Establishment and Characterization of a Human Carcinoid in Nude Mice and Effect of Various Agents on Tumor Growth

B. MARK EVERS, COURTNEY M. TOWNSEND, Jr., J. ROBERT UPP, ERICK ALLEN, STEPHEN C. HURLBUT, SUN WHE KIM, SRINIVASAN RAJARAMAN, POMILA SINGH, JEAN CLAUDE REUBI, and JAMES C. THOMPSON

Departments of Surgery and Pathology, University of Texas Medical Branch, Galveston, Texas; and Sandoz Research Institute, Berne, Switzerland

The authors have established a long-term tissue culture cell line (BON) derived from a metastatic human carcinoid tumor of the pancreas. The cells have been in continuous passage for 46 months. Tissue culture cells produce tumors in a dose-dependent fashion after SC inoculation of cell suspensions in athymic nude mice. BON tumors, grown in nude mice, are histologically identical to the original tumor; they possess gastrin and somatostatin receptors, synthesize serotonin and chromogranin A, and have a doubling time of approximately 13 days. The antiproliferative effects of the long-acting somatostatin analogue, SMS 201-995 (300 μg/kg, t.i.d.), and 2% α-difluoromethylornithine on BON xenografts in nude mice were examined. Tumor size was significantly decreased by day 14 of treatment with either agent and at all points of analysis thereafter until the animals were killed (day 33). In addition, tumor weight, DNA, RNA, and protein contents were significantly decreased in treated mice compared with controls. Establishment of this human carcinoid xenograft line, BON, provides an excellent model to study further the biological behavior of carcinoid tumors and the in vivo effect of chemotherapeutic agents on tumor growth.

Carcinoid tumors are endocrine neoplasms derived from neuroectodermal cells of the neural crest (1,2). Primarily found in the gastrointestinal (GI) tract, these tumors are uncommon, but not rare, with a reported incidence in autopsy series ranging from 0.1% to 1.4% (1). Originally called "Karzinoide" by Oberndorfer (3) to describe its apparent benign nature, modern immunohistochemical techniques have allowed identification and isolation of the vasoactive substance serotonin [5-hydroxytryptamine (5-HT)] and a host of biogenic amines and hormones produced by carcinoid tumors (1,2).

Treatment of patients with carcinoid tumors has been hampered by the lack of an animal model. In the majority of patients, treatment of metastatic carcinoid tumors has been directed largely toward relief of the debilitating sequelae of the carcinoid syndrome by means of blocking agents [for example, methysergide (4), cyproheptadine (4,5), and ketanserin (6,7)] or the inhibitory hormone, somatostatin (8–16). Antiproliferative agents that are commonly used to slow tumor growth include combinations of streptozotocin, 5-fluorouracil, doxorubicin, or cyclophosphamide (5,17–19). However, most carcinoid tumors respond poorly to cytotoxic therapy with these agents (1,4,18–19).

In this study, we report the first in vivo establishment of a long-term xenograft cell line of a human pancreatic carcinoid tumor. We have characterized tumor growth rate in vivo, the status of gastrin and somatostatin receptors, the morphology, and the production of peptides and amines. In addition, we have examined possible antiproliferative effects of the long-acting somatostatin analogue, SMS 201-995, and α-difluoromethylornithine (DFMO) (either alone or in combination) on growth of the carcinoid tumor.

Abbreviations used in this paper: DFMO, α-difluoromethylornithine; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; 5-HT, 5-hydroxytriptamine.

0016-5085/91/$3.00

Materials and Methods

Surgical Specimen Collection and Maintenance of Tumor Line

The operative specimen of a peripancreatic lymph node was sterilely obtained in April 1986 from a 28-year-old man with metastatic carcinoid tumor of the pancreas who had come into the hospital with symptoms of obstructive jaundice and diarrhea. Before exploratory laparotomy the patient had not received any interventional treatment. Final diagnosis of a metastatic carcinoid tumor was made by histological examination of the lymph node and positive staining for neuron-specific enolase and 5-HT.

A portion of the lymph node was washed in saline, minced, and tumor fragments placed in Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY) and F12K (Gibco) in a 1:1 ratio supplemented with 10% (vol/vol) fetal calf serum (FCS; Hyclone Laboratories, Logan, UT) and 1% gentamicin. The cells were grown at 37°C in an atmosphere of 95% air and 5% CO_2. Cells were routinely passed by removing the medium and overlaying the cell monolayer with 0.25% trypsin:0.1% ethylenediaminetetraacetic acid (EDTA). The elimination of fibroblasts from the stroma of the tumor tissue was accomplished by brief exposures to 0.06% trypsin:0.02% EDTA. This procedure was repeated until no further fibroblast growth was observed. Tumor cells from passage 5 were frozen after successful in vitro adaptation and removal of all fibroblasts. The cell line is presently maintained in DMEM and F12K growth medium supplemented with 10% FCS; it is passed at a 1:2 ratio when cells reach 80% confluence. Cell cultures are routinely monitored for mycoplasma contamination, and no mycoplasma growth has been detected.

Animals

Male athymic nude mice (Balb/c, 20–25 g, 3–4 weeks of age; Life Science, St. Petersburg, FL) were housed under specific pathogen-free conditions in a temperature-controlled isolation unit with 12-hour light-dark cycles in accordance with the National Research Council's Guide for the Care and Use of the Nude Mouse in Biomedical Research (20). The mice were fed a standard chow (Autoclavable Rodent Chow no. 5010; Ralston Purina, St. Louis, MO) and sterile water, both given ad libitum.

Solid Tumors in Nude Mice and In Vitro Growth Rate

BON cells (passage 8) were harvested from subconfluent cultures by a 1-minute treatment with 0.25% trypsin and 0.1% EDTA. Single-cell suspensions (5×10^6, 1×10^7, and 2×10^7) in RPMI 1640 without serum (total volume, 0.1 mL) were injected SC at a single site in the dermis of nude mice (n = 10 mice/group). Tumor size (longest perpendicular diameter) was measured biweekly with Vernier calipers (Mitutoyo Corp., Tokyo, Japan) accurate to 0.5 mm. Surface areas were calculated as the product of the two greatest perpendicular tumor diameters and were expressed as square millimeters. Tumor areas were plotted on semilogarithmic paper, and the tumor doubling time (defined as time in days required for mean tumor area to double during logarithmic growth) was determined directly from the graph.

On day 48, mice were killed and tumors from passage 8 were removed for light and electron microscopy, immunohistochemistry, and measurement of gastrin receptors.

Light and Electron Microscopy

For light microscopy, tumor tissue blocks measuring 1 × 1 × 0.5 cm were fixed in 10% neutral buffered formalin for 6–8 hours, processed routinely, and embedded in paraffin. Sections (4 μm thick) were stained with H&E and examined.

For transmission electron microscopy, tissue blocks (1 mm^3) were fixed in half-strength Karnovsky's fixative for 4 to 6 hours, postfixed in osmium tetroxide (1%), and embedded in Epon. Sections (70 nm thick) were stained with uranyl acetate and Reynold's lead citrate and examined with a Philips 410 electron microscope (Philips Medical Systems Inc., Shelton, CT).

Immunohistochemical Studies

Formalin-fixed, paraffin-embedded tissue samples were studied by means of a three-layer immunohistochemical method. The primary antibodies used were murine monoclonal antibody to chromogranin A (Boehringer Mannheim, Indianapolis, IN) and monospecific polyclonal antibodies to serotonin, substance P, pancreatic polypeptide, vasoactive intestinal peptide, glucagon, gastrin and bombesin (all from Dako Corp., Santa Barbara, CA). Tissue sections (4 μm thick) were sequentially incubated with the appropriate primary antibodies, swine anti-rabbit immunoglobulin G (IgG) or rabbit anti-mouse IgG (1:100–1:400), followed by rabbit or mouse peroxidase-antiperoxidase complexes (1:100) (Dako Corp.), with frequent washes in phosphate-buffered saline between incubations. The peroxidase reaction product was visualized by incubating with diaminobenzidine (0.05%) and hydrogen peroxide (0.01%). Controls included omission of primary antibodies and substitution of primary antibodies with nonimmune sera from the same species.

Gastrin Binding Assay

Tumors were quickly removed and washed with cold buffer A (Tris, 10 mmol/L; KCl, 2 mmol/L; $MgCl_2$, 2.5 mmol/L; and sucrose, 0.25 mol/L; pH 7.4) containing BSB [1% bovine serum albumin, fraction V (Sigma Chemical Co., St. Louis, MO)], 0.1% soybean trypsin inhibitor (Worthington Biochemical Corp., Freehold, NJ), and 0.1% bacitracin (Sigma Chemical Co.). Tumors were then stored at −70°C in an ultradeep freeze until further analysis. Specific binding sites for gastrin were measured on cell membranes prepared from tumor samples by our previously published methods (21,22).

Somatostatin Receptor Assay

Somatostatin receptors were measured on BON tumors by autoradiography on tissue sections (10 μm thick) as described before in detail for various tumors, including hormone-producing gastroenteropancreatic tumors (23). The iodinated Tyr^3 analogue of SMS 201-995 (code-named 204-090) was used as the radioligand.

Administration of SMS 201-995 and DFMO

Initially, to establish tumors, dispersed BON cells (passage 8, 1×10^7 cells) were inoculated SC in nude mice. When tumors became approximately 10 cm^2 in area, the mice were killed and tumors minced into 3-mm^2 pieces that were then implanted bilaterally into the flanks of 20 nude mice. The mice were randomly allocated to receive either saline (0.1 mL, IP, t.i.d.), 2% (wt/vol) DFMO (a gift from W. J. Hudak, Ph.D., Manager of Research Information at the Merrell Research Center, Cincinnati, OH) in drinking water, SMS 201-995 (300 μg/kg, IP, t.i.d.), or a combination of DFMO and SMS 201-995 beginning the day of tumor implantation and continuing until they were killed. Water bottles were covered to prevent light degradation. Drinking water was renewed every 2 days. SMS 201-995 (a gift of Sandoz Research Institute, Hanover, NJ) was diluted to the required concentration with saline.

Mice were weighed weekly, and tumors were measured twice weekly by the same observer. The surface areas of the tumors were calculated as described above. Mice were killed on day 33, and tumors were removed, weighed, and frozen at −70°C until assayed for DNA, RNA, and protein content.

Protein, DNA, and RNA Analysis

Tumors were thawed, homogenized (Polytron; kinematica GmbH, Kriens–Luzern, Switzerland), and extracted by the method of Ogur and Rosen (24). Protein content was determined by the method of Lowry et al. (25), with bovine serum albumin as standard. DNA content was measured by the Burton (26) modification of the diphenylamine procedure with calf thymus DNA used as the standard. RNA content was measured by means of the orcinol procedure with yeast RNA as the standard (27).

Statistics

Results are expressed as the mean ± SEM. Antiproliferative effects of SMS 201-995 and DFMO were analyzed by two-way analysis of variance. A value of $P < 0.05$ was considered significant.

Results

Tumor Growth in Nude Mice

When single-cell suspensions were injected SC into nude mice, tumors were produced in a dose-dependent fashion (Figure 1). After 2×10^7 BON cells were injected, tumors were visible by 7 days in all mice. When 1×10^7 cells were injected, tumors grew in all mice; however, they were not visible until day 10. After injection of 5×10^6 cells, 80% of tumors grew and were visible by day 17. Once the tumors began to grow, the growth rates were similar regardless of initial inoculum, and tumor-doubling time was approximately 13 days. All tumors grew as discrete encapsulated masses, without evidence of local invasion or distant metastasis.

Tumor xenografts were sent to the American Type Culture Collection (Rockville, MD) where isoenzyme determination was performed by electrophoresis, confirming that this was human tissue and not a spontaneously occurring mouse tumor.

Morphological Studies

Light microscopy. The original patient's tumor was composed of masses of ovoid cells arranged in a nonorganoid fashion in a delicate, vascular stroma. Cellular and nuclear pleomorphism was minimal (Figure 2*A*). Tumors from the nude mouse exhibited a similar histopathologic pattern with moderate nuclear pleomorphism of the cells. (Figure 2*B*).

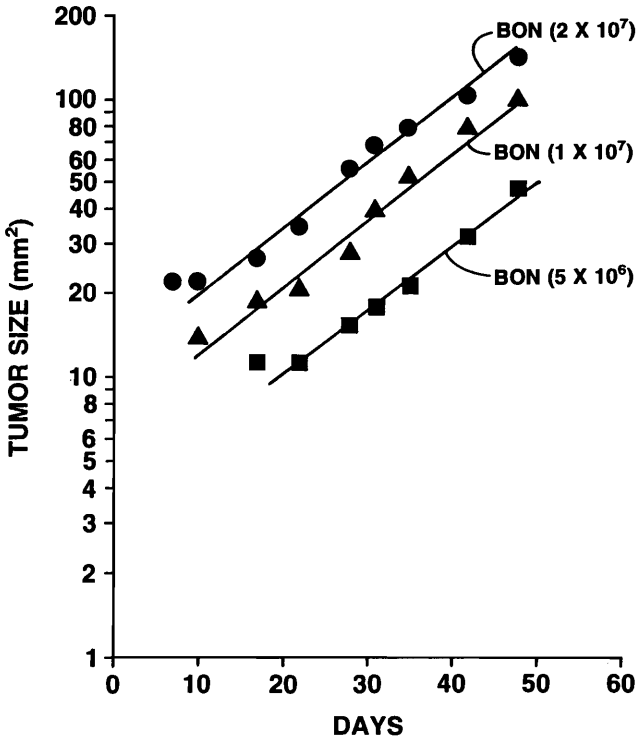

Figure 1. Logarithmic growth curves of BON tumors injected SC as single-cell suspensions (5×10^6, 1×10^7, 2×10^7 cells) in the dermis of nude mice (n = 10 mice/group).

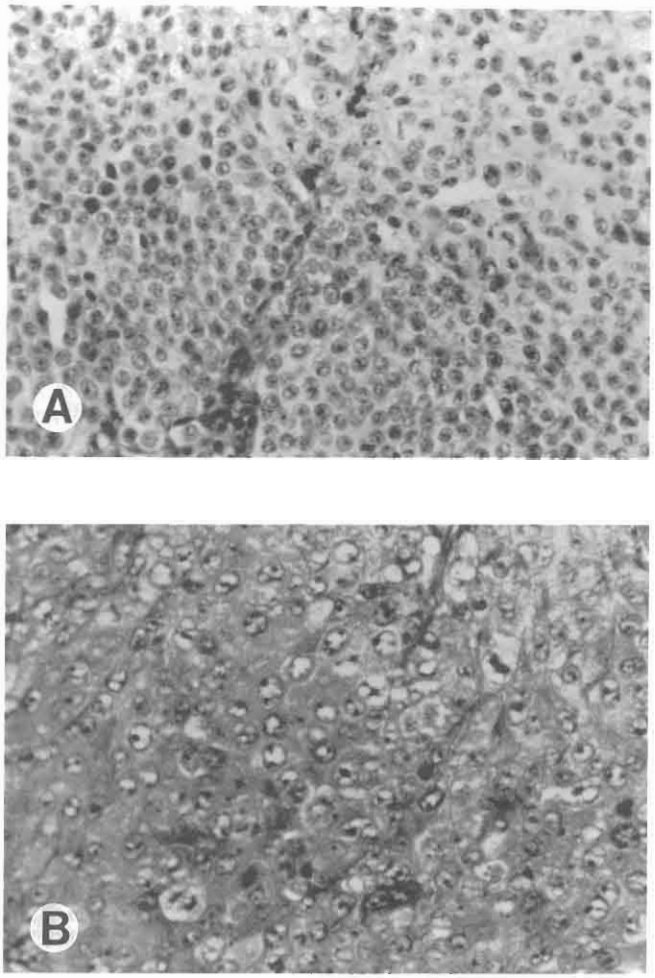

Figure 2. Light micrograph of the patient's tumor (*A*) and the nude mouse tumor (*B*) demonstrating similar histopathologic characteristics (H&E; original magnification ×500).

Electron microscopy. BON tumors from nude mice were composed of polygonal cells arranged in masses in a vascular stroma. A majority of cells exhibited an abundance of cytoplasmic secretory granules that were membrane-bound, several mitochondria, and profiles of endoplasmic reticulum. Sheaves of tonofilaments (cytokeratin) were also found in several cells, the features being characteristic of epithelial and endocrine differentiation (Figure 3).

Immunohistochemical Studies

BON xenografts stain positive for serotonin (Figure 4) and chromogranin A granules (Figure 5). The staining was appreciable in approximately 50%–60% of the BON tumor cell population exhibiting both serotonin and chromogranin A immunoreactivity. Substance P, pancreatic polypeptide, vasoactive intestinal polypeptide, glucagon, gastrin, and bombesin were not detected in the BON tumor by immunohistochemical staining.

Gastrin Binding

Specific binding sites for gastrin in the range of 10–18 fmol/mg protein with a high binding affinity (K_d = ~0.134 nmol/L) were measured from a Scatchard plot of the specific binding data on tumor membranes. The binding sites were specific for binding of gastrin and did not show any significant binding affinity for cholecystokinin or other unrelated peptides tested (bombesin, vasoactive intestinal peptide, or insulin) (data not shown).

Somatostatin Binding

Somatostatin receptors were localized on tumor tissue exclusively; however, nonhomogenous distribution can be observed, with tumor regions having higher receptor density than nontumor area. Binding of ^{125}I-[Tyr^3]-SMS 201-995 was shown to be displaced in the nanomolar range using increasing concentrations of SMS 201-995 in successive tissue sections (Figure 6). The binding was specific because unrelated peptides (e.g., luteinizing hormone–releasing hormone) did not compete with the ligand.

Effect of α-Difluoromethylornithine and SMS 201-995 on BON Growth

There were no significant differences in final body weight in treated mice compared with the control group, and mice receiving SMS 201-995 and DFMO showed no ill effects. Food and water intake was monitored, and there were no differences in

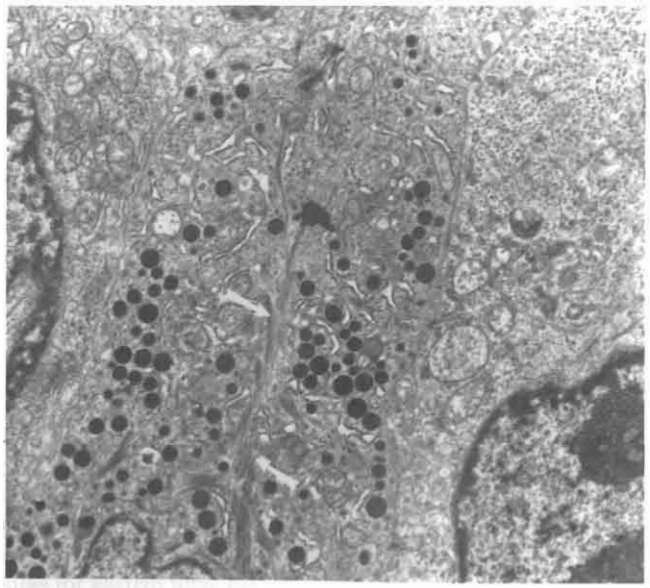

Figure 3. Electron micrograph of BON tumor demonstrating abundant dense secretory granules interspersed with sheaves of cytokeratin (*arrows*) (original magnification ×7100).

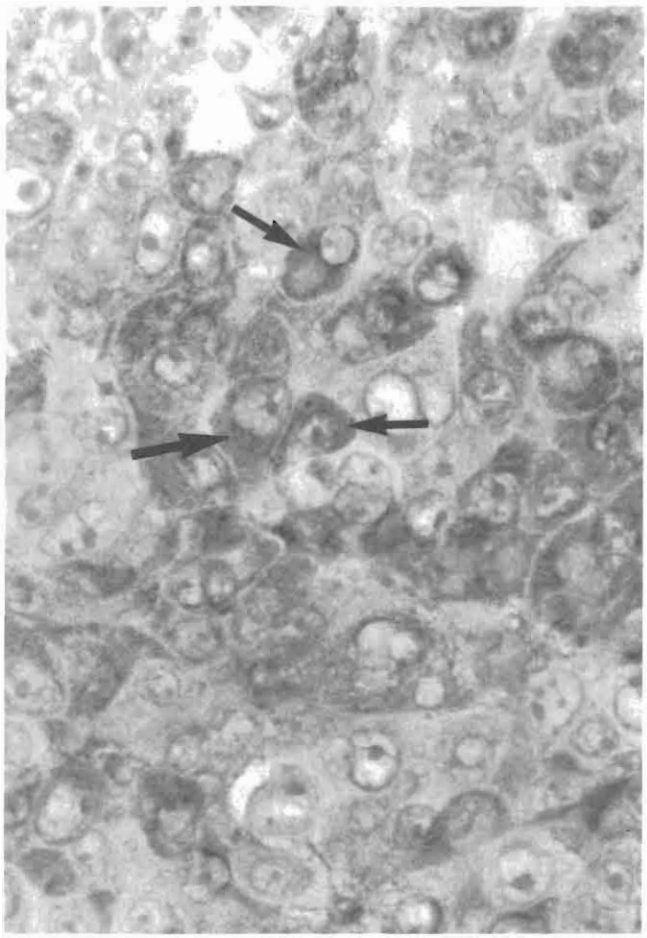

Figure 4. Light micrograph of the BON tumor stained for serotonin (*arrows*) (counterstained with hematoxylin; original magnification ×1000).

consumption between groups. Two mice (one control and one SMS 201-995–treated) died during the course of the experiment because of traumatic injections (confirmed by necropsy) and were excluded from analysis.

Both SMS 201-995 (300 μg/kg, IP, t.i.d.) and 2% DFMO, administered either as single agents or in combination, significantly inhibited tumor area by day 14; inhibition continued to the time of killing (day 33) (Figure 7). Tumor weight, DNA, RNA, and protein contents were similarly inhibited after treatment (Figure 8). The mean tumor weight of the DFMO-treated group was 42% of that of the control group, protein content was 40%, and DNA and RNA contents were 53% of controls. Mean tumor weight in the SMS 201-995–treated group was 38%, protein content was 39%, DNA content was 34%, and RNA content was 44% of the values in the control group. Although mean values of tumor area, weight, and biochemical determinations were the lowest in mice treated with combination therapy, these values were not significantly different from those in mice treated with either agent alone. Tumor weight of the combination-treated group was 23%, protein content was 18%, and DNA and RNA contents were 28% of the values in the control group.

Discussion

We have successfully established a line of human carcinoid tumor cells (BON) in athymic nude mice. When dispersed cells are injected SC in the mice, encapsulated tumors are produced that are histologically similar to the original patient tumor. BON has a stable tumor-doubling time, it stains positive for 5-HT and chromogranin A, and it has retained high-affinity gastrin receptors on the cell membrane. In addition, we have shown somatostatin receptors by autoradiography. These characteristics make BON a useful model in which to study various treatment regimens on growth of carcinoid tumors and is, to our knowledge, the first long-term human carcinoid xenograft cell line to be established in nude mice. Also, BON cells grown in tissue culture exhibit

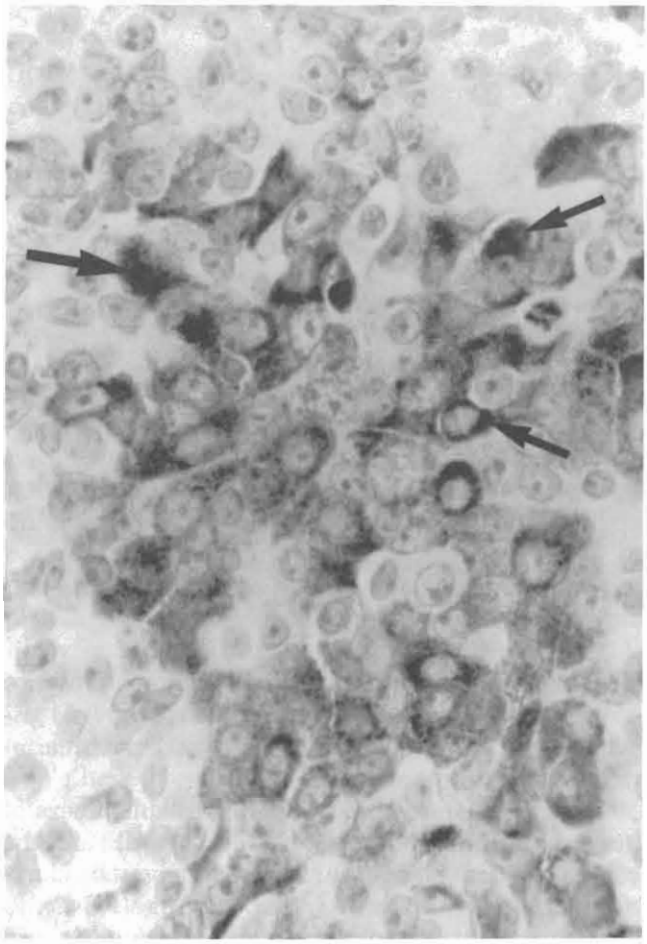

Figure 5. Light micrograph of the BON tumor stained for chromogranin A (*arrows*) (counterstained with hematoxylin; original magnification ×1000).

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.