Development and Characterization of a Novel In vivo Model of Carcinoid Syndrome

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Abstract Purpose: Carcinoid syndrome, characterized by flushing, diarrhea, and valvular heart disease, can occur following carcinoid tumor metastasis to the liver and systemic release of bioactive hormones into the systemic circulation. Treatment of this devastating disease is hampered by the lack of an in vivo model that recapitulates the clinical syndrome.

Experimental Design: Here, we have injected BON cells, a novel human carcinoid cell line established in our laboratory, into the spleens of athymic nude mice to establish liver metastases.

Results: The majority of mice injected intrasplenically with BON cells developed significant increases in plasma serotonin and urine 5-hydroxyindoleacetic acid, and several mice exhibited mesenteric fibrosis, diarrhea, and fibrotic cardiac valvular disease reminiscent of carcinoid syndrome by both echocardiographic and histopathologic evaluation. Mice pretreated with octreotide, a long-acting somatostatin analogue, or bevacizumab, a vascular endothelial growth factor inhibitor, developed fewer liver metastases and manifestations of carcinoid syndrome, including valvular heart disease.

Conclusion: We have provided an important in vivo model to further delineate novel treatment modalities for carcinoid syndrome that will also be useful to elucidate the factors contributing to the sequelae of carcinoid disease (e.g., mesenteric fibrosis and valvular heart disease).

Carcinoid tumors are uncommon neuroendocrine tumors. The incidence of carcinoid tumors is 2 cases per 100,000 patients; however, the actual incidence is thought to be higher and increasing with time (1). Carcinoid tumors show a predilection for the gastrointestinal tract, with a majority arising in the small intestine. Although generally regarded as slow-growing and indolent in nature, tumors are often multicentric and associated with an increased incidence of synchronous noncarcinoid malignancies (2, 3).

Carcinoid tumors elaborate a variety of vasoactive amines and peptides, including chromogranin A (CgA), serotonin (5-hydroxytryptamine; 5-HT), histamine, and neurotensin, among others, with site of origin dictating secretory behavior (4–6). When confined to the bowel, peptides and vasoactive amines produced by the tumor are carried through the portal vein to the liver, where they are metabolized (7). However, in the presence of liver metastases or retroperitoneal disease, release of tumor products into the systemic circulation may lead to the development of the devastating sequelae known as carcinoid syndrome, characterized by flushing, diarrhea, bronchoconstriction, and valvular heart disease (7–11). Treatment has been primarily directed toward relief of the debilitating symptoms of carcinoid syndrome using the long-acting somatostatin analogue octreotide, which inhibits the release of carcinoid secretory products (12). Antiproliferative agents, such as streptozocin, 5-fluorouracil, doxorubicin, and cyclophosphamide, may be used to slow tumor growth, but their effects are limited (12, 13). The development of more effective treatment regimens for patients with carcinoid metastasis and carcinoid syndrome has been hampered by the lack of effective in vivo models, which recapitulate the disease process in humans (9).

We established and characterized the BON cell line, a functioning human carcinoid cell line from a lymph node metastasis of a pancreatic carcinoid tumor (6, 14, 15). BON cells elaborate not only 5-HT but also produce and secrete 5-hydroxytryptophan, neurotensin, and CgA (15–18). BON cells possess functional receptors for gastrin and somatostatin and release biogenic amines and peptides in response to various secretagogues (18–20). When injected subcutaneously into nude mice, BON cells form xenografts that are reminiscent of the original tumor by histologic examination. We have shown previously that xenograft development and growth can be inhibited by treatment with several agents (e.g., IFN-α and octreotide; ref. 9); however, the xenografted tumors do not
Translational Relevance

Carcinoid syndrome, characterized by flushing, diarrhea, and valvular heart disease, can occur following carcinoid tumor metastasis to the liver. Treatment of this disease is hampered by the lack of an in vivo model that recapitulates the clinical syndrome. Here, we have injected BON neuroendocrine cells into the spleens of athymic nude mice to establish liver metastases and treated mice with vehicle, octreotide, or bevacizumab. The majority of mice injected with BON cells developed significant increases in plasma serotonin and urine 5-hydroxyindoleacetic acid, and several mice exhibited mesenteric fibrosis, diarrhea, and fibrotic cardiac valvular disease by both echocardiographic and histopathologic evaluation. Mice pretreated with octreotide or bevacizumab developed fewer liver metastases and manifestations of carcinoid syndrome. We describe a novel in vivo model of carcinoid syndrome, which will be useful to further delineate treatment modalities for carcinoid tumors and elucidate the factors contributing to the sequelae of carcinoid disease.

Materials and Methods

Materials. Rabbit monoclonal anti-neurotensin and anti-CgA antibodies were purchased from Abcam. Mouse monoclonal anti-5-HT antibody was purchased from Dako. Tissue culture media and reagents were from Mediatech. 5-HT enzyme immunoassay (EIA) was from DRG Instruments. Octreotide was from Bedfor Laboratories. Bevacizumab was from Genentech.

Cell culture and establishment of stable cell lines. BON cells are maintained in a 50:50 mixture of DMEM and F-12K supplemented with 5% fetal bovine serum in 5% CO2 at 37°C. To confirm liver metastases, we used a BON cell clone stably transfected with the plasmid pEGFP-N1 by electroporation and selected in medium containing G418 (400 μg/mL; Cellgro). Transfection was confirmed by assessment of green fluorescent protein (GFP) expression.

Animals. Male athymic nude mice (4-6 weeks; ∼25 g) were purchased from Harlan-Sprague-Dawley. Mice were housed in an American Association for Accreditation of Laboratory Animal Care-approved facility under a standard 12 h light-dark cycle. They were fed standard chow (Formula Chow 5008; Purina Mills) and tap water ad libitum and allowed to acclimate for 1 week. All studies were approved by the Institutional Animal Care and Use Committee of The University of Texas Medical Branch.

Tumor establishment. BON cells (passage 23) were harvested from subconfluent cultures by a 1 min treatment with 0.25% trypsin. Tumor cells were injected in a 200-μL volume at two sites on the subcostal flank. Two mice were housed with each site, and each site was used only once. Octreotide or bevacizumab developed fewer liver metastases and the subsequent carcinoid syndrome could be attenuated, we again performed intrasplenic injections of BON cells (1 × 10^7) into 15 athymic nude mice; intrasplenic injection of HT29 colon cancer cells into 5 mice was done as a control. Mice injected with GFP-labeled BON cells were observed each week using the IllumiTest TLS (Lightools Research). At sacrifice, spleen, liver, and heart were harvested for analysis, and plasma was collected for 5-HT detection by EIA. (b) To determine whether carcinoid liver metastases and the subsequent carcinoid syndrome could be attenuated, we again performed intrasplenic injections of BON cells (1 × 10^7) into 15 athymic nude mice; intrasplenic injection of HT29 colon cancer cells into 5 mice was done as a control. Mice were then randomized into groups (n = 5) for treatment with vehicle (distilled H2O, 200 μL intraperitoneal, every other day), octreotide (5 mg/kg, intraperitoneal, every other day), or bevacizumab (2.5 μg, subcutaneous, every day). Echocardiography was done at week 12 immediately before sacrifice; tissues were collected for analysis as above, and plasma and urine were collected for 5-HT and 5-HIAA detection by ELISA, respectively.

Echocardiography. Mice were anesthetized with isoflurane and placed on a warming blanket in the supine position. Transthoracic echocardiographic measurements, including two-dimensional, M-mode, and Doppler evaluation, were obtained in mice with the use of a 15 MHz linear transducer (Acuson Sequoia Cardiac System; refs. 22, 23).

Tissue processing, staining, and immunohistochemistry. On sacrifice, hearts and segments of livers and spleens were immediately placed in 10% neutral buffered formalin for 24 h followed by 70% ethanol for 24 h. After removal, to display cardiac valves, hearts were manually cut in a near-sagittal plane at 1 mm increments (4-5 segments per heart) with emphasis on obtaining longitudinal sections of tricuspid and mitral valves.

All tissue samples were paraffin-embedded, sectioned at 5 μm, and routinely deparaffinized and dehydrated. Sections of liver and spleen were stained with H&E; heart sections were stained with Movat pentachrome to display collagen, elastin, and glycosaminoglycans characteristic of immature connective tissue (24, 25). Immunohistochemistry was done on paraffin-embedded samples as described previously (21, 26) using DAKO EnVision Kit (DAKO). Sections were incubated overnight at 4°C with monoclonal antibodies diluted in 0.05 mol/L Tris-HCl + 1% bovine serum albumin against F4/80 (1:100), bromodeoxyuridine (1:1,000), proliferating cell nuclear antigen (1:2,000), or LC3 (1:100). After three washes with TBS-Tween 20, the sections were incubated for 30 min with secondary antibody.
labeled with peroxidase and then washed three times with TBS-Tween 20. Lastly, peroxidase substrate 3,3′-diaminobenzidine was added for immunostaining; sections were counterstained with hematoxilin. For negative immunohistochemistry controls, primary antibody was omitted from the above protocol.

**Histologic scoring.** Heart sections stained with Movat pentachrome were randomized, blinded to all observers, and presented to a pathologist for histologic assessment and scoring of cardiac lesions. After initial review of all specimens, a grading system for cardiac valvular lesions was devised as follows: grade 0, normal cardiac valve; grade 1, a single area or multiple areas of thickening involving one valve only; grade 2, areas of thickening involving two valves; grade 3, multiple areas of thickening involving two or more valves; and grade 4, multiple areas of thickening as in grade 3 but with additional degenerative/secondary changes including fibrous adherence to endocardium, metaplastic change within valve structure, or endocardial/valvular thrombus formation.

**5-HT EIA and 5-HIAA ELISA.** EIA and ELISA were done according to the manufacturer’s instructions and as described (21). Briefly, 50 μL supernatant was added to 50 μL assay diluent (1:1 dilution) in a 96-well plate. After incubation, wells were washed and conjugate was added. Substrate solution was then added, and the plates were incubated in the dark for 30 min. Stop solution was then added, and absorbance determined at 450 nm (with a correction wavelength set at 570 nm). Experiments were done in duplicate.

**Statistical analysis.** Outcomes were analyzed using Kruskal-Wallis test. Groups were assessed at the 0.05 level of significance. Multiple comparisons were conducted using Bonferroni adjustment for the number of comparisons. All statistical computations were carried out using statistical software, the SAS system, release 9.1 (27).

## Results

**BON cells metastasize to the liver and produce CgA, 5-HT, and neurotensin.** BON cells grow as tumor xenografts when injected into the flanks of athymic nude mice and show migration in vitro (28). To first determine if BON cells metastasize in vivo, single-cell suspensions of BON cells and GFP-labeled BON cells (1 × 10⁷) were injected into the spleens of 20 nude mice, and mice were sacrificed 9 weeks later. Mice injected with GFP-labeled BON cells were imaged weekly to follow tumor development (Fig. 1A). Primary tumor development was first noted by week 3, with metastatic liver lesions obvious by week 5. Following injections, 80% of the mice (n = 16 of 20) developed liver metastases, with all mice developing primary splenic tumors. Splenic primary tumors and metastases exhibited a similar histopathologic pattern as the patient’s original tumor (9). Immunohistochemistry showed heterogeneous, strong positive staining for CgA and 5-HT in the primary tumor and tumor metastases, consistent with previous immunocytochemical staining (9), whereas HT29 colon cancer metastases were negative for these products (Fig. 1B). Given that BON cells metastasize to the liver and produce hormones and bioactive amines characteristic of carcinoid tumors, we next determined whether these mice develop signs and symptoms, which recapitulate the carcinoid syndrome in humans. Before sacrifice, we noted that 8 of 20 mice developed gross diarrhea (data not shown), and at sacrifice, we noted the presence of mesenteric fibrosis in 30% (n = 3 of 20) of the mice by gross examination. A significant elevation of plasma 5-HT was noted in 45% (n = 9 of 20) of the mice (mean, 482.3 ng/mL; range, 117-841 ng/mL).

**Mice with BON cell liver metastases develop carcinoid heart disease as noted by functional evaluation and histopathologic valvular lesions.** To determine whether mice with BON cell liver metastases develop cardiac valvular abnormalities as a result of tumor development, mice were imaged with transthoracic echocardiography as described in Materials and Methods. Limitations of these studies included an inability to clearly image the tricuspid and pulmonic valves; additionally, control athymic nude mice were noted to have slight enlargement of the right ventricular diastolic volume relative to control Swiss-Webster or C57BL/6 mice of similar age, leading to an inability to use right ventricular size as a potential marker of tricuspid or pulmonic valve pathology. Despite these limitations, 6 of 15 mice with BON cell liver metastases clearly showed valvulopathy or functional cardiac impairment when compared with control mice or mice injected with HT29 colon cancer cells. Figure 2A shows a normal Doppler, M-mode, and two-dimensional echocardiographic study of a mouse injected with HT29 cancer cells; right and left ventricular sizes, appearance of
mitral and aortic valves, and laminar flow through the mitral and aortic valves are grossly normal. In contrast, 6 mice with BON cell liver metastases developed valvular or ventricular abnormalities, which were visualized by echocardiography, including irregular thickening of the mitral valve leaflets (Fig. 2B), significant right and left ventricular dilatation with irregularity of mitral valve leaflets associated with arrhythmia (Fig. 2C), as well as left ventricular hypertrophy, mitral valve thickening, and aortic regurgitation (Fig. 2D). (Video of echocardiographic studies is available in Supplementary Data.)

In initial screening studies of mice injected with BON cells, histopathologic lesions of cardiac valves were noted in 20% \((n = 4 \text{ of } 20)\) of mice; these lesions affected the tricuspid and/or mitral valve as noted by H&E and Movat pentachrome stains.

Fig. 2. Mice injected with BON cells develop cardiac functional impairment as assessed by Doppler, M-mode, and two-dimensional echocardiography. Mice were anesthetized with isoflurane and transthoracic echocardiography was done, including two-dimensional (transverse plane through mitral valve, apical view) to capture two-dimensional images of the heart and valves in cross-section (top row), Doppler, to assess the directionality and velocity of blood flow through the mitral valve (second row), and M-mode, to assess one-dimensional changes in ventricular volume and wall thickness over time (bottom row). A, mouse injected with HT29 colon cancer cells exhibiting a normal study. The appearance of mitral valve leaflets by two-dimensional analysis (white arrowhead, posterior leaflet) is thin and regular, and right and left ventricles are normal in size. An illustration is provided to the right of A for clarification. Upstrokes on Doppler analysis indicate anterograde flow through the mitral valve, whereas downstrokes represent retrograde flow (regurgitation) through the mitral valve with systole. B to D, mice injected with BON cells treated with vehicle displaying abnormalities in cardiac function. White arrowheads, abnormally thickened, irregular posterior mitral valve leaflets (B and D); black arrowhead, dilated right ventricle noted by two-dimensional echocardiography (C); white arrow, significant mitral regurgitation noted by Doppler (D). *\(^t\), left ventricular wall thickness; \(t\), left intraventricular volume by M-mode evaluation. Note the increased left intraventricular volume, decreased left ventricular wall thickness, and poor contractility with systole in C, indicative of dilated cardiomyopathy. In contrast, note the diminished left intraventricular volume and increased left ventricular wall thickness in D, indicative of hypertrophic cardiomyopathy with diastolic dysfunction.
Subsequent double-blinded studies in which the heart was multiply sectioned to maximize visualization of valves and endocardial structures, as described in Materials and Methods, showed that 100% of the mice (n = 5 of 5) with BON cell metastases treated with vehicle exhibited valvular lesions of two or more cardiac valves (grades 2-4). In contrast to normal mitral and pulmonic valves (Fig. 3A, right and left, respectively), histopathologic lesions consisted of marked thickening of valve leaflets by spindly fibroblast-like cells and extensive deposition of blue green mucopolysaccharides noted by Movat pentachrome stain (Fig. 3B, right and left). Although the cardiac valves were sampled equally in multiple sections, lesions were noted most frequently in the tricuspid (91% of hearts with lesions) and mitral (90%) valves; in comparison, aortic and pulmonic valves showed a lower incidence of lesion development (55% and 33%, respectively). In more advanced lesions (grades 2 and 4), thickened areas were large, covered extensive areas of the valve leaflets, and involved multiple valves. In addition, advanced lesions (grade 4) showed attachment of valve leaflets to the underlying endocardium (Fig. 3B, left). In areas of endocardial, valvular, and subendocardial fibrosis, rounded cells within lacunae were seen, indicative of cartilaginous metaplasia (Fig. 3C). Additionally, thrombus formation on the valvular surface was occasionally noted in advanced lesions (Fig. 3D). It was subsequently noted that all mice exhibiting advanced valvar histopathologic lesions (grades 3 and 4) had significant valvar and/or ventricular wall motion abnormalities on echocardiographic evaluation, providing a strong correlation between advanced histopathologic grade and valvular heart disease.

Mice injected intrasplenic with HT29 colon cancer cells showed liver metastases but did not exhibit valvulopathy, mesenteric fibrosis, diarrhea, or elevation of plasma 5-HT, showing that the metabolic sequelae and valvular abnormalities were specific for BON metastases and not a nonspecific consequence of liver metastases.

Treatment with either octreotide or bevacizumab significantly inhibits BON liver metastases and development of the carcinoid syndrome. Next, we determined whether BON carcinoid liver metastases and subsequent carcinoid syndrome could be attenuated by administration of octreotide, commonly used in the symptomatic management of carcinoid syndrome, or bevacizumab, which is currently being evaluated in clinical trials as adjuvant treatment for carcinoid tumors (29), but has nonetheless showed effectiveness in limiting the growth of other types of tumors, including cancers of the colon, thyroid, lung, and brain (30–33). Following intrasplenic injection of
BON cells, mice were randomized to groups of five to receive treatment with vehicle (distilled H₂O, 200 µL, intraperitoneal, once every other day), octreotide (5 mg/kg, intraperitoneal, once every other day), or bevacizumab (2.5 µg, subcutaneous, every day) within 2 h of splenic injection. Following BON or HT29 cell injections, 100% of mice (20 of 20) developed liver metastases. Mice treated with either octreotide or bevacizumab (n = 5 per group) showed significantly less liver metastasis and tumor burden compared with mice treated with vehicle (Fig. 4A and B).

Similarly, whereas all vehicle-treated mice developed significant histopathologic lesions of the heart valves (as described above), octreotide- or bevacizumab-treated mice developed significantly fewer lesions (Fig. 5A). These data correlated with echocardiographic findings; mice with advanced valvular histopathologic score (3-4) displayed significant cardiac valvular and ventricular wall motion abnormalities as in the previous study. Mesenteric fibrosis and diarrhea were noted in 20% of mice (3 of 15; data not shown). Elevation of plasma 5-HT was noted in 100% of vehicle-treated mice, 80% of octreotide-treated mice, and 20% of bevacizumab-treated mice (mean, 486.6 ng/mL; range, 7.3-1,497 ng/mL; Fig. 5B); elevation of urinary 5-HIAA was noted in 60% of vehicle-treated mice and none of the octreotide- or bevacizumab-treated mice (mean,
Discussion

Our understanding of carcinoid tumors and the clinical manifestation of the carcinoid syndrome as well as the development of better treatment options has been hampered by the absence of an appropriate in vivo model. Other investigators, including those in our laboratory who have used subcutaneous xenograft placement, have described in vivo models of carcinoid tumor development but have failed to show in vivo manifestations of carcinoid syndrome (9, 34–36). In our current study, we describe for the first time a novel in vivo model of carcinoid syndrome, which recapitulates many of the clinical sequelae noted in humans. When dispersed BON cells are injected into the spleen of athymic nude mice, the majority of the mice develop multiple multilobar liver metastases, which express CgA, 5-HT, and neurotensin, as confirmed by immunohistochemical staining. Additionally, several mice develop sequelae, which mimic human carcinoid syndrome, such as flushing, diarrhea, mesenteric fibrosis, and cardiac valvulopathy; these findings occur in mice with significant tumor burden and elevated plasma 5-HT.

The clinical effect of octreotide, and other long-acting somatostatin analogues, in patients with carcinoid syndrome remains controversial. Classicly used to inhibit the release and action of multiple hormones and attenuate exocrine secretion, its effects on tumor proliferation remain unclear (7, 37–39). We have shown that octreotide treatment inhibits the growth of subcutaneous BON carcinoid xenografts placed in the flanks of athymic nude mice (9). Consistent with these findings, our present study shows that the administration of octreotide leads to significantly less metastatic tumor burden within the liver, indicating an antiproliferative effect of octreotide. Although carcinoid tumors exhibit differential somatostatin receptor expression based on site of origin, often leading to a discrepancy in tumoral response to octreotide therapy (7, 11), BON cells are known to highly express the somatostatin receptor, likely contributing to the significant decrease in tumor progression with octreotide treatment.

Vascular endothelial growth factor is a potent endothelial cell-specific mitogen that promotes endothelial cell growth from preexisting vasculature (40). Although there is clear evidence that treatment with bevacizumab attenuates the development of colon, thyroid, lung, and brain cancer in vivo by inhibiting tumoral blood vessel development (30–33), its use has only recently been suggested in the treatment of carcinoid tumors (29). Elevated expression of vascular endothelial growth factor appears to correlate with decreased progression-free survival among patients with neuroendocrine tumors (41). In our current study, we found that treatment with bevacizumab significantly inhibited tumor growth, and tumors that did develop were far smaller in size, consistent with findings described by Zhang et al. (41).

Because survival of patients with carcinoid tumors has improved with the availability of supportive medications such as octreotide, the clinical manifestations of fibrosis are now the leading causes of morbidity and mortality associated with this disease (7, 11). Carcinoid-related mesenteric fibrosis may lead to intestinal obstruction, vascular occlusion and intestinal ischemia, or ureteral obstruction with subsequent hydronephrosis and renal failure (42–44). Carcinoid heart disease, characterized by fibrous, plaque-like thickening of the endocardium of the tricuspid and pulmonic valves most commonly, is a serious complication occurring in approximately two thirds of patients with carcinoid syndrome, leading to death in as many as one third of the cases (7, 45). The cardiac manifestations of carcinoid syndrome in humans were essentially reproduced in our model, with extensive fibrosis and thickening involving all cardiac valves, with greatest frequency noted in the tricuspid and mitral valves. Also observed were endocardial degenerative changes of cartilaginous metaplasia,
an alteration that is normally noted in aging rats near or in the aortic valve ring but is also described in other forms of endocardial fibrosis (46).

Although the association of carcinoid disease with fibrosis has been well documented, the mechanism is poorly understood. Although classically attributed to the systemic effects of elevated 5-HT, 5-HT does not promote fibroblast secretion or proliferation in vitro, nor do anti-serotonergic agents prevent the development of fibrosis in patients (7, 47–50). Therefore, focus has recently shifted to growth factors such as transforming growth factor-β, connective tissue growth factor, and platelet-derived growth factor as potential etiologic agents. The establishment of an in vivo model of carcinoid syndrome is central to the elucidation of factors contributing to this disease process. In the future, we plan to develop knockout or knock-in BON cell lines to better delineate the precise factors that promote carcinoid-associated fibrosis.

In conclusion, we describe a unique in vivo model of carcinoid syndrome, which results in liver metastases, systemic sequelae of increased 5-HT, and valves of heart disease. Our findings suggest that treatment with either octreotide or bevacizumab significantly inhibits carcinoid tumor metastasis, with bevacizumab the most effective. This model provides an important in vivo model to further delineate novel treatment modalities for carcinoid syndrome and will also be useful to elucidate the factors contributing to the sequelae of carcinoid disease, such as mesenteric fibrosis and valvular heart disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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