Microenvironment and Immunology

A Galectin-3-Dependent Pathway Upregulates Interleukin-6 in the Microenvironment of Human Neuroblastoma

Ayaka M. Silverman1,5, Rie Nakata1,5, Hiroyuki Shimada2,5, Richard Sposto1,3,5, and Yves A. DeClerck1,4,5

Abstract

Interleukin-6 (IL-6) is a pleiotropic cytokine with a broad range of physiologic and pathologic functions. Because in cancer, IL-6 contributes to a microenvironment that promotes tumor cell survival, angiogenesis, and inflammation, understanding the mechanism responsible for its production is important. In neuroblastoma, the second most common solid tumor in children, IL-6 is produced not by tumor cells but by stromal cells such as monocytes and bone marrow mesenchymal stem cells (BMMSC). Here we show that the production of IL-6 in BMMSCs is in part stimulated by galectin-3 binding protein (Gal-3BP) secreted by neuroblastoma cells. We identified a distal region of the IL-6 promoter that contains 3 CCATT/enhancer binding protein (C/EBP) binding domains involved in the transcriptional upregulation of IL-6 by Gal-3BP. Gal-3BP interacted with Galectin-3 (Gal-3) present in BMMSCs, and a Gal-3BP/Gal-3/Ras/MEK/ERK signaling pathway was responsible for the transcriptional upregulation of IL-6 in BMMSCs in which Gal-3 has a necessary function. In support of the role of this pathway in human neuroblastoma tumors, Gal-3BP was found to be present in tumor cells and in the adjacent extracellular matrix of 96% of 78 primary neuroblastoma tumor samples examined by immunohistochemistry. Considering the protumorigenic function of IL-6 in cancer, this tumor cell–stromal cell interactive pathway could be a target for anticancer therapy. Cancer Res; 72(9); 2228–38. ©2012 AACR.

Introduction

It is now well recognized that interactions between tumor cells and stromal cells in the tumor microenvironment play a determinant role in cancer initiation and progression (1–4). Therefore the mechanisms by which the tumor microenvironment influences the behavior of malignant cells have been the subject of intensive investigation over the last decade (5). The production of soluble growth factors, cytokines and chemokines by stromal cells in the presence of tumor cells is one among the several mechanisms by which the tumor microenvironment affects cancer cells (6, 7). Among these soluble factors is interleukin-6 (IL-6; refs. 8, 9). This pleiotropic cytokine has multiple protumorigenic activities including the promotion of tumor cell proliferation and survival, the stimulation of angiogenesis, the induction of a state of immune tolerance, and the activation of osteoclasts to promote osteolytic bone metastasis (10–14). IL-6 also promotes the self-seeding of circulating tumor cells (15) and contributes to a stress response that protects tumor cells from drug action (16). IL-6 is not only produced by tumor cells but also by stromal cells in the tumor microenvironment (17, 18). In neuroblastoma, for example, tumor cells do not make IL-6 but upregulate its expression in bone marrow mesenchymal stem cells (BMMSC). Stromal-derived IL-6 then promotes osteoclast activation, the formation of osteolytic bone metastasis (19), and the resistance of tumor cells to cytotoxic drugs (20). The mechanism by which IL-6 is upregulated in the tumor microenvironment is, however, not entirely understood. We reported that the production of Galactin-3 binding protein (Gal-3BP; also known as 90 kDa Mac-2 binding protein), by neuroblastoma cells was one mechanism that stimulated the expression of IL-6 in BMMSCs (21) and in monocytes/macrophages (22). Gal-3BP is a self-adhesive glycoprotein that forms oligomers of 1,000 to 1,500 kDa in the extracellular milieu and promotes cell adhesion to matrix proteins (23). Its function in cancer is not well defined, but it has been suggested that it contributes to an inflammatory reaction to tumors and infections (24). Gal-3BP affects the Th2 cytokine profile in peripheral blood monocytes of patients with asthma with a decrease in IL-4, IL-5, and IL-13 and an increase in IL-6 (25). Its role in regulating IL-6 in cancer is new and the mechanism not known. Gal-3BP interacts with multiple proteins and, in particular, Galectin-3 (Gal-3), a ubiquitous glycosylated protein present at the surface, in the cytoplasm and in the nucleus of many nonmalignant and malignant cells. It acts as a membrane-associated receptor and signal transduction protein (26). Gal-3 is synthesized by MSC and has a suppressive effect on T-cell proliferation (27, 28).

Authors' Affiliations: From the Division of Hematology-Oncology, Departments of Pediatrics, Pathology, Preventive Medicine, Biochemistry and Molecular Biology, Keck School of Medicine, University of Southern California, and The Saban Research Institute of Children’s Hospital Los Angeles, Los Angeles, California

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corresponding Author: Yves A. DeClerck, The Saban Research Institute of Children’s Hospital Los Angeles, 4650 Sunset Boulevard, MS#54, Los Angeles, CA 90027. Phone: 323-361-2150; Fax: 323-361-4902; E-mail: declerck@usc.edu

doi: 10.1158/0008-5472.CAN-11-2165
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In this study we have tested the hypothesis that the interaction between Gal-3BP made by neuroblastoma cells and Gal-3 present in BMSCs is responsible for the upregulation of IL-6 in BMSCs in the presence of neuroblastoma cells.

Materials and Methods

Cell culture

Human neuroblastoma cell lines were cultured as previously reported (20). PC3 human prostate cancer cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, penicillin, and streptomycin. The nature of the cell lines was confirmed by genotype analysis using AmpFISTR Identifier kit PCR Reagents and Gene Mapper ID v3.2 (Applied Biosystems). Human BMSCs were purchased from ALL-Cells and cultured in accordance to the instructions of the manufacturer.

Reagents

Farnesyl thiosalicylic acid (FTS) was purchased from Santa Cruz Biotechnology, Inc. (sc-205322). Recombinant human Galectin-3BP (rGal-3BP) was purchased from R&D Systems and GenWay, and these preparations had endotoxin levels of less than 1 EU/µg protein.

Conditioned medium

Conditioned medium (CM) from neuroblastoma cell lines was collected as follows. Cells were plated at 5 × 10^5 in 150 cm² tissue culture flasks or at 1 × 10^6 per well in 6-well plates and cultured for 24 to 72 hours in FBS-containing medium. Cells were then washed with PBS (KCl 0.2 g/L; KH₂PO₄ 0.2 g/L; NaCl 8 g/L; NaH₂PO₄ 1.15 g/L, pH 7.4) twice and cultured in FBS-free medium for 24 hours. The medium was collected, centrifuged to eliminate floating cells, and concentrated by ultrafiltration at 4,500 rpm in a Centriplus YM-10 microconcentrator (Millipore).

IL-6 and Gal-3BP measurements

IL-6 and Gal-3BP in the CM were detected by ELISA, using a Quantikine Immunoassay Kit (R&D Systems) for IL-6 and a Quantikine Immunoassay Kit (R&D Systems) for IL-6 and Gal-3BP. Readings were obtained in a Synergy HT (BIO-TEK) spectrometer.

Immunodepletion of Gal-3BP

Protein A Sepharose CL-4B (Amersham) was preswollen with distilled water and equilibrated with PBS containing a protease and phosphatase Inhibitor Cocktail (Thermo Scientific). Five hundred microliters of a 50% (v/v) Protein A slurry was mixed with 15 µg of a goat polyclonal antibody against human Gal-3BP or goat IgG (control) and rotated at 4°C for 3 hours. This slurry was mixed with 1 mL of 10× serum-free CM and rotated at 4°C overnight. After centrifugation, the supernatant was collected and the sepharose beads were washed 3 times in PBS containing protease inhibitors and 0.05% (v/v) Tween-20.

Quantitative reverse transcriptase PCR

Total RNA was isolated using TRIzol (Invitrogen). The RNA quality and quantity was measured by NanoDrop spectrometer (ThermoScientific). Hundred nanograms of total RNA was reverse transcribed by SuperScript III reverse transcriptase (Invitrogen) according to manufacturer’s instructions. Quantitative PCR (qPCR) was carried out in duplicate samples using an ABI 7900HT Fast Real time PCR System (Applied Biosystems) at an annealing temperature of 60°C. The relative change in gene expression was calculated by the relative standard curve method using the mean of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control.

Construction of plasmids

To construct the pGL2-IL-6-Luc plasmid, genomic DNA was extracted from normal human whole blood using a Blood and Cell Culture DNA Mini Kit (Qiagen). A fragment of the IL-6 promoter region corresponding to position –2,155 to +4 was amplified by PCR using Pfu DNA polymerase (Stratagene) and the primers shown in Supplementary Table S1. The PCR fragment generated was digested with KpnI and HindIII for 3 hours at 37°C and ligated into the multiple cloning site of pGL2 Luc (Promega) and treated with alkaline phosphatase (Roche) for 1 hour. The ligation reaction was done using the Quick Ligation Kit (New England Biolabs). IL-6 promoter deletion mutants in the pGL2-IL-6-Luc construct were generated either by Exonuclease III digestion using the Deletion Kit for kilo sequencing (Takara) in accordance with the instructions of the manufacturer (deletion –1,041) or by restriction endonuclease digestion with KpnI and NheI (deletion mutant –212) followed by Klenow fragment reaction at 37°C for 15 minutes. Construct –97 was created by PCR reaction using a forward primer: 5′-CCC GGT ACC CCT CAC CCT CCA AC-3′ and the IL-6 reverse primer described above. All constructs were sequenced to verify the absence of replication errors.

Transfection and luciferase reporter assay

Cells cultured at 1 × 10⁵ per well in 24-well plates for 24 hours in serum-containing medium were cotransfected with a pGL2 or a pGL2-IL-6-Luc plasmid (0.5 µg) and a pRV plasmid (1 ng) expressing Renilla luciferase (as transfection efficiency internal control) in the presence of Lipofectamine LTX (Invitrogen). Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) in a Lumat LB9501 luminometer (Berthold).

Electrophoretic mobility shift assay

Nuclear extracts of PC3 cells cultured as described above were obtained using a NE-PER Nuclear and Cyttoplasmic Extraction Reagent (Thermo Scientific). Extracts (8 µg) were incubated in the presence of the double stranded biotinylated oligonucleotides described in Supplementary Table S1. When indicated, cold (nonbiotinylated) oligonucleotides (10-fold excess) were added to the nuclear extracts and incubated at 4°C for 30 minutes before the addition of double stranded biotinylated oligonucleotides. Reactions were conducted in 10 mM Tris pH 7.5, 50 mM L KCl, 1 mM L dithiothreitol (DTT), 2.5% (v/v) glycerol, 5 mM L MgCl₂, 1 µg poly dl–dc, and 0.05% (v/v) NP-40. Samples were electrophoresed in a 6% native polyacrylamide gel containing 2.5% (v/v) glycerol and transferred to Immun-Blot polyvinylidene difluoride membranes (Bio-Rad). After UV cross-linking the membranes were
blocked with 5% (w/v) skim milk in 50 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl (TBS) and incubated in the presence of horseradish peroxidase-conjugated streptavidin (1 μg/mL: Vector Laboratories). The detection was done by enhanced chemiluminescence (Amersham).

**Western blot**

Western blot was carried out as previously described (21). Primary antibodies used were a goat polyclonal antibody against human Gal-3BP at a 1:1,000 dilution (R&D Systems), a mouse monoclonal antibody (mAb) against human Gal-3 at a 1:1,000 dilution (Santa Cruz Biotechnology, Inc.), rabbit polyclonal antibodies against human ERK1/2, MEK1/2, and their phosphorylated forms, p-ERK1/2 and p-MEK1/2 (Cell Signaling Technology, Inc., dilution 1:1,000) and a rabbit antibody against human actin at a 1:3,000 dilution (Sigma). Mouse mAbs against human Ras (Thermo Scientific), human N-Ras (Calbiochem), human H-Ras (Calbiochem), and human K-Ras (Santa Cruz Biotechnology, Inc.) were used at 1:200, 1:200, 1:100, and 1:100 dilutions, respectively. The detection of immune complexes and their quantification was done using the Odyssey Infrared Imaging Systems (LI-COR Biosciences).

**Gal-3 knockdown experiments**

A control short interfering RNA (siRNA) against mouse lamin A and 3 siRNA sequences against human Gal-3 (FlexiTube siRNA) were purchased from Qiagen. Sequences are described in Supplementary Table S1. Transfection was done using Lipofectamine RNAi Max (Invitrogen).

**Ras pull down assay**

Pull down assay for active Ras was done using an Active Ras Pull Down and Detection Kit (Thermo Scientific). BMMSC lysate (80 μg) and GST-Raf1-RBD (20 μg) were mixed and subjected to immunoprecipitation by centrifugation at 6,000 g for 30 seconds in the presence of glutathione beads resin. The precipitate was washed 3 times with lysis/binding/wash buffer (25 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 5 mmol/L MgCl2, 1% (v/v) NP-40 and 5% (v/v) glycerol) and proteins in the precipitate were eluted in the presence of 125 mmol/L Tris-HCl, pH 6.8, 2% (v/v) glycerol, 4% (v/v) SDS, 0.05% (w/v) bromophenol blue, and 0.05% (v/v) and 200 mmol/L DTT (reducing buffer). The eluates were then electrophoresed in a 4% to 20% gradient Tris-glycine polyacrylamide gel (Biorad) and examined for the presence of total Ras, K-Ras, H-Ras, and N-Ras by Western blot analysis.

**Immunofluorescence**

For immunofluorescence studies on cultured cells, cells were plated at 1 × 10^4 in LabTek II (Nunc) chambers for 24 hours. Cells were washed with PBS and fixed in 4% paraformaldehyde or 3% (w/v) formaldehyde. When indicated, cells were permeabilized in 0.1% (v/v) Triton X-100 for 5 minutes followed by blocking with 15% (v/v) FBS in PBS. Cells were then incubated in the presence of a goat polyclonal antibody against human Gal-3BP (1:100 dilution) and an anti-human Gal-3 mouse mAb (1:100 dilution) in PBS for 1 hour at 37°C, washed twice with 0.1% (v/v) Triton X-100 in PBS, and incubated in the presence of a Cy3–conjugated anti-mouse IgG antibody (1:300 dilution) and a fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG for 45 minutes at room temperature. The slides were washed 6 times in 0.1% (v/v) Triton X-100 in PBS and the wells were removed. The slides were mounted in Vectashield mounting medium (Vector Laboratories). For immunofluorescence studies on formalin-fixed paraffin-embedded (FFPE) specimens, 7- to 10-μm tissue sections were deparaffinized by xylene, hydrated through alcohol gradient, and immersed in PBS. The slides were microwaved for 5 minutes 3 times in VECTEX antigen unmasking solution. After blocking with 3% bovine serum albumin (BSA) and 0.1% Tween-20 in PBS for 1 hour, slides were incubated in 5% donkey serum in PBS with the primary antibody (1:100 dilution for goat anti-human IL-6 antibody or 1:200 dilution for rabbit anti-human Gal-3BP antibody) overnight at 4°C. After washing by 0.1% Tween-20 in PBS 3 times, slides were incubated with the secondary antibody (FITC-conjugated anti-goat or Cy3–conjugated anti-rabbit IgG antibody dilution 1:150) in the presence of 3% BSA and 0.1% Tween-20 in PBS for 1 hour at room temperature. Slides were washed 3 times and incubated in 300 nmol/L 4', 6-diamidino-2-phenylindole (Invitrogen) in PBS for 1 minute, washed in PBS 3 times, and mounted with Vectashield mounting medium (VECTOR).

**Immunohistochemistry**

FFPE sections of 78 primary neuroblastoma tumors were collected from the file of Children’s Oncology Group ANBL0081 Neuroblastoma Biology Study with the approval of the committee. Appropriate consent was obtained from patients/guardians at the individual contributing institutions. These sections were processed as above described and incubated in the presence of a rabbit polyclonal antibody against human Gal-3BP (ProteinTech Group, Inc.) as primary antibody (dilution 1:300) and biotin-conjugated anti-rabbit IgG and streptavidin horseradish peroxidase as secondary antibody. Immunocomplexes were visualized using 3, 3-diaminobenzidine (DAB; iView DAB detection system, BenchMark, Ventana Medical Systems, Inc.). Slides were counterstained with methyl green. The presence of Gal-3BP in these tumor samples was semiquantified by counting the number of positive cells on a total of 200 cells per sample. A score of 1 was assigned to samples having between 0% and 33% positive cells, 2 to samples having between 34% and 66% positive cells, and 3 to samples with more than 66% positive cells. Slides were blindly scored by 3 investigators (AMS, HS, and YAD). Where there was a discrepancy, a second blind analysis was carried out. After this second analysis, only 7 samples were differently scored by the 3 observers and were assigned the consensus score of 2 observers. The samples were also classified for the following parameters, age at diagnosis, clinical stage, DNA index, MYCN status, degree of neuroblastic differentiation, mitosis karyorrhexis index (MKI), and favorable versus unfavorable histology group according to the International Neuroblastoma Pathology Classification (29, 30) and overall and event-free survival (EFS).
Statistics
Data were analyzed using the Student t test, ANOVA, or univariate or multivariate linear regression methods, or Spearman rank correlation, as appropriate to the experiment or context. Two-sided P values are reported with values less than 0.05 reported as statistically significant. For studies correlating Gal-3BP scoring and patient survival, the primary endpoint for EFS analysis was time from diagnosis until the first occurrence of progressive disease, disease recurrence, or death from any cause. There were 18 total events in this patient cohort. The primary endpoint for survival analysis was time from diagnosis to death from any cause (total 10 deaths). EFS and survival plots were produced using the Kaplan–Meier estimate. The test of the differences in EFS and survival among the 3 expression groups were based on the log-rank trend test.

Results
Gal-3BP is secreted by several neuroblastoma cell lines that upregulate the expression of IL-6 by mesenchymal stem cells
We first examined the production of Gal-3BP in 9 well-characterized human neuroblastoma cell lines derived from patients with advanced disease. The presence of abundant intracellular Gal-3BP was detected by immunocytofluorescence in most cultures examined. In only one cell line (NB-19) the amount detected was very small (Fig. 1A). The cells also secreted Gal-3BP as shown by the amount of Gal-3BP detected by ELISA in the CM which varied from 0 to 60 ng/10^4 cells over 24 hours. A Western blot analysis of the CM confirmed the presence of a 93-kDa protein band identified by an anti-Gal-3BP antibody (Fig. 1B). BMMSCs were then incubated in the presence of the CM of these cell lines and examined after 24 hours for the presence of IL-6. This analysis revealed a direct correlation between the amount of IL-6 produced by BMMSCs and the amount of Gal-3BP present in the CM of neuroblastoma cells (Fig. 1C).

Gal-3BP is one among other regulators of IL-6 expression and increases the transcriptional upregulation of IL-6 in BMMSCs
To confirm the stimulatory function of Gal-3BP in neuroblastoma CM on IL-6 production, we immune-depleted Gal-3BP from the CM of CHLA-255 cells and tested this immune-depleted CM on the production of IL-6 by BMMSCs. This procedure eliminated 90% of the Gal-3BP (Fig. 2A) but only decreased the IL-6 stimulatory effect by 37% (Fig. 2B). These data indicated that Gal-3BP is only in part responsible for the stimulatory effect of neuroblastosoma CM on IL-6 expression by BMMSCs and that other factors contribute.

We then tested the effect of CHLA-255 CM on the expression of IL-6 mRNA in BMMSCs and found a 2-fold increase in IL-6 mRNA by quantitative reverse transcriptase PCR (qRT-PCR) at 4 hours after exposure to the CM. Consistent with Gal-3BP being a stimulator, we also observed a 2.5-fold increase in IL-6 mRNA upon exposure to rGal-3BP (Fig. 2C). To confirm that Gal-3BP transcriptionally upregulates IL-6 expression, BMMSCs and PC3 human prostate cancer cells (as positive control) were transfected with the pGL2-IL6-Luc construct and tested for luciferase activity upon treatment with rGal-3BP or CM from CHLA-255 cells. This analysis indicated a 2- to 4.7-fold increase in promoter activity by rGal-3BP or CHLA-255 CM and is consistent with a transcriptional mechanism of upregulation (Fig. 2D).
A distal region of the IL-6 promoter containing C/EBP binding domains is involved in Gal-3BP–mediated IL-6 transcription

We then used deletion mutagenesis in the IL-6 promoter to identify region(s) of the promoter responsible for the upregulation of IL-6 by Gal-3BP. For these experiments we used human PC3 prostate cancer cells that had a higher transfection efficiency than BMMSCs. The transfection of IL-6 promoter mutants into these cells identified a distal region of the promoter from position −2,155 to position −1,041 responsible for the stimulatory activity of Gal-3BP. When this region of the promoter was deleted, we observed no changes in baseline activity but a loss of response to treatment with rGal-3BP (Fig. 3A). Sequencing analysis of this region identified 3 C/EBP binding motifs located at positions −1,571 to −1,558, −1,293 to −1,280 and −1,270 to −1,257 (Fig. 3B). By electrophoretic mobility shift assay (EMSA), we identified the presence of a protein–DNA complex when double stranded biotinylated oligonucleotides corresponding to these 3 C/EBP binding domains were incubated with nuclear extracts of rGal-3BP–treated PC3 cells. The specificity of these complexes was confirmed by the addition of a 10-fold excess of nonbiotinylated double stranded oligonucleotides that competed for binding (Fig. 3C). Altogether the data indicated that Gal-3BP transcriptionally upregulates IL-6 by a mechanism that involves 3 C/EBP-binding motifs present in the distal region of the IL-6 promoter.

Gal-3 is necessary for Gal-3BP–mediated upregulation of IL-6

We next asked whether Gal-3, known to interact with Gal-3BP (26), was a necessary intermediate in the transcriptional upregulation of IL-6 by Gal-3BP. We had previously shown the presence of Gal-3 in BMMSCs by Western blot analysis (21).
Therefore used immunofluorescence to confirm its presence in the cytoplasm of cultured BMMSCs (Fig. 4A, a to c). When rGal-3BP was added to the culture medium, we detected Gal-3BP at the cell surface and also inside the cells where it was associated with Gal-3 (Fig. 4A, d to f) indicating the formation of intracellular Gal-3BP–Gal-3 complexes. We then tested 3 siRNA oligonucleotides (and an anti-mouse lamin A siRNA as control) for their ability to downregulate Gal-3 expression in BMMSCs and observed inhibition of Gal-3 expression with the 3 siRNA tested (Fig. 4B). Knockdown of Gal-3 expression in BMMSCs before exposure to CHLA-255 CM or to rGal-3BP resulted in a 75% to 100% inhibition of IL-6 production (Fig. 4C) indicating that Gal-3 is a necessary intermediate for the upregulation of IL-6 by Gal-3BP. In further support of a necessary role of Gal-3 for Gal-3BP–mediated IL-6 stimulation, we showed that downregulation of Gal-3 by siRNA in PC3 cells transfected with rGL-3BP (5 μg/mL) using biotinylated oligonucleotides in the presence of 10−fold excess of nonbiotinylated oligonucleotides when indicated.

**Gal-3BP activates the Ras/MEK/ERK pathway**

We then explored whether IL-6 upregulation in BMMSCs by Gal-3BP was dependent on Ras activation. Active Ras pull-down experiments showed that Ras became rapidly activated upon exposure of BMMSCs to rGal-3BP as we observed a 3.5-fold increase in the ratio Ras-GTP/total Ras after treatment with Gal-3BP (Fig. 5A). This increase involved N-Ras as K-Ras and H-Ras were not detected in BMMSCs. The increase in Ras activation was followed by an increase in mitogen-activated protein/extracellular signal–regulated kinase (MEK) and extracellular signal–regulated kinase (ERK) activation that peaked 30 minutes after treatment. Both ERK1 and ERK2 were equally activated after 30 minutes (Fig. 5B). To confirm the involvement of Ras activation in Gal-3BP–mediated IL-6 regulation, we pretreated BMMSCs with the Ras inhibitor FTS (12.5 μmol/L, a concentration that maintained 85% cell viability) before exposure to the CM of CHLA-255 cells or rGal-3BP and examined their production of IL-6 (Fig. 6A). The data revealed that pretreatment with FTS inhibited IL-6 production by 92% to 100%. Providing further evidence to support the involvement of Ras, we then showed that treatment of BMMSCs transfected with pGL2-IL-6-Luc with FTS suppressed the upregulation of the IL-6 promoter activity by rGal-3BP or CHLA-255 CM (Fig. 6B). A similar suppression of IL-6 promoter activity was observed when BMMSCs were pretreated with the MEK inhibitor PD98058 (50 μmol/L; Fig. 6C).
Gal-3BP is present in primary neuroblastoma tumors

To obtain evidence that Gal-3BP has a similar function in human neuroblastoma tumors in vivo, we semiquantitatively examined its expression by immunohistochemistry on FFPE sections from 78 samples of primary neuroblastoma tumors as described in Materials and Methods. This analysis (Fig. 7A) revealed a strong (score 3) expression in 25% of the samples, a score of 2 in 40% of the samples, and a score of 1 in 31% of the samples. Only 3 samples (4%) were found entirely negative. Gal-3BP was present not only inside the cells but also in the pericellular space and extracellular matrix surrounding neuroblastoma cells and often at the center of clusters of tumor cells forming pseudorosettes (Fig. 7A, panel c).

sections were also examined for the presence of IL-6. This analysis revealed the presence of IL-6–positive cells in close proximity of Gal-3BP expressing cells (Fig. 7B). We carried out a univariate analysis of the score of Gal-3BP expression in these tumors and specific tumor characteristics (Fig. 7C). We only detected a significantly lower score in patients with stage 4S (children <1 year with metastatic disease to skin, liver, or bone marrow and good prognosis) when compared with patients with stages 1 to 3 (nonmetastatic) or stage 4 (metastatic) disease. Kaplan–Meier estimate for survival (Fig. 7D) or EFS (Supplementary Fig. S1) did not show a difference in survival when patients were stratified according to the Gal-3BP expression score.

Figure 4. Gal-3 is necessary for Gal-3BP–mediated IL-6 expression. A, immunocytofluorescence analysis of Gal-3 (red) and Gal-3BP (green) in BMMSCs treated with rGal-3BP (5 μg/mL), fixed without permeabilization as described in Materials and Methods. a to c, untreated cells. d to f, cells treated with rGal-3BP. B, Western blot analysis of cell-associated Gal-3 in BMMSCs examined 72 hours after transfection with indicated siRNA as described in Materials and Methods. C, amount of IL-6 produced over 24 hours by BMMSCs transfected with siRNA as shown in B and incubated in the absence or presence of 5× CM from CHLA-255 cells or rGal-3BP (2.5 μg/mL). The data represent the mean IL-6 concentration (±SD) in the supernatant of triplicate samples and are representative of 3 independent experiments showing similar results. D, IL-6 luciferase promoter activity in PC3 cells transfected with a Gal-3 siRNA and treated with rGal-3BP (5 μg/mL). The data represent the mean fold increase in the FLuc/RLuc from triplicate samples from 3 separate experiments.

Control
siRNA
siRNA-1
siRNA-2
siRNA-3

Actin

rGal-3BP

Gal-3 siRNA-3

Gal-3 siRNA-2

Gal-3 siRNA-1

Gal-3

No treatment
5xCM
rGal-3BP

IL-6 (pg/mL)

Fold increase (FLuc/RLuc)

P < 0.00003

P < 0.0005

P < 0.001
In summary, our data showed that Gal-3BP is produced by neuroblastoma cells and present in the tumor microenvironment of neuroblastoma tumors, being one among the factors responsible for the stimulation of the production of IL-6 by stromal cells. They provide evidence for the first time for a Gal-3BP/Gal-3/Ras/MEK/ERK1/2 signaling pathway that is responsible for the transcriptional regulation of IL-6 in these cells.

Discussion

Here we describe a novel mechanism by which malignant cells upregulate the production of IL-6 by stromal cells. In this study, we used BMSCs as a source of stromal cells because we had previously shown that in the bone marrow microenvironment BMSCs produce IL-6 when in the presence of neuroblastoma cells (19). However, we had also shown that peripheral blood monocytes/macrophages behave similarly and respond to tumor-derived Gal-3BP by increasing their production of IL-6 (22). Although a similar IL-6 stimulatory function of Gal-3BP on peripheral blood monocytes was previously reported in patients with asthma (25), this is the first report of such a function in cancer. Thus the Gal-3BP/Gal-3/IL-6 interactive axis between tumor cells and stromal cells described here likely plays a role not only in the bone marrow microenvironment, where BMSCs are an important source of IL-6, but also in primary tumors where tumor-associated macrophages are the source of this cytokine among others. This is consistent with the observation of an abundant presence of Gal-3BP in primary human tumor specimens in proximity of IL-6 expressing cells.

Although our data provide evidence that Gal-3BP is involved in the regulation of IL-6 by stromal cells, it is clearly not the only factor secreted by neuroblastoma cells that upregulates IL-6. Immunodepletion experiments (Fig. 2) show that removal of most of Gal-3BP in the CM of...
neuroblastoma cells only partially suppresses the stimulatory effect on IL-6 production. Consistently, we had previously shown that the knockdown of Gal-3BP in neuroblastoma cells, only partially (40%–50%) suppressed the stimulatory effect on IL-6 production by BMSCs (21). The data thus indicate the presence of Gal-3BP–independent mechanisms of interaction between neuroblastoma cells and BMSCs responsible for the upregulation of IL-6 in BMSCs. One such mechanism previously reported by us is the COX-2–mediated production of prostaglandin E-2 by neuroblastoma cells that provides an amplification loop (20). The presence of other Gal-3BP–independent mechanisms is the subject of further investigation in our laboratory.

Gal-3BP is expressed in many solid tumors. It is present in tumors of the ovary, in particular serous, and mucinous tumors and clear-cell carcinoma compared with benign tumors (31). It is expressed in gastric cancer (32) and in human colon cancer (33). Its function is not entirely defined although it has been shown to promote tumor cell adhesion to ECM proteins (33), the expression of matrix metalloproteinase in prostate cancer cells (34), the formation of metastasis in lung cancer (35), and to contribute to an inflammatory reaction to tumor cells (24). We provide here evidence that it is expressed in neuroblastoma (cell lines and primary tumors) and that it contributes to the production of IL-6.

The mechanism by which Gal-3BP upregulates IL-6 has not previously been explored. Here we provide evidence that it involves a transcriptional effect. The transcriptional regulation of IL-6 expression has been well characterized and several key regulatory elements present in the proximal region of the promoter (position −271 to −54) have been identified (40–41). Little attention has, however, been placed on the more distal region of the promoter. Here we have identified a region of the promoter extending from −1,571 to −1,257 not previously associated with the transcriptional expression of IL-6. This region contains 3 C/EBP binding motifs.

We also provide evidence that the transcriptional upregulation of IL-6 by Gal-3BP is mediated by one of its binding partners, Gal-3, and that upregulation of IL-6 involves activation of the Ras/MEK/ERK pathway, a pathway that is activated by Gal-3 in breast cancer cells (36). In tumor cells, overexpression of Gal-3 coincided with an increase in K-Ras activation with a loss in N-Ras-GTP. Our data in BMSCs indicate an activation of N-Ras-GTP in the absence of K-Ras and H-Ras, which were not expressed in BMSCs. The reason for this discrepancy is not entirely clear but may be due to differences between normal and malignant cells. We also used rGal-3BP as an activator of Gal-3 rather than Gal-3 overexpression, as was the case in breast cancer cells.

Our analysis of primary tumor neuroblastoma specimens shows the expression in the majority of the samples analyzed, which is consistent with similar reports in lymphoma and ovarian cancer (31, 37). In our analysis in a cohort of 78 samples of primary neuroblastoma, however, we did not find any correlation between the amount of Gal-3BP in tissue and other biologic markers of outcome and survival. Only samples from patients with stage 4S disease, who have a favorable outcome, showed a significantly lower Gal-3BP score (P < 0.001). Considering the complexity of the interactions between neuroblastoma cells and stromal cells and the presence of other pathways upregulating stromal IL-6, the absence of correlation between Gal-3BP expression and outcome is not entirely unanticipated.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank J. Rosenberg for her excellent assistance in preparing the manuscript and Dr. L. Blavier Sarte for her technical assistance in the laboratory; Dr. A. Sakurai, Dr. R. Suganuma, and S.A. Phung for assistance with the immunohistochemistry study of Gal-3BP; K. Engell and E. Fernandez for acquiring immunofluorescence images by confocal microscopy; and the members of the Children’s Oncology Group ANBL00B1 Committee for providing the tumor specimens.

Grant Support

This work was supported by NIH grant P01 CA084103 to Y.A. DeClerck and H. Shimada, and by the Bogart Foundation Pediatric Cancer Research Program and the T.J. Martell Foundation for Leukemia, Cancer, and AIDS Research to Y.A. DeClerck.

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Received July 11, 2011; revised January 10, 2012; accepted February 15, 2012; published OnlineFirst March 2, 2012.

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Figure 7. Gal-3BP is present in neuroblastoma tumors. A, digital photomicrographs of representative neuroblastoma tumor sections stained for Gal-3BP. Panel a, absence of Gal-3BP; panel b, score 1; panel c, score 2; panel d, score 3. Bar, 50 μm. B, sections were stained for Gal-3BP (red) and IL-6 (green). Bar, 10 μm; inset, 30 μm. C, bar diagram showing the mean score (±SD) in 78 neuroblastoma samples according to categories: age at diagnosis (<18 months vs. ≥18 months); DNA index (1 or >1); Diff, grade of neuroblastic differentiation: D, differentiated; P, poorly differentiated; and U, undifferentiated; Histo: F, favorable histology and U, unfavorable histology; MKI, mitosis-karyorrhexis index; H, high (>200/5,000 cells), I, intermediate (100-200/5,000 cells), and L, low (<100/5,000 cells); MYCN: A, amplified and NA, nonamplified; and stage: 1 to 3, nonmetastatic; 4, metastatic; 4S, metastatic with good prognosis. D, Kaplan–Meier graph of survival probability by time in years from diagnosis within each of the 3 Gal-3BP expression groups.
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