Distinct Biological Roles for the Notch Ligands Jagged-1 and Jagged-2\textsuperscript{*5}

Received for publication, April 2, 2009 Published, JBC Papers in Press, April 27, 2009, DOI 10.1074/jbc.M109.003111

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Notch signaling is activated in a subset of non-small cell lung cancer cells because of overexpression of Notch3, but the role of Notch ligands has not been fully defined. On the basis of gene expression profiling of a panel of non-small cell lung cancer cell lines, we found that the predominant Notch ligands were \textit{JAG1}, \textit{JAG2}, \textit{DLL1}, and \textit{DLL3}. Given that Notch ligands reportedly have overlapping receptor binding specificities, we postulated that they have redundant biological roles. Arguing against this hypothesis, we found that \textit{JAG1} and \textit{JAG2} were differentially regulated; \textit{JAG1} expression was dependent upon epidermal growth factor receptor (EGFR) activation in HCC827 cells, which require EGFR for survival, whereas \textit{JAG2} expression was EGFR-independent in these cells. Furthermore, HCC827 cells underwent apoptosis following depletion of \textit{JAG1} but not \textit{JAG2}, whereas co-culture experiments revealed that depletion of \textit{JAG2}, but not \textit{JAG1}, enhanced the ability of HCC827 cells to chemotact THP-1 human monocytes. \textit{JAG2}-depleted HCC827 cells expressed high levels of inflammation-related genes, including interleukin 1 (IL1) and a broad range of IL1-regulated cytokines, which was attenuated by inhibition of IL1 receptor (IL1R). Our findings suggest that \textit{JAG1} and \textit{JAG2} have distinct biological roles including a previously undiscovered role for \textit{JAG2} in regulating the expression of cytokines that can promote antitumor immunity.

In mammals, there are four Notch homologues (Notch1–4) and five ligands (three Delta-like and two Jagged/Serrate) (1). Receptor and ligand are typically presented on neighboring cells; hence, ligand binding is a means of cell-cell communication. Notch participates in key aspects of organogenesis (lateral inhibition, lineage specification, and boundary formation) in the developing embryo, maintains stem cell viability and renewal in the adult, and has been implicated in multiple human cancers (1). In the developing lung, Notch receptors and ligands are expressed in a cell type-specific manner, increase in abundance from embryonic day 11.5 into adulthood, and contribute to cell lineage specification (2).

A growing body of evidence supports a role for Notch in tumorigenesis. Notch1 was first identified in humans as the Tan1 oncogene, fusing the control region of the T-cell receptor B gene to a truncated, active form of Notch1 in T-cell acute lymphoblastic leukemia (3). Subsequently, oncogenic forms of Notch2 were discovered in feline thymic lymphomas, and Notch4 was found to be an insertion site for mouse mammary tumor virus (4, 5). In NSCLC\textsuperscript{3} cell lines, the Notch3 gene on chromosome 19 is involved in balanced translocations with multiple other chromosomes, leading to Notch3 overexpression, and pharmacologic or genetic inhibition of Notch3 suppresses the proliferation of these cells (6–8). Although other examples of activation of Notch receptors by genomic rearrangement appear to be rare in human tumors, overexpression is common in a variety of solid tumors including pancreas, cervix, breast, and prostate (9–12).

Unlike the solid body of evidence supporting a role for Notch, less is known about the importance of Notch ligands in cancer. Notch ligands function as Notch signaling agonists through intercellular interactions (trans-interactions) and as Notch signaling antagonists through intracellular interactions (cis-interactions) (13). In mammalian systems, Notch ligands bind to Notch family members non-selectively; for example, Jagged-1 and Jagged-2 can both bind to Notch1, Notch2, and probably Notch3 (14, 15), suggesting a high degree of redundancy in mammalian cells to maintain Notch activity. However, mice that are null for the genes encoding Jagged-1 (\textit{Jag1}), Jagged-2 (\textit{Jag2}), or Delta-like ligand4 (\textit{Dll4}) exhibit distinct embryonic defects (13), suggesting that these ligands exert unique actions that cannot be explained entirely by their receptor binding activities. Indeed, these ligands have intrinsic ligand signaling activity independent of Notch and undergo multiple post-translational modifications, proteolytic processing, endocytosis, and membrane trafficking (13), all of which may contribute to the multifunctionality of Notch ligands.

In this study, we examined Notch ligand expression in a panel of NSCLC cell lines and found that they co-expressed

\textsuperscript{8}This work was supported, in whole or in part, by National Institutes of Health Grants P50 CA70907 and CA117965 (to J. M. K.).

\textsuperscript{9}The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1 and S2.

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\textsuperscript{3}The abbreviations used are: NSCLC, non-small cell lung cancer; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; IL1, interleukin-1; IL1R, IL1 receptor; IL1RA, interleukin-1 receptor antagonist; siRNA, short interfering RNA; TNF, tumor necrosis factor; PARP, poly(ADP-ribose) polymerase; DMSO, dimethyl sulfoxide.
multiple ligands, including JAG1, JAG2, DLL1, and DLL3. Further analysis revealed that the expression of JAG1 and JAG2 was regulated independently, and these ligands had distinct biological roles, including a novel finding that JAG2 regulated the expression of proinflammatory cytokines. Thus, JAG1 and JAG2 have non-redundant functions in NSCLC cells.

EXPERIMENTAL PROCEDURES

Reagent—Gefitinib was a gift (Astra Zeneca Pharmaceuticals, Wilmington, DE). We purchased a recombinant human IL1RA (Santa Cruz Biotechnology, Santa Cruz, CA), Hoechst 33342 (Invitrogen), polyclonal antibodies derived in goat against Jag1 (Santa Cruz Biotechnology) and in rabbits against Jag2 (Santa Cruz Biotechnology), a horseradish peroxidase-linked anti-mouse (Cell Signaling Technology, Danvers, MA), anti-goat secondary antibodies (Santa Cruz Biotechnology), and an antibody against β-actin (Sigma-Aldrich).

Cell Line—The NSCLC cell lines used in this study were from the Hamon Center Repository or purchased from the American Type Culture Collection (Manassas, VA) and were grown in 5% CO2 at 37 °C in RPMI 1640 medium with high glucose (4.5 g/liter; Invitrogen), supplemented with 10% fetal bovine serum (HyClone, Logan, UT). THP-1 cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum, 10 mM HEPES, 1.5 g/liter sodium bicarbonate, 1 mM sodium pyruvate, and 2 mM glutamine.

Transient Transfection of Cells with siRNA—The siRNA control (siCTL nontargeting pool) and the siRNA pooled oligonucleotides against human JAG1 and JAG2 were purchased (Dharmacon, Denver, CO). Cells were transfected at 50% confluency using 10 nM of each construct over a 16-h period using DharmaFECT 1 (Dharmacon).

 Stable Transfection of Cells with JAG2 Short Hairpin RNA—The short hairpin RNA plasmid constructs against human JAG2 and corresponding empty vector were purchased (Ori-gene, Rockville, MD). Cells were transfected at 50% confluency using 3 μg of each construct over a 4-h period with TransPass D1 transfection reagent (New England Biolabs, Ipswich, MA) according to the manufacturer’s protocol. After 48 h, medium was replaced with medium containing 1 μg/ml puromycin. After 14 days, the cells were trypsinized, seeded on 10-cm plates at low density, and single colonies were selected and expanded.

Expression Profiling Using Illumina Array—RNAs were labeled and hybridized to the Illumina expression array according to the manufacturer’s instructions. After 14 days, the cells were trypsinized, seeded on 10-cm plates at low density, and single colonies were selected and expanded.

Western Blotting—Cells were lysed with M-PER mammalian protein extraction reagent (Pierce). Lysates were cleared by centrifugation, and protein concentrations were quantified with 1× Quick Start Bradford dye reagent (Bio-Rad) so that equal amounts of protein (40 μg) could be resolved on 10% SDS-polyacrylamide gels. After transfer to membranes, samples were processed and visualized with ECL Western blotting reagents (Amersham Biosciences). All of the Western blotting data were representative of at least three independent experiments.

Hoechst 33342 Staining—Cells with nuclear fragmentation were quantified by staining with 10 μg/ml Hoechst 33342 for 15 min at room temperature and counting under fluorescence microscopy. Mean values for each condition were calculated based on results from 12 replicates (three independent experiments with quadruplicate samples in each experiment).

Cell Migration Assay—In vitro migration assays were performed using a 24-well Transwell unit (BD Biosciences) with polycarbonate filters. HCC827 cells transfected with siRNA for Jag1, Jag2, or control were seeded on the lower compartments of the unit in the complete medium for 16 h. Just before the migration assay, the cells on the lower compartments were washed with phosphate-buffered saline, and cul-

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ture medium was changed to serum-free medium with treatment. THP-1 cells were placed in the upper compartments in serum-free medium and incubated for 24 h. The medium was aspirated after the incubation, and the cells were removed from the upper compartment by scrubbing with a cotton swab. Cells on the underside of the membrane (migrated cells) were stained with SureStain Wright (Fisher) and then washed with water. Cells were counted by microscopy at ×20 magnification. Mean values were calculated from cell counts in five random fields for each filter. Mean values for each condition were calculated based on results from eight replicates (two independent experiments with quadruplicate samples in each experiment).

Statistical Methodology—For analysis of treatment effects and differences between genetically modified cells, the Student’s *t* test was used, and *p* values <0.05 were considered statistically significant.

RESULTS

Expression Profiling of Notch Ligands and Family Members in NSCLC Cells—We profiled gene expression in a panel of 70 NSCLC cell lines by using Illumina arrays. Of the five Notch ligands, JAG1, JAG2, DLL1, and DLL3 were expressed at levels that varied by up to 15-fold between cell lines, whereas DLL4 expression was uniformly low in all cell lines (Fig. 1). The majority of the cell lines expressed at least two ligands, and some (HCC95, HCC4018, H1755, H1666, and HCC1833) expressed four ligands. Quantitative PCR analysis of JAG1, JAG2, and Notch1–4 was performed on a subset of the cell lines, which confirmed the relative expression levels of JAG1 and JAG2 observed in the expression array studies performed on those cells and revealed that most cell lines expressed multiple Notch family members (Fig. 2).

JAG1 and JAG2 Are Regulated through Distinct Mechanisms—On the basis of reports that Notch ligands exhibit overlapping Notch binding activities (14, 15), we postulated that they have redundant biological functions in NSCLC cells and used pharmacologic and genetic approaches to test this hypothesis. The cell lines in Fig. 2 differ with respect to somatic mutations that constitutively activate EGFR (HCC827, HCC2279, H4006, H3255, and H1975) or N-Ras (H1299) or have neither mutation (H1819) (20). Comparison of their expression levels determined by quantitative PCR analyses revealed that JAG1, NOTCH1, and NOTCH2 were more highly expressed in EGFR mutant cells (*p* values = 0.025, 0.013, and 0.03, respectively) (Fig. 2A), whereas NOTCH3, NOTCH4, and JAG2 were not differentially expressed (Fig. 2B). Because increased JAG1 expression correlated with the presence of mutant EGFR, we examined whether JAG1 was regulated by EGFR. JAG1 RNA and protein levels decreased sharply in HCC827 cells and H4006 cells treated with the EGFR-specific tyrosine kinase inhibitor gefitinib (Fig. 3, A and B). Gefitinib treatment had no effect on JAG1 levels in H1975 cells, which have a resistance mutation (T790M) (18) that inhibits EGFR binding to gefitinib (Fig. 3, A and B), indicating that the decrease in JAG1 levels by gefitinib required EGFR inhibition. In contrast, gefitinib had no effect on JAG2 levels in these cells (Fig.
To examine the role of EGFR using a different approach, EGFR-wild-type H1299 cells, which expressed low levels of JAG1 and JAG2 (Fig. 2), were treated with EGF or transfected with wild-type EGFR or empty vector (EV). Relative EGFR expression in the transfectants was quantified by quantitative PCR and Western analysis (inset). The quantitative PCR values represent the means of replicate samples and are normalized to the L32 ribosomal RNA gene. *, empty vector versus EFGR (DMSO treatment), p < 0.05; +, DMSO versus gefitinib (EGFR transfectants), p < 0.05.

FIGURE 3. Distinct regulation of JAG1 and JAG2 by EGFR. A, quantitative PCR analysis of cells treated with gefitinib (1 μM) or vehicle (DMSO). The values represent the means of replicate samples and are normalized to the L32 ribosomal RNA gene. *, p < 0.05. B, Jagged-1 Western blot in HCC827 and H1975 cells treated with gefitinib. C, quantitative PCR analysis of gene expression in H1299 cells treated with EGF (100 ng/ml) or transfected with wild-type EGFR or empty vector (EV). Relative EGFR expression in the transfectants was quantified by quantitative PCR and Western analysis (inset). The quantitative PCR values represent the means of replicate samples and are normalized to the L32 ribosomal RNA gene. *, empty vector versus EFGR (DMSO treatment), p < 0.05; +, DMSO versus gefitinib (EGFR transfectants), p < 0.05.

FIGURE 4. JAG1 and JAG2 are mutually suppressive, and JAG1 maintains the survival of HCC827 cells. A, Western analysis of HCC827 cells transfected with JAG1, JAG2, or scrambled control (SCR) siRNA. B, cell numbers of scrambled (SCR) or JAG1 siRNA transfectants over time. *, p < 0.05, **, p < 0.01. C, percentages of apoptotic cells (bar graph; *, p < 0.05) as determined by Hoechst 33342 staining (images) to identify fragmented nuclei (arrows). Results are the means of replicate samples. The Western blot shows cleaved PARP (arrow) in JAG1 siRNA transfectant. Transfecting siRNAs to selectively deplete JAG1 or JAG2 from cultured cells. Transfection of HCC827 NSCLC cells with JAG1- or JAG2-specific siRNAs achieved greater than 90% reductions in Jagged-1 and Jagged-2, respectively (Fig. 4A). Of note, Jagged-2 protein levels increased in JAG1 siRNA-transfected cells, and Jagged-1 protein levels increased in JAG2 siRNA-transfected cells (Fig. 4A), indicating that JAG1 and JAG2 levels were regulated reciprocally.

JAG1 Maintains the Survival of HCC827 Cells—Relative to the control transfectants, the JAG1 siRNA-transfected HCC827 cells exhibited a reduction in cell density over time (Fig. 4B) and an increase in nuclear fragmentation and cleavage of poly-(ADP-ribose) polymerase (PARP) (Fig. 4C), which is consistent with apoptotic cell death. In contrast, JAG2 siRNA-transfected cells exhibited minimal evidence of apoptotic cell death based on the absence of PARP cleavage (Fig. 4C) and nuclear fragmentation (data not shown). Collectively, these findings suggest that HCC827 cell survival was dependent upon JAG1 but not JAG2.

JAG1 and JAG2 Levels Are Regulated Inversely—We next sought to examine the biological roles of JAG1 and JAG2 by transfecting siRNAs to selectively deplete JAG1 or JAG2 from cultured cells. Transfection of HCC827 NSCLC cells with JAG1- or JAG2-specific siRNAs achieved greater than 90% reductions in Jagged-1 and Jagged-2, respectively (Fig. 4A). Of note, Jagged-2 protein levels increased in JAG1 siRNA-transfected cells, and Jagged-1 protein levels increased in JAG2 siRNA-transfected cells (Fig. 4A), indicating that JAG1 and JAG2 levels were regulated reciprocally.

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JAG2 Inhibits the Ability of HCC827 Cells to Recruit Monocytes—In addition to their ability to proliferate in an uncontrolled manner, NSCLC cells recruit inflammatory cells, fibroblasts, and endothelial cells, which constitute the tumor stroma (21). These cells are required for tumor growth and
metastasis (22). To determine whether JAG1 and JAG2 are involved in this process, we examined the chemotraction of THP-1, a human monocytic cell line (23), by HCC827 cells following transfection with JAG1 or JAG2 siRNAs. HCC827 cells and THP-1 cells were seeded into the lower and upper chambers, respectively, of Transwell plates. These chambers were separated by a porous membrane, allowing bidirectional diffusion of secreted, soluble mediators. Following 24 h of incubation, the numbers of THP-1 cells that had migrated across the porous membrane were counted. Relative to control siRNA, JAG2 siRNA transfection enhanced the ability of NSCLC cells to recruit THP-1 cells, whereas JAG1 siRNA had no effect (Fig. 5). Thus, JAG2 inhibited monocytic recruitment by HCC827 cells.

**JAG2 Inhibits the Expression of Inflammation-related Genes in an IL1R-dependent Manner**—To explore the mechanism by which JAG2 inhibits monocyte recruitment, JAG1 and JAG2 siRNA-transfected HCC827 cells were transcriptionally profiled using a quantitative PCR expression array (RT2 Profiler array, SABiosciences) containing 84 inflammation-related genes (gene lists are in supplemental Table 2). Of these, 33 genes were measurable in all three samples (Fig. 6A); the other genes either were undetectable or did not achieve a technically satisfactory amplification in all samples. JAG1 and JAG2 siRNA transfectants exhibited striking differences; relative to the control transfectants, JAG2 siRNA enhanced the expression of the majority of the measurable genes, whereas JAG1 siRNA reduced the expression of a smaller subset (Fig. 6A). Those increased by JAG2 siRNA include, among others, a broad spectrum of CXC chemokines (CXCL1, CXCL3, CXCL5, CXCL6, and CXCL9), CC chemokines (CCL2, CCL5, CCL13, CCL20, and CCL24), interleukins (IL1α, IL1β, IL1F8, IL1F9, and IL17c) and their receptors (IL1RN, IL10Rα, IL13Rα, CCR1, CCR8, and CXCR1). To validate these findings, quantitative PCR was performed on RNA samples from an independent experiment using different primers from those on the array, which confirmed that JAG2 siRNA enhanced the expression of all four genes tested (CCL20, IL1α, IL1β, and CCL5), whereas JAG1 siRNA had no detectable effect on any of them (Fig. 6B). Thus, JAG2 was unique in its ability to regulate the expression of inflammation-related genes.
Given the widespread nature of the gene expression changes induced by JAG2 siRNA, we postulated that JAG2 regulates one or more genes that are central nodes in the inflammatory process. To test this, we analyzed their positions within known or predicted global protein interaction networks (interactomes) by using the HiMAP software program. Interactomes identified by this approach are organized into a series of modular structures characterized by centrally located nodes (called hubs) that have multiple connections with other proteins (18). Although this approach is purely exploratory and carries no statistical weight, findings in yeast show that centrality within a protein interactome predicts the biological importance of a protein (24).

Of the 33 measurable inflammation-related genes from the RT2 Profiler PCR array, 28 mapped within a single interactome based on HiMAP analysis (Fig. 7). The hubs within the network with the highest number of links (≥10) included TNFα, IL1α, IL1β, IL1R, CCL2, and CCL5, all of which were prominently up-regulated by JAG2 siRNA (Fig. 6A). Based on the centrality of IL1R and its ligands within the network, we postulated that IL1R mediates JAG2-induced suppression of inflammation-related genes. To test this, we examined the effects of treatment with IL1R antagonist (IL1RA), a naturally occurring, physiological inhibitor of IL1R (25). In co-culture assays, IL1RA treatment abrogated the recruitment of THP-1 cells by JAG2 siRNA-transfected HCC827 cells (Fig. 8A). Furthermore, IL1RA treatment attenuated the effects of JAG2 siRNA on three (CCL20, IL1α, and TNFα) of the 10 inflammation-related genes analyzed (IL1R, CCL3, CCL5, CCL11, IL1α, IL1β, CCL2, CCL5, CCL20, and TNFα) (Fig. 8B). Collectively, these findings suggest that IL1R had a pivotal role in the biological effects of JAG2 in HCC827 cells.

**DISCUSSION**

Notch signaling has been implicated in multiple facets of cancer biology, including, among others, stem cell renewal, cancer cell proliferation, tumor angiogenesis, and metastasis (1). The findings presented in the current study advance our understanding of the role of Notch in cancer by demonstrating that the roles of JAG1 and JAG2 in NSCLC cells are quite distinct, including regulation of diverse proinflammatory cytokines, a biological property of Notch signaling that, to our knowledge, has heretofore not been reported.

Given the reports that Jagged-1 and Jagged-2 bind to Notch family members with similar specificities (14, 15), the finding that they had distinct biological functions in HCC827 cells raises the possibility that they mediate their actions in part through Notch-independent mechanisms. Providing further support for this possibility was evidence that treatment with a γ-secretase inhibitor N-(N-(3,5-difluoro-phenacetyl)-L-alanyl))-S-phenylglycine t-butyler ester, which reportedly inhibits Notch activity (26), only minimally decreased HCC827 cell density (data not shown). All of the Notch ligands, with the exception of Delta-like ligand-3 and Jagged-2, have PDZ-binding motifs at their extreme C termini (27). These motifs are dispensable for ligand activation and Notch inhibition (28–31), but they are required for Notch ligands to affect oncogenic transformation (28). Although it is unclear whether such interactions might have caused the changes in cytokine expression observed here, some PDZ domain proteins such as calcium/calmodulin-dependent serine protein kinase (CASK), Bridge-1, and gluta-
mate receptor interacting protein 1-β (GRIP1-β) act as transcriptional activators (32–34), whereas others, such as afadin/A6 and Acrvinp1, interact with Ras and Smad3 (28, 35–37), respectively, which regulate diverse transcriptional programs.

We observed that JAG1 and JAG2 were regulated inversely in HCC827 cells. Notch signaling interacts with a number of different signaling systems, and many of these affect Notch ligand expression. In particular, fibroblast growth factor, platelet-derived growth factor, transforming growth factor-β, vascular endothelial growth factor, hedgehog, and Wnt have been found to modulate Notch ligand expression (13), any of which might have mediated the mutual suppression between JAG1 and JAG2 in HCC827 cells. Equally intriguing is the possibility of a Notch-mediated feed-forward loop that sensed the loss of Notch ligand through the disengagement of Notch trans-interactions, to which it responded by increasing the expression of other Notch ligands.

Findings presented here suggest that following JAG2 inhibition, HCC827 cells can induce THP-1 monocyte recruitment. Because the cells in this study were co-cultured in separate compartments and communicated through a porous barrier, we conclude that these interactions were mediated by secreted factors and did not require cell-cell contact. In fact, JAG2 depletion dramatically increased the expression of a broad spectrum of inflammatory mediators. Within the interactome of inflammation-related genes that were regulated by JAG2 depletion, IL1 receptor occupied a central position, and treatment with IL1RA abrogated the recruitment of monocytic cells and the expression of certain inflammatory mediators. Consistent with
these findings, IL1 has been reported to regulate the expression of a vast array of proinflammatory cytokines and chemokines (38). Collectively, these findings suggest that IL1 is a critical mediator of inflammation by JAG2, adding to the complexity of cell-cell interactions known to regulate macrophage function (14), and these findings constitute the first report, to our knowledge, that Notch pathways can regulate tumor cell-induced inflammation.

NSCLC remains the primary cause of cancer-related death in Western countries, which is largely because of the fact that once the disease has metastasized, NSCLC cells are resistant to the current treatment options. Even in the setting of tumors with activating EGFR somatic mutations, which confer a unique sensitivity to treatment with EGFR tyrosine kinase inhibitors that leads to rapid and often sustained tumor shrinkage (39–41), the initial tumor response is typically followed by disease recurrence. The problem of disease recurrence has not been obviated by the addition of standard chemotherapeutic agents to EGFR tyrosine kinase inhibitors. Given the importance of Notch signaling in the development of a variety of malignancies, pharmacologic strategies are under development to inhibit the Notch signaling pathway in cancer patients. The findings presented here suggest that such strategies may benefit NSCLC patients by inhibiting cancer cell viability and possibly by enhancing antitumor immunity.

Acknowledgment—We thank Dror Berel for technical assistance.

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