Constitutive activation of STAT5A and STAT5B regulates IgM secretion in Waldenström’s macroglobulinemia

Lucy S. Hodge, Steven C. Ziesmer, Zhi-Zhang Yang, Frank J. Secreto, Anne J. Novak, Stephen M. Ansell

Division of Hematology and Internal Medicine, Mayo Clinic, Rochester, MN

Address correspondence to:

Stephen M. Ansell, M.D., Ph.D.
Division of Hematology
Mayo Clinic
200 First St SW
Rochester, Minnesota 55905
Phone: (507) 284-0923
Fax: (507) 266-4972
Ansell.stephen@mayo.edu

Supported in part by grants from the International Waldenström Macroglobulinemia Foundation and the Leukemia and Lymphoma Society Translational Research Program.
Key Points

1.) STAT5 is constitutively phosphorylated in malignant B cells obtained from patients with Waldenstrom’s macroglobulinemia

2.) Inhibition of STAT5 signaling significantly decreases IgM production and may be useful therapeutically for patients with high IgM
Abstract

Activation of the JAK-STAT signaling pathway has been associated with the pathogenesis and progression of both solid and hematologic malignancies. We have detected constitutive activation of STAT5 in malignant B cells derived from patients with Waldenstrom’s macroglobulinemia (WM). While shRNA-mediated knockdown of the STAT5A and STAT5B isoforms did not affect cellular proliferation, loss of STAT5 significantly decreased IgM secretion. A similar dose-dependent inhibition of IgM secretion was observed when WM cell lines were treated with a small molecule inhibitor of STAT5. These data suggest that STAT5 is involved in regulating IgM production in WM, and that inhibition of STAT5 may represent a novel therapeutic strategy for lowering IgM levels in WM patients.
Introduction

Signaling events initiated by cytokines within the bone marrow microenvironment are necessary for the viability and development of normal B-cells. Such events are equally important for the maintenance of many B cell malignancies as well, including Waldenstrom’s macroglobulinemia (WM), a lymphoplasmacytic lymphoma characterized by high levels of serum IgM. One of the most prominent signaling cascades activated by cytokines is the Janus kinase family (JAK)/signal transducers and activators of transcription (STAT) pathway composed of four JAK proteins (JAKs1-3, Tyk2) and seven STAT proteins (STATs 1-6, including STAT5A and STAT5B). Hyperactivation of these proteins, either in response to heightened cytokine signaling or secondary to activating mutations within the JAK/STAT pathway, leads to carcinogenesis, with numerous reports suggesting an association between constitutive STAT5 activation and uncontrolled cellular proliferation in hematologic malignancies.

Despite a high degree of amino acid sequence homology, STAT5A and STAT5B are distinct transcription factors, with evidence indicating both overlapping and non-redundant functional activities for these isoforms in solid tumors. However, the activation and downstream function of the STAT5 isoforms in lymphoma have not been well characterized. Here, we have explored the relationship between STAT5 phosphorylation and the biologic activity of WM tumor cells. Our data indicate that STAT5 is hyperactive in malignant WM B cells, promotes IgM secretion, and investigations into the targeting of both STAT5A and STAT5B clinically are warranted.
Methods

Cell Lines and Reagents

The WM cell lines, MWCL-1, BCWM.1 and RPCI-WM1 were cultured as described. CD19+CD138+ WM cells were isolated from the bone marrows of consenting patients using positive selection beads. The STAT5 inhibitors, N’-((4-Oxo-4H-chromen-3-yl)methylene) nicotinohydrazide and N1-(11H-indolo[3,2-c]quinolin-6-yl)-N2,N2-dimethylethane-1,2-diamine (IQDMA) were purchased from Calbiochem.

Immunohistochemistry

Paraffin-embedded bone marrow specimens were stained with anti-human pSTAT5 and visualized as outlined previously. All slides were observed with light microscopy (Olympus AX70, 200x aperture 0.46, 400x aperture 0.75, 600x aperture 0.80; Olympus America) with images being captured with a SPOT RT camera and software (Diagnostic Instruments). A novel methodology termed SIMPLE (Sequential Immunoperoxidase Labeling and Erasing) was used to stain the sections for pSTAT5 together with either CD20 or CD138 as previously described. Images were prepared with Adobe Photoshop (Adobe Systems Incorporated). IRB approval was obtained to collect bone marrow cells from patients with Waldenstrom’s macroglobulinemia for use in translational research projects. Informed consent was obtained in accordance with the Declaration of Helsinki.

Flow Cytometry

WM cells were fixed and permeabilized prior to staining with STAT antibodies or respective isotype controls (Phosflow, BD Biosciences). Cells were analyzed on a FACSCalibur, and data was processed using FlowJo software (TreeStar Inc).
**shRNA-mediated knockdown of STAT5A and STAT5B**

STAT5A- and STAT5B-targeting shRNAs in the doxycycline-inducible pTRIPZ vector system were purchased from Open Biosystems. Recombinant lentiviral particles were expressed following transient transfection of HEK293T cells using the TransLenti Viral Packaging System (Open Biosystems). Viral supernatant was then used to transduce MWCL-1 cells and, after 48 hours, cells were selected with puromycin. Specificity and efficiency of doxycycline-induced knockdown was determined after 72 hours by western blotting.

*Enzyme-linked immunoabsorbent assay (ELISA)*

Cell-free supernatants were collected, and the concentration of IgM was determined according to the manufacturer’s protocol (Human IgM ELISA Kit; Bethyl Laboratories, Inc).

*Proliferation Assay*

Cells were cultured with either a STAT5 inhibitor or doxycycline for 48 or 72 hours, respectively. Tritiated thymidine (³H-TdR; 5.0 Ci/mmol [185 GBq/mmol] (Amersham) was added 18 hours prior to scintillation counting.

*Immunoprecipitation and Immunoblotting*

Cell lysates from BCWM.1 and MWCL-1 cells were incubated overnight with either anti-STAT5A or anti-STAT5B antibodies followed by the addition of Protein G Dynabeads (Invitrogen) for an additional 24 hours. Beads were collected using a magnet and heated for ten minutes in LDS Sample Loading Buffer. Protein was separated by SDS-PAGE. Following transfer onto nitrocellulose membranes, blots were probed using antibodies targeted towards either pSTAT5 (Y694/Y699) (Abcam), STAT5A or STAT5B
Western blotting for STAT1, STAT3 and actin was performed using antibodies from Santa Cruz Biotechnology, Inc.

Statistical analysis

Statistical analysis was performed with GraphPad Prism v5.0d (GraphPad Software) using the Student’s t-test. Significance was set at $p<0.05$. 

Results and Discussion

Constitutive activation of STAT5 in WM

Constitutive STAT signaling is associated with the pathogenesis and progression of many malignancies. A flow cytometry-based characterization of the baseline activation status of several STAT proteins in malignant CD19+CD138+ cells obtained from the bone marrows of six patients with WM, as well as in three WM cell lines, revealed significant phosphorylation of STAT5 (Figure 1A). Phosphorylation was greatest in the patient samples, possibly a result of in vivo exposure to STAT5-activating cytokines prior to harvesting. No other STAT protein was consistently phosphorylated. To confirm the hyperactivation of STAT5 in WM, immunohistochemical staining for pSTAT5 was performed on bone marrows obtained from both healthy controls and WM patients. While pSTAT5 staining was detected in all marrows, staining was more intense and uniform in the specimens obtained from patients with WM. (Figure 1B). Sequential staining of bone marrow specimens revealed STAT5 phosphorylation in both CD20+- and CD138+-expressing cell populations (Figure 1C). Furthermore, immunoprecipitation experiments revealed baseline phosphorylation of both the STAT5A and STAT5B isoforms in the BCWM.1 and MWCL-1 cell lines (Figure 1D). Together these data indicate that STAT5 is constitutively phosphorylated in malignant WM B cells.

Biological effects of STAT5 knockdown in WM

Hyperactivation of STAT5 has been reported in other hematological malignancies including Hodgkin lymphoma, acute myeloid leukemia and acute lymphocytic leukemia,
and inhibition of STAT5 phosphorylation either chemically or using siRNA-mediated approaches has been observed to significantly enhance apoptosis and limit cellular proliferation.\(^4,\)\(^15\) We have employed both methodologies here to assess the biological relevance of constitutive STAT5 phosphorylation in WM. Decreasing the expression of either STAT5A or STAT5B using isoform-specific, doxycycline-inducible shRNA significantly decreased IgM levels as compared to MWCL-1 cells transduced with an empty vector shRNA (Figure 2A, 2B). These results were subsequently confirmed in BCWM.1 cells (data not shown). Interestingly, the STAT5A- and STAT5B-mediated decreases in IgM secretion do not appear to occur secondary to decreases in overall cell number, as inhibition of neither STAT5A nor STAT5B had an effect on the level of cellular proliferation (Figure 2C). STAT5 has been previously reported to drive cellular proliferation through activation of cyclin D1.\(^16\) However, it is possible that both isoforms need to be inhibited to see effects on proliferation, as knocking down only STAT5A or STAT5B may lead to compensation by the other isoform, an effect that has been observed following inhibition of other STAT proteins.\(^17,\)\(^18\)

These experiments were then repeated using known small molecule inhibitors of both isoforms of STAT5, N’-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide and IQDMA.\(^19,\)\(^20\) A dose-dependent decrease in IgM secretion was observed following incubation of WM cell lines with both small molecule inhibitors, providing the first data to indicate that STAT5 regulates IgM secretion by malignant B cells (Figures 2E, 2G). The small molecule inhibitors also significantly decreased cellular proliferation, albeit at slightly higher concentrations than those needed to affect IgM levels. (Figures 2F, 2H). These results support the existence of a compensatory mechanism between STAT5A
and STAT5B such that both isoforms need to be inhibited before cellular proliferation is affected.

In conclusion, we have identified high levels of STAT5 activation in malignant WM B cells. As inhibition of both STAT5 isoforms either alone or together led to significant reductions in IgM levels, addition of a STAT5 inhibitor to current treatment protocols could provide a useful clinical strategy for lowering IgM levels in patients with WM.
Acknowledgments

Supported in part by grants from the International Waldenström Macroglobulinemia Foundation and the Leukemia and Lymphoma Society Translational Research Program.
Authorship Contributions

LSH, AJN and SMA designed experiments. LSH and SCZ performed experiments. LSH, AJN, ZY, FJS, AJN and SMA analyzed data. LSH and SMA wrote the manuscript with input from all authors.
Disclosure of Conflict of Interest

The authors have no conflicts of interest to disclose.
References


Figure Legends

**Figure 1. Activation of STAT5 in malignant WM B cells.** (A) Baseline STAT phosphorylation was measured in freshly sorted CD19\(^+\)CD138\(^+\) cells obtained from the bone marrows of patients with WM (n=6) and MWCL-1, BCWM.1 and RPCI-WM1 cells. Following fixation and permeabilization, tyrosine phosphorylation of STATs 1, 3, 4, 5, and 6 was determined via FACS analysis. Normalization for non-specific antibody binding was performed by dividing the mean fluorescence intensity associated with the specific phosphor-antibody signal by the mean fluorescence intensity of the isotype control. Data are presented as the normal mean fluorescence intensity (\(\Delta MFI\)). Significance of STAT5 phosphorylation relative to other STAT proteins was determined by the Student’s t-test for both primary cells and WM cell lines, *\(p<0.05\), **\(p<0.01\). (B) Immunohistochemical staining of pSTAT5 (brown) in bone marrow sections obtained from four consenting patients with WM (WM1-WM4) and 4 normal bone marrows (NM1-4) was performed using a polyclonal anti-pSTAT5 antibody as described in “Methods”. Slides were visualized on an Olympic Provus AX70 light microscope, and images shown are original magnification X400. (C) Costaining of pSTAT5 (red) with CD20\(^+\) (blue) and CD138\(^+\) (yellow) expressing B cells in WM bone marrow. (D) Immunoprecipitation for STAT5A (5A) and STAT5B (5B) was performed on unstimulated BCWM.1 (B) and MWCL-1 (M) cells followed by western blotting and detection of STAT5A, STAT5B and pSTAT5.

**Figure 2. Biological relevance of STAT5 activation in WM.** (A) Expression of STAT5A and STAT5B were individually inhibited through the use of a doxycycline-
inducible shRNA system transduced into MWCL-1 cells. Following a 72-hour treatment with doxycycline (500 ng/mL), cells were lysed and immunoblotted for STAT1, STAT3, STAT5A, STAT5B and actin, and protein expression was compared to that observed in cells transduced with an empty vector shRNA (EV). Western blotting was performed on three separate experiments. Representative blots are shown. (B) Following 72-hour induction of STAT5A or STAT5B shRNA with 500 ng/mL doxycycline, IgM was measured by ELISA (B) and cellular proliferation was measured by ³H-TdR incorporation (C) and levels were compared to those detected in the empty vector-transduced cells (EV). (D) Effect of a small molecule inhibitor of STAT5 on STAT5 activation status in BCWM.1 and MWCL-1 cells. Shaded histograms represent isotype control, dotted lines represent baseline STAT5 phosphorylation and solid lines represent STAT5 phosphorylation following a 24 hour treatment with 10 μM N’-((4-Oxo-4H-chromen-3-yl)methylene) nicotinohydrazide. Representative plots of three separate experiments are shown. (E-H) BCWM.1 and MWCL-1 cells were treated with increasing concentrations of N’-((4-Oxo-4H-chromen-3-yl)methylene) nicotinohydrazide or IQDMA for 48 hours. IgM was measured by ELISA (E, G) and cellular proliferation was measured by ³H-TdR incorporation (F, H). All experiments were performed in triplicate. *p<0.05 as determined by the Student’s t-test.
Figure 2.
Constitutive activation of STAT5A and STAT5B regulates IgM secretion in Waldenstrom’s macroglobulinemia

Lucy S. Hodge, Steven C. Ziesmer, Zhi-Zhang Yang, Frank J. Secreto, Anne J. Novak and Stephen M. Ansell