LYMPHOID NEOPLASIA

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

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Key Points

- STAT5 is constitutively phosphorylated in malignant B cells obtained from patients with Waldenström’s macroglobulinemia.
- Inhibition of STAT5 signaling significantly decreases IgM production and may be useful therapeutically for patients with high IgM levels.

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 Waldenström’s macroglobulinemia (WM), a lymphoplasmacytic lymphoma characterized by high levels of serum immunoglobulin M (IgM). One of the most prominent signaling cascades activated by cytokines is the Janus kinase family/signal transducers and activators of transcription (JAK/STAT) pathway composed of 4 JAK proteins (JAK1-JAK3, Tyk2) and 7 STAT proteins (STAT1-STAT6, 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 nonredundant 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 and promotes IgM secretion, and that 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, ×200 aperture 0.46, ×400 aperture 0.75, ×600 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 Photoshop (Adobe Systems Inc.). Institutional review board approval was obtained to collect bone marrow cells from patients with WM 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, before staining, with STAT antibodies or respective isotype controls (Phosflow, BD Biosciences). Cells were analyzed on a FACSCalibur, and data were processed using FlowJo software (TreeStar Inc.).

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shRNA-mediated knockdown of STAT5A and STAT5B

STAT5A- and STAT5B-targeting short hairpin RNAs (shRNAs) in the doxycycline-inducible pTRIPZ vector system were purchased from Open Biosystems. Recombinant lentiviral particles were expressed after 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 immunosorbent assay

Cell-free supernatants were collected, and the concentration of IgM was determined according to the manufacturer’s protocol (Human IgM Enzyme-Linked Immunosorbent Assay 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 before 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 10 minutes in 4X Laemmli Loading Buffer (Bio-Rad). Protein was separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. After transfer onto nitrocellulose membranes, blots were probed using antibodies targeted toward pSTAT5.
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 t test. Significance was set at P < .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 6 patients with WM, as well as in 3 WM cell lines, revealed significant phosphorylation of STAT5 (Figure 1A). Phosphorylation was greatest in the patient samples, possibly as a result of in vivo exposure to STAT5-activating cytokines before harvesting. No other STAT protein was consistently phosphorylated. To confirm the hyperactivation of STAT5 in WM, immunohistochemical staining for pSTAT5 was performed on bone marrow specimens obtained from both healthy controls and WM patients. Although 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.

(Y694/Y699) (Abcam), STAT5A, or STAT5B (both Santa Cruz Biotechnology, Inc.). Western blotting for STAT1, STAT3, and actin was performed using antibodies from Santa Cruz Biotechnology, Inc.
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 shRNA-mediated approaches, has been observed to significantly enhance apoptosis and limit cellular proliferation.\textsuperscript{1,14} 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 in MWCL-1 cells, using isoform-specific, doxycycline-inducible shRNAs, significantly decreased IgM levels as compared with cells transduced with an empty vector shRNA (Figure 2A-B). These results were subsequently confirmed in BCWM1 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.\textsuperscript{15} 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 after inhibition of other STAT proteins.\textsuperscript{1,16,17}

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.\textsuperscript{18,19} A dose-dependent decrease in IgM secretion was observed after 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 (Figure 2E,G). The small molecule inhibitors also significantly decreased cellular proliferation, albeit at slightly higher concentrations than those needed to affect IgM levels (Figure 2F,H). 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

This work was supported in part by grants from the International Waldenström Macroglobulinemia Foundation and the Leukemia and Lymphoma Society Translational Research Program.

Authorship


Conflict-of-interest disclosure: The authors declare no competing financial interests.

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