Conservation of the Notch1 signaling pathway in gastrointestinal carcinoid cells

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Submitted 29 March 2005; accepted in final form 14 June 2005

Kunnimalaiyaan, Muthusamy, Kelly Traeger, and Herbert Chen. Conservation of the Notch1 signaling pathway in gastrointestinal carcinoid cells. Am J Physiol Gastrointest Liver Physiol 289: G636–G642, 2005; doi:10.1152/ajpgi.00146.2005.—Gastrointestinal (GI) carcinoid cells secrete multiple neuroendocrine (NE) markers and hormones including 5-hydroxytryptamine and chromogranin A. We were interested in determining whether activation of the Notch1 signal transduction pathway in carcinoid cells could modulate production of NE markers and hormones. Human pancreatic carcinoid cells (BON cells) were stably transduced with an estrogen-inducible Notch1 construct, creating BON-NIER cells. In the present study, we found that Notch1 is not detectable in human GI carcinoid tumor cells. The induction of Notch1 in human BON carcinoid cells led to high levels of functional Notch1, as measured by CBF-1 binding studies, resulting in activation of the Notch1 pathway. Similar to its developmental role in the GI tract, Notch1 pathway activation led to an increase in hairy enhancer of split 1 (HES-1) protein and a concomitant silencing of human Notch1/HES-1/achaete-scute homolog 1. Furthermore, Notch1 activation led to a significant reduction in NE markers. Most interestingly, activation of the Notch1 pathway caused a significant reduction in 5-hydroxytryptamine, an important bioactive hormone in carcinoid syndrome. In addition, persistent activation of the Notch1 pathway in BON cells led to a notable reduction in cellular proliferation. These results demonstrate that the Notch1 pathway, which plays a critical role in the differentiation of enteroendocrine cells, is highly conserved in the gut. Therefore, manipulation of the Notch1 signaling pathway may be useful for expanding the targets for therapeutic and palliative treatment of patients with carcinoid tumors.

human achaete-scute homolog-1; neuroendocrine tumors

GASTROINTESTINAL (GI) carcinoids are rare neuroendocrine (NE) tumors with a reported incidence of 1–8/100,000 people (19, 20, 31). These tumors are characterized by their production of hormonal substances such as 5-hydroxytryptamine (serotonin (5-HT)), chromogranin A, neuron-specific enolase (NSE), and synaptophysin. Besides surgery, there are no other curative options available. The growth of carcinoid and other NE tumors has been shown to be dependent on growth factors and hormones and at least in part controls tumor cell proliferation. Therefore, to study the effects of Notch1 activation in GI carcinoid cells in vitro, we utilized an established pancreatic carcinoid cell line, BON, derived from a metastasis of a human pancreatic carcinoid tumor (12). In the present study, we show that BON cells have no detectable Notch1 protein at baseline. Stable expression of a Notch1 fusion protein resulted in high levels of functional Notch1 as measured by CBF-1 binding activity. Activation of Notch1 led to a downstream induction of HES-1 and a suppression of hASH1 expression. Notch1 activation also caused reductions in the levels of 5-HT, chromogranin A, NSE, and synaptophysin. Furthermore, whereas NE markers and hormone levels were significantly reduced, the induction of Notch1 also inhibited proliferation of BON cells. Taken together, our results suggest that Notch1 activation plays a critical role in the significant reduction of various hormones and at least in part controls tumor cell proliferation.

MATERIALS AND METHODS

Cell culture. BON cells were provided by Drs. Mark Evers and Courtney Townsend, Jr. (University of Texas, Galveston, TX), and maintained in DMEM-F-12K (Life technologies; Grand Island, NY) supplemented with 10% fetal calf serum (Sigma; St. Louis, MO), 100 IU/ml penicillin, and 100 µg/ml streptomycin (Life Technologies) in a humidified atmosphere at 37°C in 5% CO2 (3). BON-NIER cells were maintained in the same conditions as BON cells except that the medium was phenol free and contained 500 µg/ml G-418 (Life Technologies).

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inhibit human achaete-scute homolog 1 (hASH1) transcription. upregulates hairy enhancer of split 1 (HES-1). Increased levels of HES-1 then active form, Notch1 intracellular domain (NICD). Activated Notch1 then to Notch1 transmembrane receptor protein leads to cleavage of Notch1 to its

Fig. 1. Schematic diagram of the Notch1 signaling pathway. Binding of Delta to Notch1 transmembrane receptor protein leads to cleavage of Notch1 to its active form, Notch1 intracellular domain (NICD). Activated Notch1 then upregulates hairy enhancer of split 1 (HES-1). Increased levels of HES-1 then inhibit human achaete-scute homolog 1 (hASH1) transcription.

**BON-NIER cell line.** BON cells were stably transduced with the retroviral vector pLNClX/NIER. This construct is a chimera with the human estrogen receptor hormone binding domain (ER) fused to the active, intracellular portion of Notch1 (amino acids 1,759–2,556-NIC, where NIC is the Notch1 intracellular subunit) (Virote Suriapong and Doug Ball, Johns Hopkins University). Briefly, BON cells were infected with equal volumes of retroviral supernatant from PA317 packaging cells transfected with the retroviral vector pLNClX/NIER. The infection of BON cells was augmented by 2 μg/ml polybrene. After 48 h, the medium was replaced by selection medium containing 0.5 mg/ml G-418. The resulting resistant cells, termed BON-NIER cells, were pooled and maintained in phenol red-free DMEM-F12K (1:1) supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 500 μg/ml G-418. To induce Notch1 activity in BON-NIER cells, 1 μM β-estradiol was added to the media 24 h after the flask were seeded. An equivalent dilution of ethanol, the carrier for the β-estradiol, was used to treat control cells.

**Western blot analysis.** Protein lysates were extracted from cells harvested at the time indicated in results using standard protocols (30). Protein concentration was quantified with a bicinchoninic acid assay kit (Pierce; Rockford, IL). Denatured cellular extracts (50 μg) were separated with 10% SDS-PAGE and transferred onto nitrocellulose membranes (Schleicher and Schuell; Keene, NH) by electroblotting. Membranes (Schleicher and Schuell; Keene, NH) were incubated at 37°C for 2 h. Then, 750 μl of DMSO (Sigma) were added to each well following manufacturer’s instructions. Absorbance was determined using a spectrophotometer at a wavelength of 540 nm. For the viable cell count, cells were counted after the addition of trypan blue using a hemocytometer at different time intervals. Experiments were performed in triplicate at least twice.

**RESULTS**

**Notch1 expression in BON and BON-NIER cells.** BON cells were stably transfected with an estradiol-inducible Notch1 construct, creating BON-NIER cells. BON-NIER cells did not differ from parental BON cells in phenotype. To determine the baseline levels of Notch1 protein in BON and BON-NIER cells, we utilized Western blot analysis. Figure 2A shows that Notch1 protein was not detectable in BON cells treated with control or estradiol (lanes 1 and 2). However, BON-NIER cells treated with control (in the absence of estradiol) expressed high levels of the Notch1:ER fusion protein (Fig. 2A, lane 3). The addition of estradiol to BON-NIER cells resulted in slower mobility (Fig. 2A, lane 4) of Notch1 fusion protein. It has been demonstrated by others using a similar Notch1:ER fusion construct that the slower mobility is due to the phosphorylation of the activated fusion protein (27). The Notch1:ER fusion protein was still present at 4 days of estradiol treatment. This allowed the Notch1:ER fusion protein to now functionally interact with downstream pathway members.
Treatment of BON-NIER cells with estradiol leads to functional Notch1 signaling. As shown in Fig. 2A, estradiol treatment of BON-NIER cells appeared to alter the Notch1:ER fusion protein, as indicated by the slower mobility in the gel. However, it is not known whether or not this fusion protein is equivalent to functional Notch1. The best-characterized downstream effector of Notch 1 in mammals is its binding partner, C-promoter binding factor 1 (CBF)/recombinant signal binding protein-Jκ (RBP-Jκ) (15, 27). Therefore, we utilized a well-described system for determining Notch1 activity using the luciferase reporter plasmid containing either wild-type CBF-1 binding sites (4xCBF1Luc) or mutated CBF-1 binding sites (4xmtCBF1Luc) (15). Binding of active Notch1 to this construct leads to high levels of luciferase expression (15, 27).

Similar to other cell lines that lack functional Notch1, BON cells treated with control or estradiol showed similar basal activity by transient transfection with either plasmid (Fig. 2B). This was also observed in BON-NIER cells treated with control. This suggests a lack of functional Notch1 activity in BON and BON-NIER cells. However, treatment of BON-NIER cells with estradiol led to a marked induction of Notch1 activity as shown by the large increase in the relative fold induction of luciferase after transfection with the 4xCBF1Luc construct. This represented a near 100% increase over basal levels (Fig. 2B).
The Notch1 signaling pathway is conserved in human GI carcinoid cells. hASH1, a basic helix-loop-helix transcription factor, has been previously shown to be highly expressed in NE tumors (4, 7, 9, 11, 29). Activation of Notch1 has been shown in multiple organisms to downregulate the expression of hASH1 and its homolog. The mechanism of Notch1-mediated hASH1 repression through upregulation of HES-1 has been well characterized (2). We (11) have previously shown that the induction of HES-1 in small cell lung cancer cells can reduce hASH1 expression. Thus we were interested in whether activation of Notch1 in BON cells led to changes in HES-1 and hASH1 expression.

BON cells treated with control or estradiol for 4 days had minimal amounts of HES-1 protein (Fig. 2A). Likewise, BON-NIER cells treated with control had minimal levels of HES-1. However, Notch1 induction in BON-NIER with estradiol treatment led to an increase in HES-1 protein (Fig. 2C). In addition, activation of Notch1 also resulted in alterations of hASH1 levels. Whereas hASH1 is normally not found in normal human adult tissue, we and others (29, 32, 33) have previously shown that hASH1 protein is present at high levels in medullary thyroid cancer and small cell lung cancer cells. BON cells express large amounts of hASH1 protein at baseline (Fig. 2C). Treatment with estradiol did not affect the high levels of hASH1 in BON cells. Similarly, BON-NIER cells had high levels of hASH1 protein in control conditions. However, activation of Notch1 by estradiol treatment in BON-NIER cells led to a marked reduction in hASH1 protein levels at 2 days. After 4 days of Notch1 induction, hASH1 protein was barely detectable in BON-NIER cells.

**Effects of Notch1 activation on NE hormone production.** In small cell lung cancer cells, loss of hASH1 by treatment with hASH1 antisense oligonucleotides resulted in a significant decrease in NE markers (4). On the basis of the developmental role of Notch1 in regulating hASH1 expression as well as the data showing that NE phenotype is dependent on hASH1, we would predict that Notch1 overexpression could suppress NE hormone production in BON cells. BON cells normally produce high levels of 5-HT and chromogranin A and moderate levels of synaptophysin (24, 38). As shown in Fig. 2D, BON and BON-NIER cells both express high levels of these molecules. Whereas estradiol treatment did not alter the levels of neuroendocrine markers in BON cells, Notch1 activation through estradiol treatment in BON-NIER cells led to a modest decrease (5–7%) in the levels of chromogranin A, NSE, and synaptophysin, especially after 4 days (Fig. 2D). These reductions in neuroendocrine markers and hormone levels were detected as early as 4 days after Notch1 induction.

**Persistent effect of the Notch1 signaling pathway on GI carcinoid tumor cells.** To determine the effects of long-term induction of the Notch1 signaling pathway, we treated the carcinoid cells with estradiol for up to 12 days. Treatment with estradiol in BON-NIER cells led to the continuous activation of the Notch1 pathway, and that can be seen by the increase in HES-1 production and decrease in hASH1 protein levels (Fig. 3A). Most interestingly, the levels of NE hormones such as chromogranin A, synaptophysin, and NSE were more significantly reduced, by between 17% and 41% (Fig. 3B). This suggests that persistent Notch1 pathway activation is required to reduce NE hormones levels.

**Notch1 activation decreases 5-HT levels in BON cells.** The most important bioactive hormone produced by carcinoid cells is 5-HT. An increase in 5-HT levels accounts for the symptoms associated with the carcinoid syndrome. Therefore, given the alterations in other NE markers, we were interested in the level of 5-HT after Notch1 activation in BON cells. Similar to the other NE markers, there were only modest changes in 5-HT levels after 2 and 4 days of Notch1 activation (Fig. 4). Nevertheless, the levels of 5-HT were reduced by 40% at day 8 and by 43% and 50% at days 10 and 12, respectively, in BON-NIER cells treated with estradiol. However, BON cells treated with either control (ethanol) or estradiol and BON-NIER cells treated with ethanol showed no significant change in 5-HT levels, with the exception of BON cells treated with estradiol at days 2 and 12. As shown in Fig. 4, at 2 and 12 days, estradiol appeared to induce 5-HT production in BON cells. Thus we
can speculate that the reduction in 5-HT levels seen with Notch1 induction after 12 days in estradiol-treated BON-NIER cells could actually be more than 50%, given that estradiol may have induced 5-HT levels above baseline.

**Effect of Notch1 induction on BON cell proliferation.** Activation of the Notch1 signaling pathway has been shown to inhibit growth and induce apoptosis in B cells and other hematopoietic lineages in vitro (21). Moreover, transient expression of activated Notch1 by recombinant adeno- viruses in small cell lung cancer cell lines has been shown to cause a profound growth arrest (32). To determine whether Notch1 activation in BON cells affects cellular proliferation, we utilized MTT assays and viable cell counts. As shown in Fig. 5, there were no differences in proliferation rates of BON cells treated with control or estradiol and BON-NIER cells treated with control over a 12-day period. However, the induction of Notch1 in BON-NIER cells by estradiol treatment led to a reduction of 60% at day 12 in cellular proliferation (Fig. 5). Notably, persistent activation of the Notch1 pathway was required to maintain the inhibition of carcinoid growth. Similar results were obtained by the MTT assay (data not shown).

**DISCUSSION**

Notch1 is a multifunctional transmembrane receptor that plays multiple important roles in cellular differentiation and development (2). Several studies have also illustrated that Notch1 signaling is essential in the developing GI tract (1, 2, 16, 17). Recently, Notch1 has been shown to play a critical role in the enteroendocrine differentiation of cells in the pancreas and GI tract (1, 16, 17). Similar to its role in developing nervous tissues in *Drosophila* and *Caenorhabditis elegans*, Notch1 signaling is thought to mediate a process called lateral inhibition within the gut. During GI tract development, multipotent cells destined to differentiate into GI endocrine cells express the Notch1 ligand Delta. Delta then binds to Notch1 receptors on neighboring undifferentiated cells, leading to cleavage of Notch1 by presenilin-1-dependent γ-secretase activity (13) and release of NIC, which migrates to the nucleus. NIC, the active form of Notch1, then transactivates various target genes, such as HES-1 (2, 11), leading to a cascade that inhibits the expression of proendocrine genes such as hASH1. Thus the overall effect is to limit the number of cells that can differentiate into enteroendocrine cells.

In the present study, we found that Notch1 is not detectable in human GI carcinoid tumor cells. The induction of Notch1 in human BON carcinoid cells led to high levels of functional Notch1, as measured by CBF-1 binding studies, resulting in activation of the Notch1 pathway. However, there was no morphological change observed with Notch1 activation. Similar to its developmental role in the GI tract, Notch1 pathway activation led to an increase in HES-1 protein and a concomitant silencing of hASH1. These results illustrate that the Notch1 pathway, which plays a critical role in the differentiation of enteroendocrine cells in the gut, is highly conserved in GI carcinoid tumor cells.

We and others have previously shown that components of the Notch1 signaling pathway are conserved in other NE tumor cells. hASH1 has been shown to be highly expressed in a variety of NE
tumors, including small cell lung cancer, medullary thyroid cancer, pheochromocytomas, and thymic carcinoids (4, 8, 29). Overexpression of HES-1 in small cell lung cancer cells leads to a significant reduction in hASH1, predominantly through transcriptional inhibition (11). During GI enteroendocrine development, Notch1 is thought to extinguish hASH1 expression through the induction of HES-1 (2, 11). Similar to our results in BON cells, Notch1 induction in small cell lung cancer cells leads to only modest increases in HES-1 with dramatic reductions in hASH1 (32). Recently, however, Sriuranpong and colleagues (33) have shown that Notch1 can also degrade hASH1 protein through a HES-1-independent manner. Thus this mechanism may account for the discrepancy seen between the degree of HES-1 and hASH1 modulation by Notch1 activation in BON cells.

Several studies have shown that Notch1 induction in a variety of neoplasms inhibits tumor development and/or growth. Activation of the Notch1 signaling pathway has been shown to inhibit growth and induce apoptosis in B cells and other hematopoietic lineages in vitro (5). Moreover, transient expression of activated Notch1 by recombinant adenoviruses in small cell lung cancer cell lines has been shown to cause a profound growth arrest (32). Notch1 has been reported to function as a tumor suppressor in the skin (23, 26). Notch1 signaling has also been shown to inhibit growth of human hepatocellular carcinoma cells (25). Similarly, in our studies, Notch1 induction suppressed proliferation of BON cells.

Whereas the growth suppressive effects of Notch1 activation have been well characterized in other tumor types, to our knowledge, this is the first report of Notch1 signaling altering tumor endocrine phenotype. Activation of Notch1 signaling led to reductions in the levels of 5-HT, NSE, synaptophysin, and chromogranin A in BON cells. This is not totally unexpected given the role of Notch1 in enteroendocrine differentiation. However, the mechanism by which Notch1 exerts these hormone suppressive effects is not clear. It is possible that the reduction in NE hormones could be due to the downregulation of hASH1. Borges and colleagues (4) have shown that treatment of small cell lung cancer cells with hASH1 antisense oligonucleotides results in a reduction in hASH1 protein and NE marker levels as measured by immunohistochemistry. It is also possible that Notch1 might activate other known signaling pathways that regulate NE hormone production. Therefore, further studies are needed to identify the mechanism by which Notch1 reduces hormone levels.

We (30) have recently shown that activation of the Raf-1 signaling pathway in BON carcinoid cells results in marked reductions in the number of neuroendocrine secretory granules by electron microscopy and in the production of 5-HT and chromogranin A through a MEK-dependent mechanism. We (29) have also shown that the induction of raf-1 in medullary thyroid cancer cells leads to significant reductions in chromogranin A and calcitonin production. Zhang and coauthors (38) have shown that phorbol ester treatment of BON cells results in a persistent release and cellular depletion of chromogranin A through a PKC-dependent pathway. Furthermore, Kim and colleagues (18) have shown that mechanical stimulation of BON cells led to increased levels of 5-HT secretion. They determined the mechanism of this enhanced hormone release was stimulation of a G protein-coupled receptor leading to mobilization of intracellular calcium (18). Recently, 5-HT secretion by BON cells has been studied by acetylcholine and calcium stimulation (34). Here, we speculate from our findings that Notch1 may be interacting with one or more of these pathways to regulate 5-HT secretion. Nevertheless, a key challenge in the future is to determine how Notch1 activation regulates 5-HT secretion.

Carcinoid and other GI NE tumors frequently metastasize to the liver and are second to colorectal carcinoma as the most common source of isolated hepatic metastases (6, 10, 31). Although surgical resection can be potentially curative, most patients are not candidates for hepatectomy due to the degree of hepatic involvement by NE tumors. Therefore, besides surgery, there are no curative treatments for GI carcinoid tumors and their hepatic metastases, emphasizing the need for development of strategies for other forms of therapy. Interestingly, these in vitro data illustrate that activation of the Notch1 signaling pathway reduces tumor cell proliferation as well as hormone production by carcinoid tumor cells. Therefore, manipulation of the Notch1 signaling pathway may be useful for expanding the targets for therapeutic and palliative treatment of patients with carcinoid tumors.

In conclusion, our findings demonstrate that activation of Notch1 in human GI carcinoid tumor cells suppresses hormone production and cellular proliferation (Fig. 6). Furthermore, Notch1 signaling is likely to be a critical integrator of signals that control the growth as well as regulation of NE hormones in carcinoid cells. We believe that the data presented here may prove helpful in expanding the role of Notch1 signaling in other cancer types. Furthermore, these findings may permit development of strategies to activate the Notch1 pathway that in turn may be useful as a therapeutic alternative in the treatment and palliation of GI carcinoid tumors.
ACKNOWLEDGMENTS

We thank Drs. Mark Evers and Courtney Townsend, Jr., for the BON cell line, Drs. Virote Sriuranpong and Doug Ball for the pLNCX/NIER construct, and Dr. David Hayward for the CBF1-1 reporter constructs. We thank Yi-Wei Zhang for the technical assistance. We thank other members of the Chen laboratory for critical reading of the manuscript.

GRANTS

This study was supported in part by a Research Scholars Grant from the American Cancer Society, National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-063015, DK-064735, and DK-066169, the American Surgical Association Foundation Fellowship Award, and the George H.A. Clowes, Jr., Memorial Research Career Development Award of the American College of Surgeons.

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