Ly3 and Ly10 cell lines were a gift from Dr. B. Hilda Ye (Albert Einstein College of Medicine, Bronx, NY) and Dr. L.M. Staudt (National Cancer Institute, Bethesda, MD), respectively. Human GC-like DLBCL DHL-4 cell line was a gift from Dr. M. Jensen (Seattle Children’s Research Institute, Seattle, WA). Ly3 and Ly10 cells were maintained in Iscove’s modified Dulbecco’s medium supplemented with 10% FBS and 10% human plasma, respectively. DHL-4 cells were maintained in RPMI medium supplemented with 10% FBS. The lentiviral vectors were produced as described previously (6). Ly3 cells were then transduced at a multiplicity of infection (MOI) of 1. To select the transduced cells, the culture medium was replaced after 24 hours with fresh medium containing 1 μg/mL puromycin. All the experiments were carried out on cells stably transduced with the lentivirus expressing the STAT3 shRNA no. 842 except those for Fig. 2D, where cells were stably transduced with lentivirus...
expressing STAT3 shRNA no. 840 (6). Mycoplasma testing and flow cytometric analysis of a panel of several surface markers expression were done as quality and authenticity control testing of the cell lines studied in this work (data not shown). Tests were carried out within the past 6 months.

**Drugs and cytokines**

STATTIC and S31-201 were provided by EMD Chemicals and were dissolved in 100% dimethyl sulfoxide to prepare a 40 mmol/L stock and 25 mmol/L stock, respectively, and stored at −20°C. Recombinant human interleukin (IL)-6 and IL-10 (R&D Systems) were reconstituted in sterile 1X PBS containing 0.1% bovine serum albumen to prepare a 10 μg/mL stock and stored at −20°C.

**MTS assays**

MTS (inner salt) assay (Promega) was carried out according to instructions from the supplier. Absorbance was measured at 490 nm with a Chameleon plate reader (Bioscan).

**Flow cytometry**

For apoptosis analysis cells were stained with Annexin V and propidium iodide (PI) using Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences Pharmingen). The percentage of viable and dead apoptotic cells was determined by flow cytometry using Annexin V/propidium iodide staining. Values represented as bar graphs are the mean of 3 independent experiments with the standard deviation. Bar graphs are the mean of 3 independent experiments with the standard deviation.

**Figure 1.** Effects of shRNA-mediated STAT3 inhibition in vitro. A, control or STAT3 shRNA lentivirus Ly3 cells were grown for 48/72 hours in full or serum-free medium. The percentage of viable and dead apoptotic cells was determined by flow cytometry using Annexin V/propidium iodide staining. Values represented as bar graphs are the mean of 3 independent experiments with the standard deviation. B, control or STAT3 shRNA lentivirus Ly3 and Ly10 cells were plated in the presence of medium containing 10% FBS in 96-well plates and incubated with 4 ng/mL interleukin (IL)-6 or IL-10 for 48 hours. Following this, the percentage of cell proliferation was determined by MTS assay. Values represented as bar graphs are the mean of 3 independent experiments with the standard deviation. C, after 48 hours of culture, cells were collected to isolate RNA and quantitative real-time PCR was carried out to see changes in mRNA levels of STAT3 target genes. Bars represent relative expression values normalized to the β-actin levels. Values represented as bar graphs are the mean of 3 independent experiments with the standard deviation.
were collected from each sample using a CyAn ADP Violet (Dako) cytometer and calculated using the Summit software (Dako) for Fig. 1A, whereas for Fig. 4A a Cytomics FC500 (Beckman Coulter) was used and the geometric mean fluorescence intensity was analyzed for live population. The percentage of dead cells in Fig. 1A was calculated considering all the Annexin V-positive plus the PI-positive and the Annexin V/PI–positive cells.

Animal model studies
Ly3 cells (1 × 10^7) were resuspended in HBSS and injected s.c. into the flank of 4- to 6-week-old athymic nude mice (Taconic Laboratories), NOD/SCID (nonobese diabetic/severe combined immunodeficient) mice, or NOD/SCID IL2Rnull mice (The Jackson Laboratories). All mice were maintained under specific pathogen-free conditions and were used in compliance with protocols approved by the local Institutional Animal Care and Use Committee.

Immunofluorescence staining
Frozen tumor sections were analyzed as described before (8) using primary rabbit anticleaved Caspase-3 antibody (1:50; Cell Signaling), followed by secondary antibody (goat anti-rabbit, Alexa Fluor 488 labeled, 1:200; Molecular Probes), and both primary rat anti-mouse Gr1 and CD31 antibodies (1:20; BD Biosciences), followed by secondary goat anti-rat antibody conjugated with Alexa Fluor 555 (1:200; Invitrogen).

Western blot analysis
Tumor samples were homogenized in RIPA lysis buffer, which included protease and phosphatase inhibitors. Western blot analysis was carried out as described before (9) using the indicated antibody: rabbit polyclonal anti-STAT3 and anti–phospho-STAT3 (Tyr705; Cell Signaling Technology); mouse monoclonal anti–c-Myc and rabbit polyclonal anti–Mcl-1 (Santa Cruz Biotechnology); rabbit polyclonal anti-survivin (Novus Biologicals); mouse monoclonal β-actin antibody (Sigma-Aldrich).

Cytokine antibody array
Tumor samples were digested in HBSS containing DNAse and collagenase and processed in the strainer. Single-cell suspensions were then cultured in medium for 24 hours. Following that, supernatants were analyzed using human cytokine antibody array (Raybiotech) according to the manufacturer’s protocol.

Quantitative real-time PCR and PCR arrays
Total RNA was isolated and purified from cells by RNeasy Kit (Qiagen) and reverse-transcribed using the Omniscript Reverse Transcription Kit (Qiagen). For Fig. 1C, cDNAs were analyzed by quantitative real-time PCR (qRT-PCR) using primers provided by SABiosciences and iQ SYBR Green supermix (Bio-Rad). For Fig. 3C, cDNAs were analyzed using a PCR array system (SABiosciences) according to the manufacturer’s protocol. PCRs were carried out on a DNA Engine thermal cycler equipped with Chromo4 detector (Bio-Rad).
Figure 3. STAT3 silencing induces tumor apoptosis and affects infiltration of tumor microenvironment cells in Ly3 xenografts. A, left, tumors from control lentivirus or STAT3 shRNA lentivirus NOD/SCID IL2Rnull mice were harvested 6 days after injection, stained with cleaved Caspase-3 antibody and analyzed by immunofluorescence. Nuclei were stained with Hoechst. A (right), 2 separate cohorts of nude mice were injected s.c. with control lentivirus Ly3 cells or STAT3 shRNA lentivirus Ly3 cells. Tumor samples from each group were harvested at the indicated time and when the volume reached the indicated value; whole-cell lysates were prepared and subjected to Western blotting individually or pooled with tumors harvested from the same group in the same time; β-Actin levels served as loading controls. The integrated density values (IDV) were determined by the densitometry software Alphalmager. Values below each band indicate the ratio between the IDV of that band with the IDV of the correspondent β-actin band. The statistical significance was calculated using the normalized IDVs and the statistical analysis was carried out with a Student t test (unpaired t test with Welch’s correction). B, 6 days after injection tumors from control lentivirus or STAT3 shRNA lentivirus NOD/SCID mice were isolated, pooled, and processed to carry out a cytokine array. Signals were quantified by densitometry and normalized to positive controls. The percentage of cytokine secretion in STAT3 shRNA lentivirus is relative to 100% control lentivirus. C, after 48 hours of culture cells were collected to isolate RNA and PCR array was carried out to see changes in mRNA levels of inflammatory cytokines and receptor genes. D, tumors from control lentivirus or STAT3 shRNA lentivirus NOD/SCID IL2Rnull mice were harvested 6 days after injection, stained with CD31 (left) or Gr1 (right) antibody and analyzed by immunofluorescence. Nuclei were stained with Hoechst 33342.
Statistical analysis

Statistical analyses were carried out using the statistical software GraphPad Prism 4. Statistical significance was set at a level of $P < 0.05$.

Results and Discussion

We investigated the effect of STAT3 silencing in 3 different animal models of Ly3 lymphoma (nude, NOD/SCID, and NOD/SCID IL-2Rnull mice). For this purpose, we established stably transduced STAT3 shRNA-expressing lentivirus Ly3 cells and control lentivirus Ly3 cells. The stable expression of STAT3 shRNA resulted in 40% to 50% reduction of total STAT3 protein levels compared with the control lentivirus cells (Supplementary Fig. S2A).

In vitro experiments revealed that STAT3 downregulation was associated with a higher percentage of dead cells in STAT3 shRNA cells compared with the control cells, and this percentage was even higher in serum starvation conditions and after 72 hours of culture compared with 48 hours, suggesting a role for STAT3 in the survival of those cells (Fig. 1A). STAT3 silencing inhibited the growth of Ly3 cells (approximately 40% inhibition) even in the presence of IL-6 or IL-10, which are major activators of STAT3 signaling and are important in the pathophysiology of DLBCL (ref. 10; Fig. 1B). The percentages of Ly3 cell proliferation at 48 hours were comparable to those at 72 hours (Supplementary Fig. S2B). Similar results were obtained in Ly10 cells, showing that this strategy also affected another ABC-like cell line (Fig. 1B). qRT-PCR revealed that silencing of STAT3 resulted in downregulation of different STAT3 target genes in a cell-dependent manner. In particular, we observed significant reduction in mRNA levels of Mcl-1, Bcl-xL, and survivin in STAT3 shRNA lentivirus Ly3 cells, as well as significant reduction of cyclin D2 and upregulation of STAT1 in STAT3 shRNA lentivirus Ly10 cells (Fig. 1C). STAT3 inhibition also reduced adhesion of Ly3 cells to the bone marrow stroma layer and migration toward SDF-1α (data not shown), an important factor that mediates proliferation, survival, chemotaxis, migration, and adhesion into bone marrow stroma, and has been shown to be a chemoattractant factor for B-lymphoma cells (11). We extended this strategy to a cell line representing the GC subtype, DHL-4, which shows very low or undetectable levels of phospho-STAT3 by Western or flow cytometric analysis (data not shown). Cell growth and STAT3

Figure 4. Small-molecule STAT3 inhibitors affect the growth of Ly3 cells in vitro and in vivo. A, Ly3 parental cells were plated in the presence of medium containing 10% FBS and treated with STAT3 inhibitors at the indicated concentrations. After 24 or 48 hours, phospho-STAT3 level or cell proliferation were analyzed by flow cytometry or MTS assay, respectively. Values represented as bar graphs are the mean of 3 independent experiments with the standard deviation. B, Ly3 parental tumor-bearing mice were treated intra tumor with vehicle or STATIC at 3.75 mg/kg every day. Two hours after the last treatment, samples from each group were harvested. Whole-cell lysates were prepared and subjected to Western blot analysis for phospho-STAT3 level detection. β-Actin levels served as loading controls.
target gene expression were not affected in these cells cultured in vitro (data not shown), showing that GC-like DHL-4 cells do not depend on STAT3 in terms of proliferation. These data are in agreement with a previous study, which showed that STAT3 is downregulated in GC-like cells (3). Thus, on the basis of our observations, we raise the possibility that GC-like cells may generally not be dependent on STAT3; however, this suggestion needs to be validated with additional GC-like cells.

Tumors in control lentivirus Ly3-bearing mice grew progressively (Fig. 2A), whereas tumors in STAT3 shRNA lentivirus Ly3-bearing mice regressed 4 to 5 days after injection (Fig. 2B and C). These results were comparable to those obtained with Ly3 stably transduced with lentivirus expressing a STAT3 shRNA with different sequence than the original one (Fig. 2D). Similar results were observed in Ly10 xenografts (Supplementary Fig. S3). STAT3 shRNA Ly3 tumor regression was associated with Caspase-3–dependent apoptosis and significant reduction of STAT3 target genes at the protein level such as Mcl-1, c-Myc, and survivin (approximately 40%–60% inhibition; Fig. 3A). Survivin expression is an unfavorable prognostic factor in DLBCL (12). We also observed 30% reduction of IL-10 production, and the secretion of many other cytokines was altered as well (Fig. 3B). c-Myc rearrangements and elevated IL-10 plasma levels are associated with poor prognosis in DLBCL (13, 14). Whereas both STAT3 shRNA and control lentivirus Ly3 cells grown in vitro had the same protein levels of c-Myc, STAT3 silencing resulted in inhibition of IL-10–inducible upregulation of c-Myc (Supplementary Fig. S2C) associated with downregulation of IL-10–dependent STAT3 activation (data not shown) and inhibition of IL-10–inducible cell growth (Fig. 1B, left and Supplementary Fig. S2B). These data suggest a role of IL-10/STAT3/cMyc in Ly3 cell proliferation and reveal a difference between in vitro and in vivo, where IL-10 was observed among the cytokines that were produced the most in control tumors and reduced in STAT3 shRNA lentivirus tumors.

It is notable that partial STAT3 downregulation (40% inhibition of total STAT3 levels was observed by Western blot analysis) results in such dramatic antitumor effects in vivo. This implies that Ly3 cells depend strongly on STAT3 for proliferation and survival in vivo. Whereas in vitro studies did show Ly3 dependency on STAT3 for proliferation and survival, these effects were not as dramatic as the in vivo results, suggesting that the tumor microenvironment has a critical role in STAT3-dependent tumor cells. STAT3 signaling is important in cross-talk between tumor cells and stroma cells (15), and through control of expression of multiple factors is involved in induction of angiogenesis and immune evasion (16). Blocking STAT3 in Ly3 cells affected secretion and expression of several factors responsible for migration of diverse stroma cells and protumor microenvironment remodeling (Fig. 3B and C). The most striking observation is the requirement of STAT3 signaling in lymphoma cells for induction of tumor angiogenesis, as shown by significant reduction of CD31+ endothelial cell infiltration (Fig. 3D). Moreover, blocking STAT3 signaling affected infiltration of Gr1+ cells, a subpopulation of myeloid cells previously associated with tumor immune suppression and angiogenesis (8, 17). Proangiogenic gene signatures of large B-cell lymphoma were previously associated with poor prognosis (18). Furthermore, blocking STAT3 signaling in Ly3 cells induces expression of genes involved in T-cell migration, such as CXCL10, which was shown to be upregulated after blocking of STAT3 in a mouse melanoma model, resulting in increased infiltration of T cells (19).

Finally, we investigated whether small-molecule STAT3 inhibitors had antilymphoma activity like the shRNA. We tested 2 commercially available inhibitors, STATTIC and S31-201. The viability of Ly3 cells grown in vitro was affected by both inhibitors tested in a dose-dependent manner and the antiproliferative effect was associated with inhibition of STAT3 phosphorylation (Fig. 4A). The antilymphoma activity of STATTIC was also observed in a Ly3 NOD/SCID IL2Rnull mouse model (Fig. 4B). The inhibition of tumor growth was significant, but not as dramatic as that mediated by shRNA, most probably due to low efficiency of intratumoral drug bioavailability. Nevertheless, the tumor growth inhibition was associated with decreased levels of phospho-STAT3 in all tumor samples isolated from STATTIC treated mice compared to vehicle treated mice.

Previous reports with STAT3 siRNA or kinase inhibitors such as Janus activated kinase inhibitors in these tumor cells were limited to proliferation analysis in vitro (5, 20). This is the first demonstration in vivo that direct STAT3 inhibition in ABC-like DLBCL suppresses tumor growth. Our data also suggest that direct inhibition of STAT3, rather than indirect inhibition targeting upstream signaling, may be more effective as a therapeutic strategy for these tumor types. Moreover, partial inhibition of target genes would be the most likely response in the treatment of patients, due to limited delivery efficiency of the siRNA into tumors. Thus, our results show that even partial downregulation of STAT3 could achieve complete suppression of the tumorigenesis of ABC-like DLBCL Ly3 cells.

Although developing a small-molecule STAT3 inhibitor as well as siRNA delivery for clinical trials is still a challenge, our in vitro and in vivo studies show that STAT3 is a good target for therapy in DLBCL and also establish that STAT3 siRNA-based gene therapy is a feasible approach for DLBCL. Our data encourage continued development of new STAT3 inhibitors as well as STAT3 siRNA delivery strategies for treatment of DLBCL.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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