G-protein-coupled Receptor Agonist BV8/Prokineticin-2 and STAT3 Protein Form a Feed-forward Loop in Both Normal and Malignant Myeloid Cells*

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Background: Signaling pathways underlying BV8-mediated oncogenesis remain unknown.
Results: BV8-STAT3 forms a feed-forward loop in both normal and malignant myeloid cells and promotes tumor growth.
Conclusion: JAK2/STAT3 signaling plays critical roles in BV8-mediated myeloid cell-dependent oncogenesis.
Significance: This study identifies a novel role of BV8-STAT3 signaling in mediating cross-talk between tumor microenvironment and tumor cells.

An important role of BV8 in mobilization of myeloid cells and myeloid cell-dependent angiogenesis has been established. Recently, it has also been shown that granulocyte colony-stimulating factor (G-CSF)-induced BV8 expression is STAT3 dependent in CD11b+Gr1+ myeloid cells. However, the BV8 downstream signaling pathway(s) intrinsic to myeloid cells crucial for angiogenesis, and potentially also for development of cancers of myeloid origin, remains largely unknown. Here we show that BV8 activates STAT3, which is critical for regulating genes important for both tumor cell proliferation/survival and tumor angiogenesis, in both normal and malignant myeloid cells. Further, BV8-induced STAT3 activation requires Janus-activated kinase 2 (JAK2) activity as shown by both genetic and pharmacologic inhibition. Knocking down BV8 in human myeloid leukemia cells inhibits STAT3 activity and expression of STAT3 downstream angiogenic and pro-proliferation/survival genes, leading to a decrease in tumor cell viability. BV8 shRNA expressing leukemia cells exhibit reduced STAT3 activity and tumor growth in vivo. Taken together, we have delineated a signaling pathway downstream of BV8 that plays critical roles in both the tumor microenvironment and malignant myeloid cells for angiogenesis and tumor cell proliferation/survival.

BV8/prokineticin-2 is a small and secreted protein, which belongs to a family of peptides with a five-disulfide bridge motif (1–3). BV8 and its homologous protein, endocrine gland-de-
genesis and identifies a novel role of BV8 in promoting oncogenesis intrinsic to malignant cells of myeloid origin.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Recombinant human BV8 and G-CSF were obtained from PeproTech (Rocky Hill, NJ) and R&D Systems (Minneapolis, MN), respectively. JAK2 inhibitor AZD1480 was provided by AstraZeneca (Waltham, MA) and dissolved in dimethyl sulfoxide (DMSO) for *in vitro* studies. For *in vivo* experiments, AZD1480 was dissolved in water supplemented with 0.5% hypromellose and 0.1% Tween® 80. Antibodies recognizing phospho-STAT3 (Tyr-705), phospho-JAK2 (Tyr-1007/1008), and JAK2 were purchased from Cell Signaling Technology (Danvers, MA). Antibodies recognizing STAT3 (C-20), Bcl-x<sub>1</sub> (B cell lymphoma-extra large) (H-50), VEGF (A-20), poly(ADP-ribose) polymerase-2 (PARP) (H-250), and BV8 (H-51), as well as human BV8 shRNA lentiviral particles (sc-61409-V), were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FLAG-M2 and anti-β-actin were from Sigma. Human STAT3 and control shRNA lentiviral particles were also purchased from Sigma.

**Cell Lines**—Acute human myelogenous leukemia cell line, KG1, was kindly provided by Dr. Carlotta Glackin (Beckman Research Institute, City of Hope National Medical Center, Duarte, CA). Human U937 monocyte leukemia cell line and mouse B16 melanoma cell line were purchased from the American Type Culture Collection. Mouse renal cell carcinoma cell line, Renca, was provided as a generous gift by Dr. Alfred Chang (University of Michigan Medical Center, Ann Arbor, MI). Mouse endothelial cell lines derived from prostate were kindly provided by S. Huang and J. Fidler (M.D. Anderson Cancer Center, Houston, TX). All cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS).

**Transduction of shRNA Lentiviral Particles and Transfection of Plasmids**—Transduction of lentiviral particles into KG1 and U937 cells to generate stable cell lines that expressed human BV8, STAT3, or control shRNA was performed according to the manufacturer’s instructions (Sigma). BV8 or STAT3 expression in pooled puromycin-resistant cells was examined by real-time PCR and Western blotting. Stable cell lines were maintained in RPMI 1640 with 10% FBS containing 5 ng/ml puromycin (Sigma). pRC/CMV/STAT3C-FLAG (active form of STAT3) and pRC/CMV were transfected into KG1 cells using the Amaxa cell line nucleofector kit R according to the manufacturer’s instructions (Sigma). pRC/CMV/STAT3C-FLAG (active form of STAT3) and pRC/CMV were transfected into KG1 cells using the Amaxa cell line nucleofector kit R according to the manufacturer’s instructions (Lonza).

**Mice and in Vivo Experiments**—Animal use procedures were approved by the Institutional Animal Care and Use Committees of the Beckman Research Institute at City of Hope. Athymic nude (NCR<sup>−</sup> nu/nu), BALB/c, and C57BL/6 mice were purchased from NCI, National Institutes of Health (Bethesda, MD), Jak2<sup>fl</sup> and Stat3<sup>fl</sup> mice were kindly provided by Drs. Kay-Uwe Wagner (University of Nebraska Medical Center, Omaha, NE) (21) and S. Akira (Osaka University, Japan), respectively. Both Jak2<sup>fl</sup> and Stat3<sup>fl</sup> mice were crossed with Mx1-Cre mice, which were obtained from The Jackson Laboratory. Mice with Jak2<sup>−/−</sup> or Stat3<sup>−/−</sup> hematopoietic cells were generated by treating Mx1-Cre/Jak2<sup>fl</sup> or Mx1-Cre/Stat3<sup>fl</sup> mice with poly(I-C) as described previously (22). Deletion of Stat3 and Jak2 was verified by real-time RT-PCR. For *in vivo* KG1 tumor challenge, 1 × 10<sup>6</sup> of KG1 cells expressing either control or BV8 shRNA were injected intraperitoneally into 7–8-week-old nude mice, which were euthanized at day 60. Tumor volumes were determined at the end of the study, and tumor tissues were collected for further analysis. For Renca tumor challenge, 2.5 × 10<sup>6</sup> of Renca cells were subcutaneously injected into the flank of 7–8-week-old BALB/c mice. When the average tumor volume reached ~150 mm<sup>3</sup>, AZD1480 or vehicle was administered daily by oral gavage at the dose of 50 mg/kg of body weight for 6 consecutive days. For B16 tumor challenge, 1 × 10<sup>6</sup> of B16 cells were subcutaneously injected into the flank of mice with *Stat3<sup>−/−</sup>* or *Jak2<sup>−/−</sup>* hematopoietic cells. Mice were euthanized 14 days after tumor inoculation. The preparation of single-cell suspension from mouse spleen and the enrichment of splenic CD11b<sup>+</sup> myeloid cells were performed as described previously (22).

**BV8, G-CSF Stimulation, and AZD1480 Treatment in Vitro**—To determine STAT3 activation in KG1 and U937 cells, tumor cells were first starved with serum-free medium for 24 h and subsequently treated with 200 ng/ml human BV8 for the indicated time. Alternatively, freshly isolated mouse splenic CD11b<sup>+</sup> myeloid cells were treated with 100 ng/ml human BV8 in RPMI 1640 medium with 1% FBS. Recombinant human BV8 proteins show cross-reactivity to mouse and rat proteins (PeproTech, Rocky Hill, NJ). For G-CSF stimulation, KG1 cells expressing either control or STAT3 shRNA cultured in Hanks’ balanced salt solution with 0.02% bovine serum albumin (BSA) were stimulated with 50 ng/ml human G-CSF for 6 h. To examine the effects of AZD1480 in vitro, splenic CD11b<sup>+</sup> cells from naive C57BL/6 were treated with AZD1480 (1 μM) or DMSO in RPMI 1640 medium with 1% FBS for 1 h and subsequently stimulated with BV8 (100 ng/ml) for various times. To test cell viability, KG1 or U937 cells were cultured in the presence of 200 ng/ml BV8 in RPMI 1640 with 1% FBS for 48 h.

**Cell Viability, Cell Cycle, Apoptosis, and Western Blotting Analyses**—Viable cell number was determined by counting cell number manually after 0.04% trypan blue staining. Each of the viability tests was repeated at least six times. For cell cycle analysis, KG1 cells in the culture medium (1 × 10<sup>6</sup>/ml) were stained with propidium iodide according to the manufacturer’s instructions (BD Biosciences). For apoptosis assays, KG1 cells expressing control or BV8 shRNA were grown in serum-free RPMI 1640 medium for 48 h. Cells were stained with annexin V, Allophycocyanin Conjugate (eBioscience, San Diego, CA) and assessed for the percentage of annexin V-positive population using an Accuri C6 flow cytometer. For Western blotting assay, harvested cells were lysed in the lysis buffer (25 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 5% glycerol, and 1 mM EDTA). Tumor tissues collected from KG1 xenograft were first homogenized using a homogenizer (Fisher Scientific) and then lysed in the lysis buffer. An equal amount of protein was subjected to SDS-PAGE and blotted with indicated antibodies.

**Immunofluorescence**—Immunofluorescent staining of tumor sections was described previously (16). Anti-mouse CD31 (BD Biosciences) was used to detect blood vessels in xenograft tumors. The representative images were captured at 200×.
Feed-forward Loop between BV8 and STAT3

magnification. Quantification data were obtained by counting CD31-positive vessels manually on three random fields (200×) per slide, three slides per tumor, with four tumors per group.

Real-time RT-PCR—Total RNA was extracted using an RNase kit (Qiagen). cDNA was synthesized by using an iScript cDNA synthesis kit (Bio-Rad). Real-time PCR was conducted using iQ SYBR Green supermix (Bio-Rad) and performed by using a Chromo-4 real-time detector (Bio-Rad). Gene-specific primer sets for human/mouse BV8, human HIF1A (hypoxia inducible factor-1α), CDC25A, BCL2, VEGF, and IL1B were purchased from Qiagen. Either GAPDH or 18S rRNA housekeeping gene was used as internal control.

Immunoprecipitation—KG1 cells (10 × 10⁶) expressing control or STAT3 shRNA and subjected to ChIP assay as described previously (18). 4 μg of anti-STAT3 rabbit polyclonal antibody or control rabbit IgG were used for immunoprecipitations. The following primers, 5′-tcatttttgttgagggaaacaggc-3′ and 5′-aagcgctgttctcagttgccagc-3′, were used to amplify the human

FIGURE 1. BV8 induces STAT3 activation in both malignant and normal myeloid cells. A, Western blotting showing phospho-STAT3 (p-STAT3) and STAT3 protein amounts in KG1 and U937 cells treated with human BV8 (200 ng/ml) at the indicated time points. B, Western blotting showing increased expression of phospho-STAT3 (p-STAT3) proteins in tumor-primed, splenic CD11b⁺ myeloid cells upon treatment with BV8 (100 ng/ml) for 6 or 16 h. Cells were enriched from splenocytes of B16 tumor-bearing mice; mean ± S.E., n = 3.

FIGURE 2. STAT3 regulates BV8 expression in both malignant and normal myeloid cells. A, BV8 and STAT3 gene expression levels in stable cell lines (KG1 and U937) expressing indicated shRNA were analyzed by real-time RT-PCR. B, left, real-time RT-PCR showing BV8 gene expression in KG1 or U937 cells expressing control or STAT3 shRNA; *, p < 0.01. Right, immunoprecipitation (IP) by BV8 antibodies followed by Western blotting (IB) showing expression levels of KG1 cell-derived BV8 protein. C, BV8 mRNA levels in G-CSF-treated KG1 cells expressing control or STAT3 shRNA shown by real-time RT-PCR; *, p < 0.05. D, upper, BV8 mRNA levels in KG1 cells transfected with control plasmid or plasmid encoding STAT3C; *, p < 0.05. Lower, Western blotting to detect FLAG-tagged STAT3C protein in cells transfected with the indicated plasmids. E, ChIP assays showing STAT3 binding to the BV8 promoter in KG1 cells stably expressing control or STAT3 shRNA. Immunoprecipitated DNA fragments were detected by PCR (left) or quantitative real-time RT-PCR (right). Bottom left, a graph showing the relative intensity of DNA bands in the PCR gel (top), which were obtained by ImageJ software and then plotted as a percentage of the band intensity in each sample to the corresponding input (100%) (bottom left). Bottom right, data from real-time PCR assay were first calculated as (DNA amount in anti-STAT3 immunoprecipitation minus DNA in IgG immunoprecipitation)/(DNA in input), and then compared with the data from the anti-STAT3 antibody immunoprecipitation sample of STAT3 shRNA-expressing cells, which is designated as 1; mean ± S.E., n = 3; *, p < 0.05. F, real-time RT-PCR measuring BV8 mRNA levels in splenic CD11b⁺ cells freshly isolated from tumor-bearing mice with Stat3⁺⁻ or Stat3⁺⁺ hematopoietic cells (mean ± S.E., n = 3).
Western blotting analysis showed robust STAT3 activation in these CD11b\(^+\) myeloid cells upon 6 or 16 h of treatment with BV8 (Fig. 1B).

**STAT3 Regulates BV8 Expression in Both Normal and Malignant Myeloid Cells**—A recent study indicated that STAT3 is a key transcription factor for G-CSF-induced BV8 expression in mouse CD11b\(^+\) Gr1\(^+\) myeloid cells (12). However, whether STAT3 also regulates BV8 in cancer cells of myeloid origin has not been characterized. We therefore generated stable KG1 and U937 cell lines expressing BV8 or STAT3 shRNAs to address whether STAT3 is required for BV8 expression in the malignant cells of myeloid origin. Real-time RT-PCR showed that knockdown efficiency of BV8 or STAT3 gene in both cell lines reached 85–90% (Fig. 2A). BV8 mRNA expression level was drastically reduced in both KG1 and U937 cells expressing STAT3 shRNA (Fig. 2B, left). Importantly, levels of BV8 protein secreted to the culture medium of BV8 or STAT3 shRNA-expressing cells were also significantly reduced when compared with the control shRNA-expressing cells (Fig. 2B, right). To confirm whether G-CSF-induced BV8 expression required STAT3 in malignant cells of myeloid origin, we treated KG1 cells expressing STAT3 or control shRNA with G-CSF and checked their BV8 expression. Real-time PCR showed that BV8 expression was not induced by G-CSF in STAT3 shRNA-expressing cells (Fig. 2C). Furthermore, BV8 expression was significantly up-regulated by overexpression of STAT3C, a constitutively active mutant form of STAT3 (23) in KG1 cells (Fig. 2D). In addition, we evaluated STAT3 binding activity to the human BV8 promoter in both control and STAT3 shRNA-expressing KG1 cells using ChIP analysis. The data indicated that the binding of STAT3 to the BV8 promoter was reduced in STAT3 knockdown cells (Fig. 2E). Consistently, BV8 expression was abolished in Stat3-deficient mouse myeloid cells (Fig. 2F). Taken together, we show that BV8 induces STAT3 activation and STAT3 regulates BV8 expression, thereby forming a feed-forward loop in both normal myeloid cells and human malignant cells of myeloid origin.

**BV8-induced STAT3 Activation Requires JAK2**—BV8 binds to G-protein-coupled receptors, PKR1 and PKR2, and our previous studies have shown that sphingosine-1-phosphate (S1P),
which binds to another G-protein-coupled receptor S1PR1, activates STAT3 through JAK2 (17). Therefore, we investigated whether JAK2 is involved in and necessary for BV8-induced STAT3 activation. Western blotting analysis showed that phosphorylation of JAK2 was triggered after BV8 stimulation in myeloid cells isolated from B16 tumor-bearing mice or mouse endothelial cells, which correlated with STAT3 activity (Fig. 3A). In addition, BV8 was not able to induce Stat3 activity in Jak2-deficient myeloid cells (Fig. 3B). Moreover, our results showed that AZD1480, a JAK2 inhibitor (24, 25), blocked BV8-induced STAT3 activation in myeloid cells (Fig. 3C). These data indicate that JAK2 is required for BV8-induced STAT3 activation. We further investigated whether JAK2 is required for STAT3 up-regulation of BV8 expression. Treatment with JAK2 inhibitor AZD1480 in Renca mouse model reduced phospho-JAK2 and phospho-STAT3 levels and also led to a reduction in BV8 expression in myeloid cells (Fig. 3D). In addition, we found that BV8 expression was significantly reduced in Jak2-deficient myeloid cells (Fig. 3D).

Silencing BV8 Expression Reduces Myeloid Leukemia Cell Viability and Expression of Proliferation/Survival and Angiogenic Genes—To address whether BV8 could also promote tumor growth through increasing tumor cell survival, proliferation, and angiogenic potential, we knocked down BV8 in human myeloid leukemia cells by shRNA and subsequently tested the viability of these cells. Results from these experiments indicated that silencing BV8 expression in the leukemia cells significantly reduced their viability (Fig. 4A). Consistently, treatment with BV8 considerably enhanced the viability of leukemia cells (Fig. 4B). To address whether BV8 promotes the viability of myeloid leukemia cells through STAT3, we knocked down STAT3 by shRNA in KG1 cells and treated them with BV8. In this case, BV8 was unable to increase the viability of leukemia cells (Fig. 4C). These data demonstrated that BV8
promotes myeloid leukemia cell viability in a STAT3-dependent manner. We next carried out cell cycle analysis using flow cytometry to determine whether the modulation of tumor cell growth by BV8 was the result of cell cycle arrest or induction of apoptosis or the simultaneous activation of these two modes. The results revealed that the percentage of KG1 cells expressing BV8 shRNA was increased in sub-G₀ and G₀/G₁ phase, which was accompanied by a corresponding reduction in the percent-age of cells in S and G₂/M phase (Fig. 4D). Relevant to this, the apoptotic cell percentage was increased in KG1 cells expressing BV8 shRNA when compared with KG1 cells expressing control shRNA (Fig. 4E).

Furthermore, expression of genes crucial for proliferation, survival, and angiogenesis in the tumor cells, such as CDC25A, BCL2, HIF1A, VEGF, and IL1B, was significantly reduced in myeloid leukemia cells expressing BV8 shRNA (Fig. 4F).

**Inhibiting BV8 Expression in Myeloid Leukemia Cells Reduces STAT3 Activity and Tumor Growth**—BV8 has emerged as a therapeutic target to inhibit tumor angiogenesis. Anti-BV8 antibody reduced tumor growth and angiogenesis (11). Thus, we knocked down BV8 in human myeloid leukemia cells by shRNA and studied its effect in tumor growth. First, STAT3 activity was significantly reduced in BV8 shRNA-trans- fected human myeloid leukemia cells (Fig. 5A). Next, we implanted these tumor cells in nude mice. Tumor volume was significantly reduced in mice implanted with BV8 knockdown leukemia cells when compared with mice implanted with control leukemia cells (Fig. 5B). In addition, inhibiting BV8 also attenuated tumor expression of VEGF and Bcl-xL (Fig. 5C). Furthermore, we demonstrated that blood vessels were also decreased in the xenograft KG1 tumors transfected with BV8 shRNA (Fig. 5D).
DISCUSSION

Our current study has identified a novel JAK2-dependent BV8-STAT3 feed-forward signaling loop intrinsic to myeloid-derived leukemia cells, which contributes to tumor cell proliferation/survival (Fig. 5E). Although STAT3 activation has been traditionally characterized as rapid in the context of certain cytokines and growth factors (14, 15), our previous studies have demonstrated the distinct STAT3 activation mode by G-protein-coupled receptor signaling S1P/S1PR1 that elicits persistent STAT3 activation in tumors (17). BV8 and its cognate receptor signaling, to our knowledge, would be the second example of G-protein-coupled receptor signaling that activates STAT3, through JAK2. Similar to S1P/S1PR1, activation of STAT3 by BV8/BV8 receptor is slower and long lasting relative to IL-6, the quintessential STAT3 activator.

In tumor cells, it takes 6 h to see high levels of STAT3 activation by BV8, which may indicate an indirect effect. However, in endothelial cells, the activation of JAK2 is within an hour. Because the time frame could still be within the limit of direct activation, i.e. in the absence of de novo protein synthesis that causes secondary effects/activation, the activation by BV8 can be direct. At the same time, our data do not rule out completely the involvement of expression of some immediate early genes that might contribute to JAK2 activation, thereby leading to STAT3 activation.

In addition, STAT3 not only up-regulates BV8 transcriptional activation as reported previously (12) but also release of functional BV8 proteins from tumor cells to the tumor milieu to further activate STAT3. Although a role of BV8 in promoting myeloid cell-facilitated tumor angiogenesis has been demonstrated (11), our study unravels a novel role of tumor cell-derived BV8 in providing direct growth advantage to tumor cells. Therefore, a JAK2-dependent BV8-STAT3 signaling in tumors can be an efficient therapeutic target for the treatment of myeloid-derived human cancers.

Although a crucial role of STAT3 for diverse human cancer has been established, being a transcription factor, STAT3 remains a challenging target for current therapeutic interventions. STAT3 is well known to be the signal transducer for many cytokines and growth factors, which activate STAT3 through their tyrosine kinase activity. For that reason, several frontline drugs for cancer therapies, such as Sunitinib and Sorafenib, are tyrosine kinase inhibitors that can inhibit STAT3 at proper doses (26, 27). Unlike the conventional tyrosine kinase inhibitors, the receptors for BV8 are G-protein-coupled receptors, which are among the most druggable targets. Our studies thus open up new opportunities to target STAT3 with inhibitors of BV8 receptors, including both antibody and small molecule inhibitors, for the treatment of myeloid leukemia. Because other signaling pathways, such as ERK/MAPK, PI3K/AKT, Wnt/β-catenin, and NF-κB pathways, are also critical for proliferation/survival of the leukemia cells (28–31), combining inhibitors against BV8 receptors, and these pathways may be necessary to effectively treat myeloid cell leukemia. Our studies also show that JAK2 is critical for BV8/receptor-mediated STAT3 activation, suggesting that the use of JAK2 inhibitor(s) may enhance the efficacy of BV8/receptor inhibitors. Ultimately, when an effective direct STAT3 inhibitor(s) is available, the combination use of drugs against STAT3 and its upstream activators, which include BV8/receptor, may be an optimal option for treating myeloid leukemia. Further studies focusing on combinational therapy are therefore warranted.

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