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Development of Neuroendocrine Tumors in the Gastrointestinal Tract of Transgenic Mice

Heterogeneity of Hormone Expression

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Expression of hormones in endocrine tumors and derived cell lines of transgenic mice carrying insulin-promoted oncogenes has been investigated by histochemical, immunohistochemical, ultrastructural, and radioimmunologic means. Tumors of the pancreas, small intestine, mesentery, and liver were examined. Insulin-immunoreactive cells were prevalent in pancreatic tumors, with a significant subpopulation of pancreatic polypeptide-immunoreactive elements. Conventional ultrastructural and immunogold analysis identified insulin-storing beta granules in pancreatic tumor cells. In contrast, the largest immunoreactive subpopulation of intestinal tumors expressed secretin (53% of total cells), followed by proglucagon-related peptides (15%), glucose-dependent insulinotropic polypeptide (7%), gastrin (7%), pancreatic polypeptide (2%), neurotensin (2%), and somatostatin (1%). No detectable immunoreactivity for either insulin or serotonin was observed. Electron microscopy and immunogold labeling showed that intestinal tumor cells contained secretin-storing S-type granules. Lymph node and liver tumors contained secretin-immunoreactive cells with ultrastructural features similar to those of intestinal tumors. In addition, high levels of circulating insulinlike and secretinlike immunoreactants were detectable. Analogous hormone profiles were identified in tumor cell lines and culture media. Large T-antigen immunoreactivity was detected in all the nuclei of neoplastic cells, as well as in insulin-immunoreactive elements of non-neoplastic islets and pancreatic ducts and in some secretin-immunoreactive cells of small intestinal mucosa. These data indicate that neuroendocrine tumors arise both in beta cell and S-cell subpopulations of transgenic mice. (Am J Pathol 1990, 136:1349–1363)

Human neuroendocrine tumors include a wide spectrum of neoplasms that produce monoamines and/or peptides common to both the endocrine and nervous systems.[1] The analysis of the specific hormones they express may provide information about their anatomic origin, secretory properties, and clinical behavior.[2] Recently, animal models of neuroendocrine tumors have been derived using transgenic mice expressing oncogenes in neuroendocrine cells.[3–9] The general strategy entails the construction of a hybrid gene composed of the regulatory region from a hormone gene linked to the coding region of an oncogene. This hybrid gene is then transferred into the mouse germ line *via* microinjection of fertilized eggs and the resulting transgenic mice are able to express the oncogene in the cell types that normally express the hormone regulatory sequences.[10]

A well-characterized example is the hybrid oncogene made with the promoter of the rat insulin II gene linked to the potent viral oncogene SV40 T antigen, which results in tumorigenic transformation of pancreatic B cells in transgenic mice.[4–6] Nevertheless, unexpected patterns of expression have been reported.[7–9]

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In this study we analyze the tumors arising in transgenic mice carrying transgenes consisting of the rat insulin promoter (RIP) linked to either the SV40 early region (Tag) or to polyoma small T antigen (PyST).[11] To investigate the role of polyoma small T antigen in transformation, a lineage of transgenic mice harboring a hybrid gene linking the rat insulin promoter to polyoma small T antigen (RIP2PyST1) was created.[11] No abnormality could be detected in these mice; however when mice from RIP2-PyST1 lineage were mated with RIP1Tag2 mice[4] and offspring harboring both transgenes (double transgenics) were examined, they were found to have frequent intestinal tumors in addition to pancreatic B-cell tumors. Analysis of insulin gene expression and time course of tumor development suggested that the intestinal and pancreatic tumors arose as separate entities.[11] We applied conventional histologic methods at light and electron microscopic levels, coupled with histochemistry, immunohistochemistry, and radioimmunoassay of serum to assess the origin of tumors, their endocrine nature, and hormone profile. This analysis reports the first example of secretin-producing tumors in addition to characterizing a novel secretin-producing cell line derived from these tumors. Our final aim was to establish these transgenic mice as a possible model for human endocrine neoplasms of the gut and pancreas.

Materials and Methods

Mice

Thirteen mice were examined in detail. Eight double transgenic mice representative of the established lineage RIP1-Tag2/RIP2PyST1 (Grant et al[11]) of different ages (RIP1-Tag2/RIP2PyST1 #1, 2, 3, 4, and 7, 10 to 14 weeks old; RIP1Tag2/RIP2PyST1 #5, 6 and 8, 6 to 7 weeks old) and 5 wild type (C57B1/6J), age-matched controls were deeply anesthetized by ether inhalation, bled by cardiac puncture, and killed by cervical dislocation. Blood was centrifuged at room temperature and plasma was kept at −20°C until assayed for peptide hormones.

Light Microscopy

A complete autopsy was performed and all visceral organs were removed, fixed by immersion in Bouin's fluid for 4 to 6 hours at room temperature, and processed into paraffin wax. Serial sections (3 to 5 μm) were stained with hematoxylin and eosin for conventional histology, with periodic acid-Schiff (PAS)/alcian blue (AB) (pH 2.5) for mucin detection, and with Grimelius' silver impregnation method for argyrophil endocrine cells.[12] Immunohistochemical tests for large T antigen and a range of different endocrine cell products (Table 1) were performed using the peroxidase anti-peroxidase (PAP) method[13] or the avidin-biotinylated peroxidase complex (ABC, Vector, Burlingame, CA) method.[14] Before immunostaining for large T antigen, sections were treated with the proteolytic enzyme subtilisin (protease type XXIV, Sigma Chemicals, Poole, UK) (0.003% weight/volume in phosphate-buffered 0.15 mol/l [molar] saline, PBS, pH 7.4) for 5 to 15 minutes at room temperature.[15] Colocalization studies were performed using 3-μm serial or reverse-face sections.[16] Specificity tests for the immunostains consisted of absorption of each antiserum with its homologous antigen (10 nmol/ml), omission of the first layer, and use of control tissue with or without the pertinent antigen.[17]

Table 1. *Antisera Used in this Study*

Antiserum to	Dilution	Source
Large T antigen	1:2,000	S. Alpert, USA
Chromogranin A (rat)	1:2,000	H. Winkler, Austria
Insulin	1:5,000	Sorin Biomedica, Italy
Glucagon	1:5,000	Hammersmith Hospital, UK
Pancreatic polypeptide	1:16,000	R. Chance, USA
Somatostatin	1:10,000	RIA UK Ltd, UK
Serotonin	1:10,000	Sera Lab, UK
Glicentin	1:10,000	A. Moody, USA
GLP-I	1:2,000	Hammersmith Hospital, UK
GLP II	1:2,000	Hammersmith Hospital, UK
Secretin	1:25,000	Hammersmith Hospital, UK
GIP	1:15,000	Hammersmith Hospital, UK
Neurotensin	1:16,000	Hammersmith Hospital, UK
Gastrin/CCK	1:10,000	Hammersmith Hospital, UK
Total glucagon*	1:15,000	Hammersmith Hospital, UK
Pancreatic glucagon*	1:320,000	Hammersmith Hospital, UK
Secretin*	1:375,000	Hammersmith Hospital, UK
Insulin*	1:1,000,000	Hammersmith Hospital, UK

* Antisera used in radioimmunoassay tests only.
Anti-serotonin is a mouse monoclonal antibody; all other antisera raised in rabbit except for anti-insulin, which was raised in guinea pig.

Electron Microscopy

For ultrastructural analysis, small samples from pancreatic (n = 4) and intestinal tumors (n = 4) and from liver (n = 2) and lymph node (n = 1) metastases were fixed by immersion in glutaraldehyde (2.5% volume/volume in 0.1 mol/l phosphate buffer, pH 7.4) for 2 to 4 hours at 4°C. Tissue was further postfixed in osmium tetroxide (1% volume/volume, 0.1 mol/l phosphate buffer, pH 7.4) for 1 hour at 4° C. The specimens were then processed into Araldite. Semithin sections (0.5 to 1.0 μm) were cut from osmicated tissue and stained with toluidine blue (1% volume/volume in 3% weight/volume aqueous borax). Semithin sections (0.4 to 1.0 μm) from nonosmicated and osmicated tissue were also immunostained with PAP or ABC methods to assess the preservation of secretin after resin embedding. Areas of interest were trimmed and then sectioned (60 to 100 nm) with a Reichert ultracut E ultramicrotome (Reichert-Jung Optische Werke AG, Wien, Austria).

Ultrathin sections were collected on uncoated nickel 300 mesh grids, desiccated, counterstained with uranyl acetate and Reynold's lead citrate and then observed in a Zeiss 10 CR transmission electron microscope (Carl Zeiss, Oberkochen, West Germany). Immunogold staining was performed using gold-labeled protein A or IgG (Bio Clin, Biochemical Services, Sheffield, UK) methods.[18,19]

Radioimmunoassay

Plasma samples (10 μl) from all groups were measured in duplicate for secretin, pancreatic glucagon, total glucagon, and insulin by radioimmunoassay as previously described.[20-23]

Cell Lines

BTC-2 and STC-1 neuroendocrine cell lines derived from RIP1Tag2/RIP2PyST1 tumors[11] were harvested from 10-cm Petri dishes after trypsinization. Cells were either extracted in 0.5 mol/l acetic acid for 15 minutes at 100 C° or fixed in paraformaldehyde (4% weight/volume, 0.1 mol/l phosphate buffer, pH 7.4). Fixed cells were thoroughly washed in PBS, than processed into paraffin wax for light microscopy, and analyzed with the same procedures adopted for tissue specimens (see above). Cell extracts and culture media from both lines were assayed for peptides as described for mice plasma samples (see above).

Results

We analyzed RIP1Tag2/RIP2PyST1 double transgenic mice[11] in two age groups of younger (RIP1Tag2/RIP2-PyST1#5, 6 and 8; 6, 7 and 7 weeks of age, respectively) and older animals (RIP1Tag2/RIP2PyST1#1, 2, 3, 4, and 7; 14, 13, 13, 10 and 13 weeks of age, respectively). Ages were selected on the basis of the time course analysis;[11] we expected to find early stages of tumor progression in young mice and advanced tumors in older mice.

General Morphologic Findings

At autopsy, the three young mice showed no gross abnormalities during macroscopic inspection. Microscopic examination revealed tumor nodes in the pancreas of all three mice, in the small intestine of one (RIP1Tag2/RIP1-PyST1 #5), and no metastases in any mouse.

The five older mice presented round, pale masses in the abdomen mostly related to the pancreas, the mesentery, and the wall of the small intestine. Small, whitish spots were also detected on the surface of the livers in four of the five older mice. All the remaining organs and the central nervous system appeared to be normal. Microscopic examination revealed the intestinal and pancreatic masses to be tumor nodules, the mesenteric lymph node enlargements and the white spots on the liver surface to be nodes and nests of metastatic tumor cells. In addition, hyperplastic-dysplastic lesions were found in pancreatic islets and multiple micrometastases were detected in the single liver apparently lacking macroscopic tumor. The remaining organs were histologically normal.

Pancreas

A total of 47 tumor nodules were found in the eight organs examined. Each organ contained multiple tumors that increased in number with age (young mice, n = 9, mean value 3; older mice, n = 38, mean value 7). In all pancreata analyzed, most islets of Langerhans showed hypercellularity and some degree of cytologic atypia. Tumor growths were distinguished from islet dysplasia using the following histologic parameters: presence of cell atypia, loss of internal islet structure (eg, formation of trabeculae with pseudoglandular spaces) and increased islet size. True tumor nodules presented severe cellular atypia, complete loss of internal structure, and a minimum diameter of 0.5 mm. All lesions not showing all of the above changes reported for tumors were interpreted as dysplastic islets. Large tumors (maximum diameter, 0.6 cm) were not encapsulated with margins compressing the surrounding exocrine parenchyma. Tumor structure was either solid with or without lacunar spaces or presented a gyriform pattern. The cells often showed frank cytologic atypia with increased nucleus/cytoplasm ratio, irregular nuclei, and presence of often atypical mitoses. The whole microscopic picture resembled that described for RIP1Tag2 transgenic mice pancreata.[4-6]

Grimelius' silver impregnation method[12] revealed the expected slight argyrophilia, with clear brown staining of the central, insulin-producing B cells and deep brown/black color of the cells at the periphery in both normal and dysplastic islets. The tumors were composed of weakly argyrophilic cells with a prevalent tan, clear/brown stain typical of insulin-producing B cells (data not shown).

In the infrequent normal islets, immunohistochemical analysis revealed the expected proportions of A (glucagon-producing), B (insulin-producing), D (somatostatin-producing), and PP (pancreatic polypeptide-producing) cells.[24] The dysplastic islets revealed a complete derangement of these proportions and were mainly composed of B cells. A few immunoreactive cells for these four peptides were also detected in the wall of small and large ducts. All the tumors were composed mainly of insulin-immunoreactive cells (Figure 1a) and a consistent subpopulation of PP-immunoreactive cells were detected

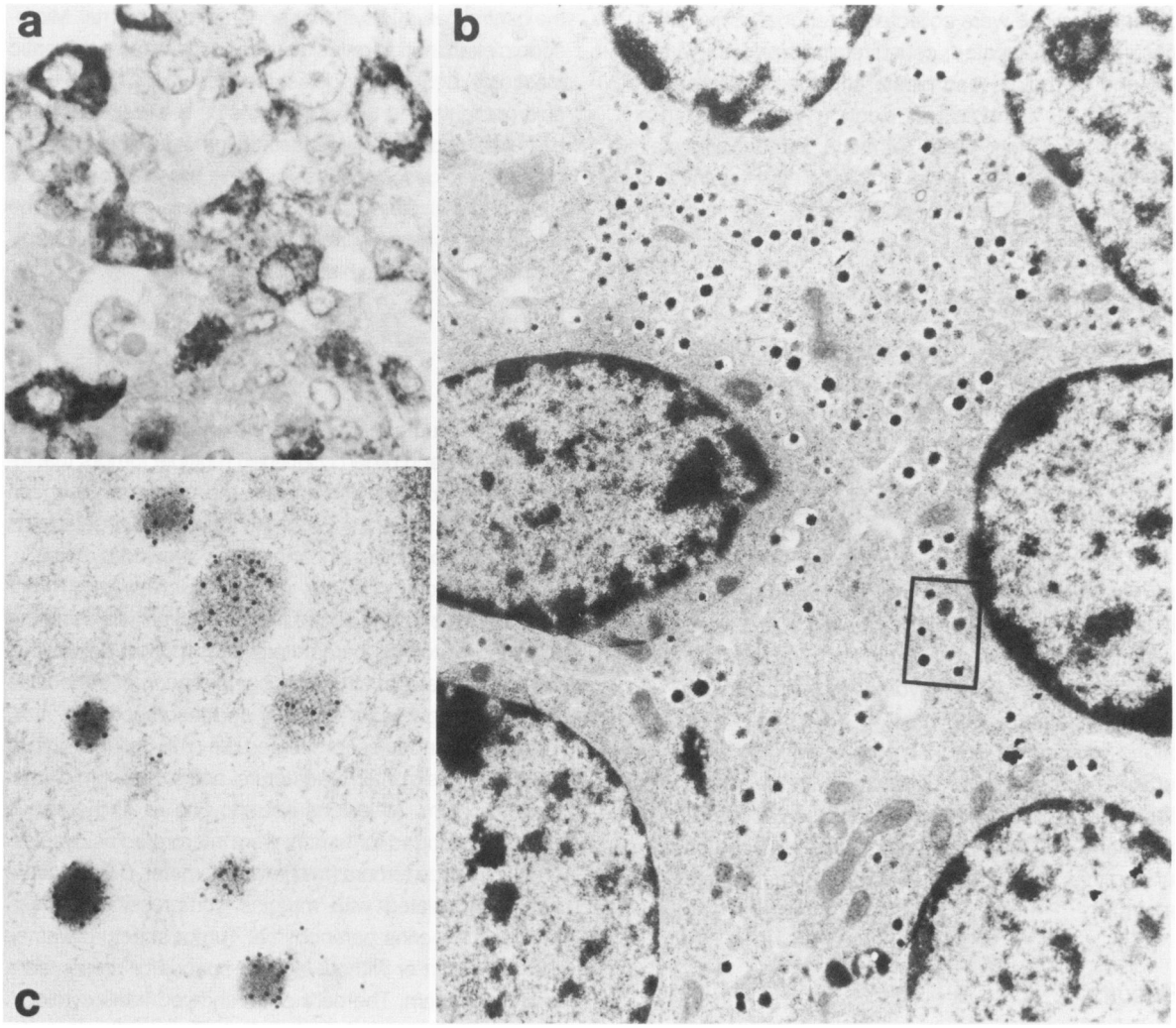

Figure 1. *RIP1Tag2/RIP2PyST1 pancreas tumors.* **a:** *RIP1Tag2/RIP2PyST1 #4. Variable insulin immunoreactivity in the cells of a large tumor. PAP method, hematoxylin counterstain, ×900.* **b** *and* **c:** *RIP1Tag2/RIP2PyST1 # 7. Immunoelectron microscopy preparations with anti-insulin. General view of a pancreas tumor.* **b:** *Note the typical, haloed, electron-dense granules that are heavily labeled with gold particles (10 nm diameter).* **c:** *Enlargement of the square in* (b) *to show the gold labeling. Protein A gold method, osmium postfixed sample, uranyl acetate, and lead citrate counterstain, ×10,000* (b), *×62,000* (c).

in 20 tumors (42%). A few somatostatin- or glucagon-immunoreactive cells also were found sometimes at the periphery of some growths. Rare secretin-immunoreactive cells were detected in 3 of 47 tumors tested (RIP1Tag2/RIP2PyST1 #2, 4, and 7). All tumor cells showed large T-antigen immunoreactivity, while it was present in only the insulin-immunoreactive elements of islets and ducts.

Electron microscopy was performed on samples taken from tumors of four older mice (RIP1Tag2/RIP2-PyST1 #1, 2, 3, and 7). All were mainly composed of cells showing typical round, medium-sized (mean diameter, 190 nm) mature B granules of two major forms[25,26]: haloed and with a dense core, sometimes geometrical in shape; and less frequently, with a scarcely electron-dense, pale core. All granules reacted strongly with anti-insulin antibodies using immunogold methods. The gold particles were detected mainly in the dense part of these granules (Figure 1b and c). Some cells appeared to be less granulated, sometimes presenting a variable degree of cytoplasmic engulfment by round, enlarged mitochondria (oncocytoid pattern). A few multilamellar bodies were also observed.

Intestine

Seventy-two small intestinal tumor nodes from the five older mice were analyzed along with a single intramucosal growth from one young animal (RIP1Tag2/RIP2PyST1 #5). The whole range of proliferative patterns were observed from very early intramucosal lesions to large invasive tumors (Grant et al[11]). Initial lesions were small nests

of neoplastic cells located within the lamina propria of the villi, either filling this space with variable effacement of the normal structure of the villi (expansive growth pattern) or surrounding the crypts and superficially infiltrating the muscularis propria (infiltrative growth pattern). Large tumors had an umbilicated gross appearance, often reducing the size of the intestinal lumen. Microscopically, this corresponded to complete eradication of the villous pattern of the mucosa. The neoplastic masses bulged into the lumen and were covered by the thin, sometimes ulcerated mucosal layer. All levels of infiltration were seen, with larger masses dissecting the muscle layers to various extents, often reaching the serosal surface. Very frequently, groups of neoplastic cells were seen inside lymph and blood vessels, and Peyer's patches. The infiltration *via* the lymphatics sometimes extended a considerable distance from the primary lesion. The tumors most frequently were either ribbonlike or solid with formation of pseudoglandular spaces. The stroma was scanty and, when present, highly vascularized. High-grade cytologic atypia was a common feature, with abnormal, enlarged nuclei, prominent nucleoli, and spotted chromatin. Mitoses were frequent and often atypical. No significant lesion such as epithelial dysplasia could be found either in the normal mucosa surrounding the tumors or at a distance from them. There was no apparent inflammatory infiltrate near the neoplasms.

The tumor cells did not stain with PAS/AB (data not shown), indicating a lack of mucus secretion, but retained strong positivity for the Grimelius' silver method, thereby revealing their endocrine nature. Further evidence of endocrine differentiation was provided by the diffuse positivity to antibodies to chromogranin A (rat), a general marker of endocrine granules.[16,27-30] Serial sections from 73 intestinal tumors of six mice were immunostained with a panel of antisera to gastrointestinal hormones (Table 1). The number and pattern of distribution of immunoreactive cells in each section was determined (Table 2). The tumors expressed multiple hormones, up to a maximum of eight peptides in the same growth, although a distinct hormone profile emerged. Overall the most abundant immunoreactive subpopulation expressed secretin (53% total cells), followed by glicentin (10%), glucose-dependent insulinotropic polypeptide (7%), gastrin (7%), glucagonlike polypeptide I (5%), pancreatic polypeptide (2%), neurotensin (2%), and somatostatin (1%). There was no detectable immunoreactivity to either insulin or serotonin. Notably, the single tumor observed in the young mouse RIP1Tag2/RIP2PyST1 expressed 80% secretin immunoreactivity, 1% pancreatic polypeptide, and no other hormones. In 37 of 73 (51%) tumors, 66% to 100% of the cells were immunoreactive for the above peptide antisera; in 14 (19%), 33% to 66% were immunoreacitve, while in 23 (30%) neoplasms only 1% to 33% of the total number

Table 2. *Hormone Immunoreactivities in Serial Sections of Intestinal Tumors*

Mouse		SEC	GLI	GIP	GAS	GLP	NT	PP	SOM	5HT	INS
Young											
RIP1Tag2/RIP2PYST1 #5	Tumors	1/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
	cells, mean %	80						1			
Old											
RIP1Tag2/RIP2PyST1 #1	Tumors	9/9	8/8*	7/9	4/9	8/9	1/9	4/9	9/9	0/9	0/9
	cells, mean %	53	11.5	10	1	10	1	1	2		
RIP1Tag2/RIP2PyST1 #2	Tumors	12/14	11/12*	2/14	5/14	10/14	3/14	1/14	8/14	0/14	0/14
	cells, mean %	50	9	7.5	10	2	4	5	1.5		
RIP1Tag2/RIP2PyST1 #3	Tumors	19/21	14/20*	15/20*	16/21	15/21	12/20*	9/21	20/21	0/21	0/20*
	cells, mean %	28	9	11	15	8	1	1	1		
RIP1Tag2/RIP2PyST1 #4	Tumors	12/12	6/9*	9/10*	10/12	7/12	10/12	8/12	10/12	0/12	0/10*
	cells, mean %	61	16	10	15	8	3	1	2		
RIP1Tag2/RIP2PyST1 #7	Tumors	13/16	7/10*	4/13*	1/16	1/16	4/13*	2/16	15/16	0/15*	0/13*
	cells, mean %	44	13	1	1	1	1	1	1		
	Total tumors	66/73	46/67	37/67	36/73	41/73	30/69	25/73	62/73	0/71	0/67
	Total cells, mean %	53	10	7	7	5	2	2	1		

SEC, secretin; GLI, glucagonlike polypeptide I; GIP, glucose-dependent insulinotropic polypeptide; GAS, gastrin-CCK; GLP, glucagonlike polypeptide; NT, neurotensin; SOM, somatostatin; PP, pancreatic polypeptide; 5HT, serotonin; INS, insulin; tumors, neoplasms tested; cells, mean %, mean % of positive cells for each tumor; *the number of tumors tested is reduced due to the lack of smaller growths in the last sections.

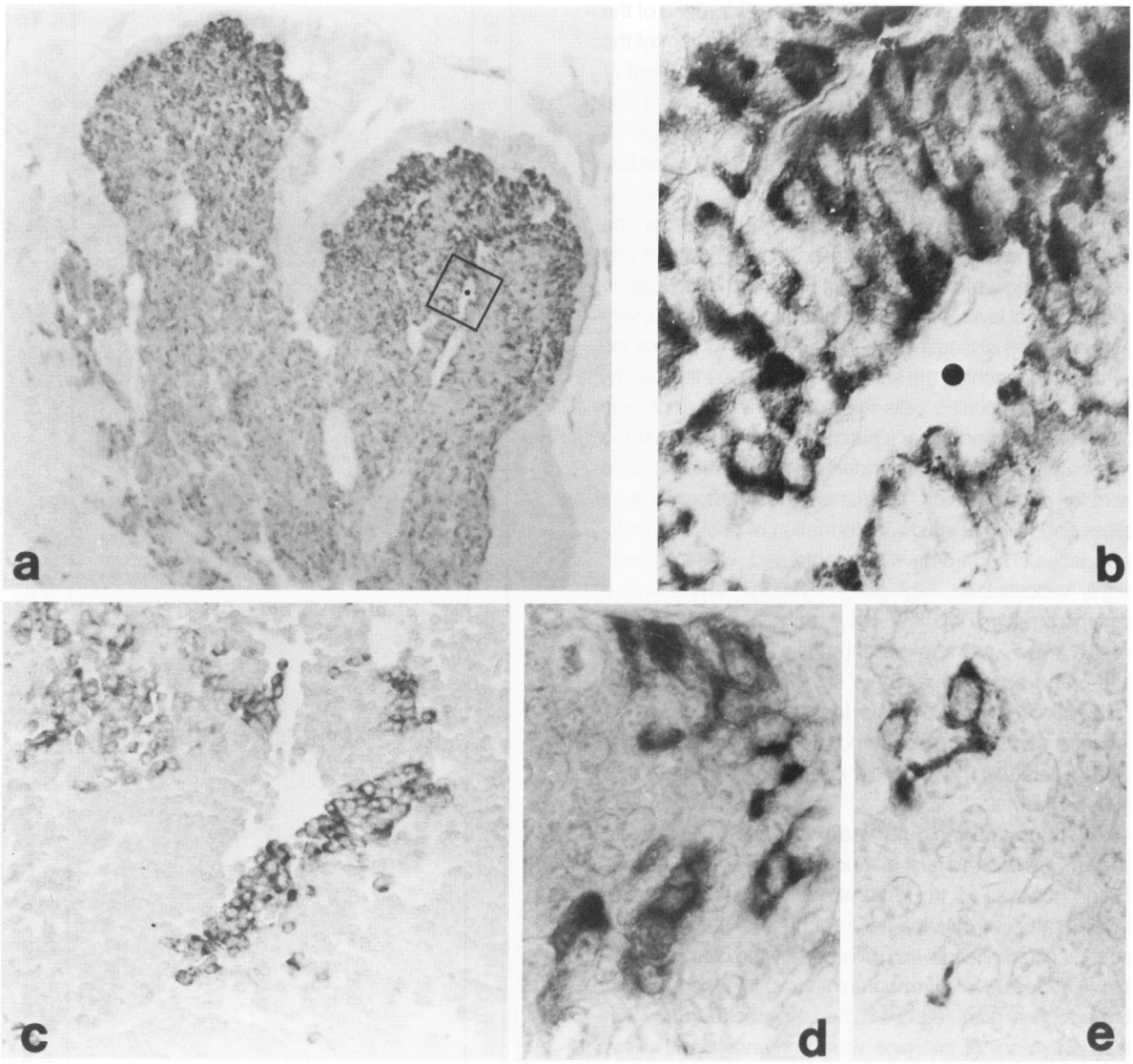

Figure 2. *Intestinal tumors, immunohistochemical findings.* **a–d:** *RIP1Tag2/RIP2PyST1 #2* **(a, b),** *#3* **(c)** *and #4* **(d, e).** *Immunoreactivity for secretin* **(a, b),** *glicentin* **(c),** *gastrin* **(d),** *and somatostatin* **(e)** *in advanced tumors. Highly differentiated secretin cells are the major tumor population in the intramucosal part of intestinal neoplasms* **(a);** **(b)** *= higher magnification of the square in* **(a),** *(the black dot is in the middle of the same pseudoglandular space) to show the strong immunoreaction. Cells positive for glicentin* **(c),** *more frequently, and gastrin* **(d),** *when present, are isolated cell clusters denoting a clonal alternative state of endocrine differentiation, in respect to the majority of the surrounding S-type cells. Somatostatin cells* **(e)** *are prevalently single, isolated elements: note the typical cytoplasmic elongations. ABC method, hematoxylin counterstain* **(c–e),** *× 100* **(a),** *×300* **(c),** *×900* **(b, d, e).**

of tumor cells reacted positively. Strongest immunoreaction was generally observed in intramucosal tumors or in the submucosal part of large masses, with a progressive reduction of the staining density in the lower layers and a net increase of nonimmunoreactive elements. This pattern was typically observed with the secretin antiserum (Figure 2a and b). The overall distribution of hormone immunoreactivity in intestinal tumors of older mice showed two distinct types. In general, secretin and the different parts of the proglucagon molecule[31] (Figure 2c) were expressed in large clusters of cells, unlike the more scattered distribution of cells immunoreactive to other hormone antisera (Figure 2d). Typically, somatostatin-immunoreactive elements could be found as strongly stained, isolated cells (Figure 2e). The antihormone sera used in this study (Table 1) proved to be specific in immunoabsorption tests with homologous and related peptide fragments (10 nmol/ml diluted antibody) on normal and tumor samples (Table 3).

Large T-antigen immunoreactivity was detected in the nuclei of all tumor cells and in few scattered mucosal cells of the small intestine[11] in all the older mice and in two of the three young mice (RIP1Tag2/RIP2PyST1 #5 and #6). Mucosal T-antigen–positive cells appeared to be located

Table 3. *Results of Absorption Tests for Immunohistochemistry*

Antiserum to	Glucagon	GLP I	GLP II	Secretin	GIP
Glucagon	−	+	+	+	+
GLP I	+	−	+	+	+
GLP II	+	+	−	+	+
Secretin	+	+	+	−	+
GIP	+	+	+	+	−
Somatostatin	+	+	+	+	+
			Antigen		

All absorption tests were performed using 10 nmoles of antigen/ml of optimally diluted antibody on samples of normal gut mucosa and tumor from RIP1Tag2/RIP2PyST #1 transgenic mouse. + immunostaining not affected; − immunostaining fully quenched.

mainly in the median upper part of the crypts and on the villi and did not react when counterstained with PAS/AB (data not shown), thus suggesting that they did not store mucous substances. Analysis of conventional serial or reverse-face paired sections showed that some mucosal T-antigen–positive cells could be stained for rat chromogranin A (Figure 3a and b), thus indicating their endocrine nature, and also for secretin (Figure 3c and d). However this analysis did not reveal reactivity for sera against the proglucagon fragments in these cells. Only a small proportion of the chromogranin A- or secretin-immunoreactive cells bore large T-antigen immunoreactivity. No obvious atypia was detected in such cells, nor in large T-antigen unreactive endocrine cells.

Electron microscopy was performed on samples taken from large masses belonging to four older mice (Figure 4a to c). The tumors appeared to be composed of cells with a variable number of electron-dense, thin-haloed, targetlike endocrine granules (mean diameter, 170 nm). Some granules with irregular size and shape were also observed. Most of the cells seemed to have lost secretion polarity, with granules partially distributed along the cytoplasmic membrane. Some showed a very low number of granules, sometimes presenting large, round mitochondria engulfing the cytoplasm (oncocytoid pattern). Multilamellar bodies were also present. Immunohistochemical tests performed on osmicated and nonosmicated specimens with sera against secretin permitted

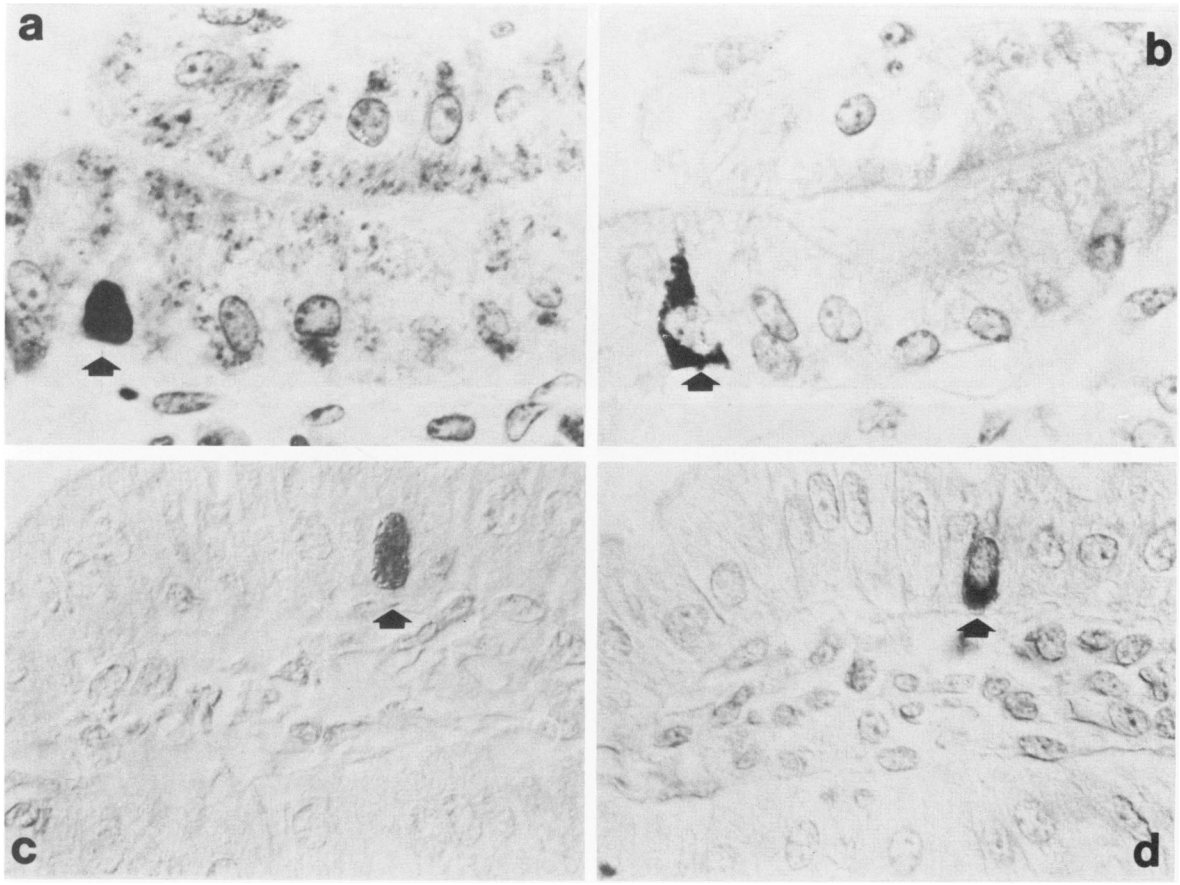

Figure 3. *Intestinal mucosal cells that express the transgene.* **a** *and* **b**: *RIP1Tag2/RIP2PyST1 #4. Nuclear large T-antigen immunoreactivity* **(a)** *and cytoplasmic immunoreactivity for rat chromogranin A* **(b)** *in the same mucosal cell. The arrow indicates the basal membrane of the cell.* **c** *and* **d**: *RIP1Tag2/RIP2PyST1 #4. Nuclear large T-antigen immunoreactivity* **(c)** *and cytoplasmic immunoreactivity for secretin* **(d)** *in the same mucosal cell. The arrow indicates the basal membrane of the cell. Reverse face, consecutive sections, ABC method, hematoxylin counterstain, ×900* **(a–d)**.

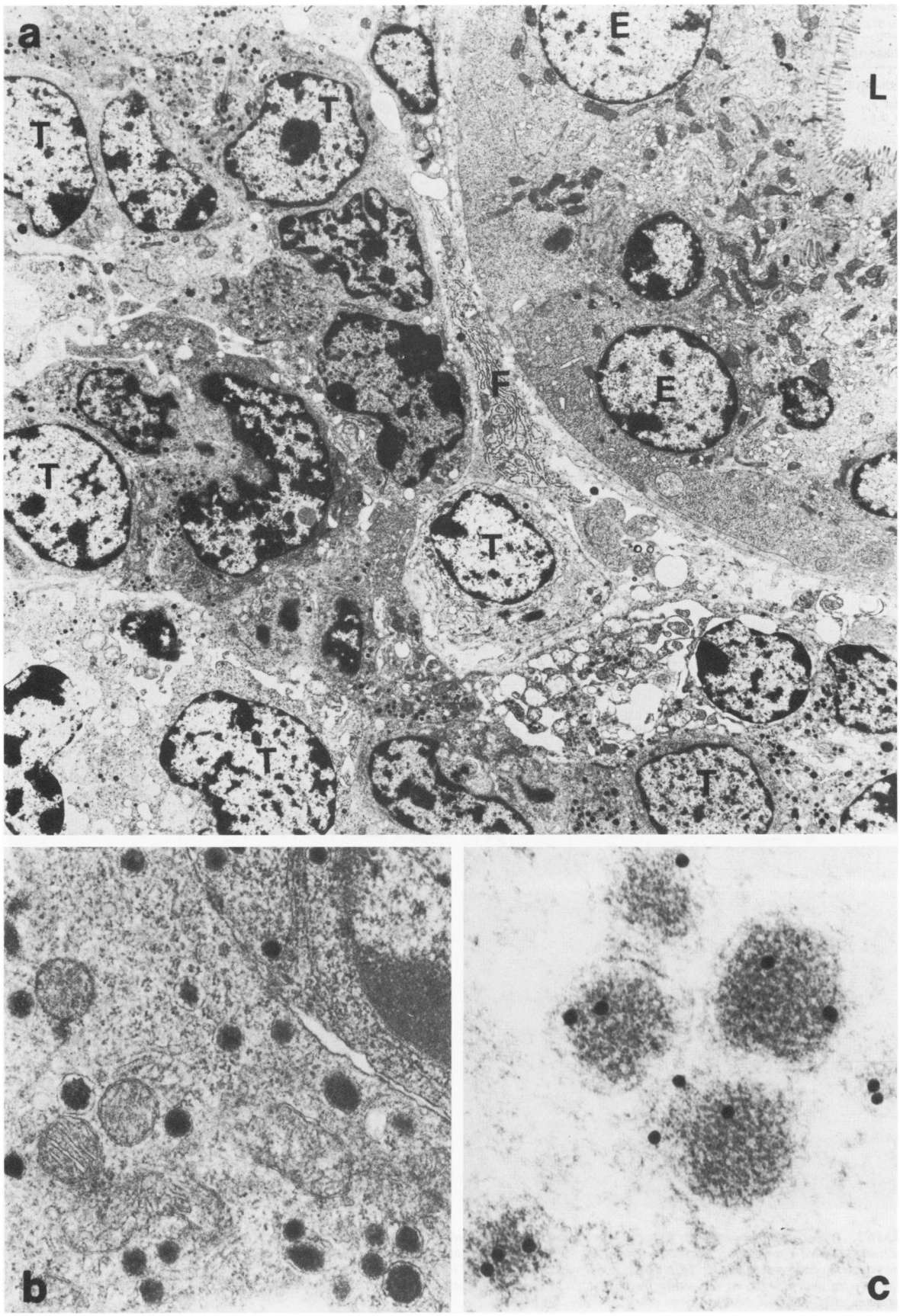
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Figure 4. *Intestinal tumors ultrastructure.* a: *RIP1Tag2/RIP2PyST1 #7. General view of tumor cells infiltrating the lamina propria of the small intestine. Note in the upper right corner part of a crypt (L, lumen; E, normal enterocytes), and cytoplasmic bundles of a fibroblast (F) near the basal membrane of an enterocyte. Tumor cells (some of them are labeled T on the nucleus) show a fairly variable number of electron-dense endocrine granules. Osmium postfixed sample, uranyl acetate and lead citrate counterstain, ×3200.* b: *RIP1Tag2/RIP2PyST1 #4. Morphology of the electron-dense granules. Note the different diameters and the constant presence of a narrow halo. Osmium postfixed, uranyl acetate and lead citrate counterstain, ×35,000.* c: *RIP1Tag2/RIP2PyST1 #3. Immunogold (15 nm diameter gold particles) labeling for secretin in a tumor cell. IGGS method on osmium postfixed samples, ×160,000.*

a specific labeling of these granules (Figure 4c). All these features, together with positive immunohistochemical tests, suggested that these tumors were mainly composed of secretin-producing (S-type) cells.[32,33]

Mesenteric Lymph Nodes and Liver Tumors

Eighteen mesenteric lymph nodes with tumors from four of the older transgenic mice (RIP1Tag2/RIP2PyST1 #1, 2, 3 and 7) and 57 liver nodes from all older mice were analyzed with hematoxylin and eosin, Grimelius' silver impregnation method,[12] and with sera against large T antigen, secretin, and insulin. The proliferations sometimes effaced the entire lymph node structure or comprised single or small nests of cells, often arranged in a ribbonlike structure in the liver. Multiple liver localizations were a common finding, sometimes embolizing the vascular tree in a miliary fashion (eg, RIP1Tag2/RIP2PyST1 #7, liver tumors, n = 36) (Grant et al[11]). All of the growths in both anatomic sites were composed of strongly argyrophilic, large T-antigen–immunoreactive cells (Figure 5a). Seventeen of eighteen lymph node tumors and 57 of 58 liver tumors were immunostained with anti-secretin serum (Figure 5b). Insulin immunoreactivity was detected in only one liver tumor (mouse RIP1Tag2/RIP2PsST1#7) and in no lymph nodes. This liver tumor may have originated from primary insulin-immunoreactive pancreatic tumors also found in the same mouse (see above). One lymph node growth failed to react with either serum (RIP1Tag2/RIP2-PyST1 #3).

Samples taken from one enlarged lymph node (RIP1-Tag2/RIP2PyST1 #7) and from white foci in the livers of two mice (RIP1Tag2/RIP2PyST1 #2 and 7) were analyzed with electron microscopy and were composed of cells with the same ultrastructural features as those described for the S-type intestinal tumors (Figure 5c).

With one possible exception in the liver, the above data taken as a whole suggested that the intestinal tumors were the prevalent site of origin of the lymph nodes and liver tumors.

Plasma Sample Assays

All radioimmunologic data are detailed in Table 4. Plasma samples taken from seven (the three young and four of the older) transgenic mice (RIP1Tag2/RIP2PyST1 #2 to 8) and five age-matched controls (C57B1/6J) were assayed for insulin, secretin, and pancreatic glucagon. In transgenic mice, the insulinlike circulating immunoreactant was elevated approximately threefold in young mice (controls: mean value, 100 pmol/l; young transgenic mice: mean value, 380 pmol/l), and fivefold in older (transgenic mice: mean value, 580 pmol/l).

The secretinlike immunoreactant was below the assay detection limit in controls in all three young transgenic mice and in one of the older transgenic mice (RIP1Tag2/RIP2PyST1#4), but was significantly elevated in the three older transgenic mice assayed (Table 4). All mice with increased circulating secretinlike immunoreactant had extensive tumor disease of the small intestine with prevalent secretin immunoreactivity (compare Table 2).

Circulating pancreatic glucagonlike immunoreactants showed no significant difference between the two groups (controls: mean value, 128 pmol/l; transgenic mice: mean value, 150 pmol/l).

Tumor Cell Lines

We studied the cell lines derived from RIP1Tag2/RIP2-PyST1 pancreatic (BTC-5) and intestinal (STC-1) tumors.[11] Paraffin-embedded cells of both lines were positive for the Grimelius' silver impregnation method[12] and for antibodies to rat chromogranin A, thus indicating their endocrine properties. In BTC-5 line, immunohistochemical tests for hormones were strongly positive for insulin in the majority of the cells and a few scattered elements could also be stained for secretin and glucagon. In the STC-1 line, a high proportion of cells immunoreactive for both sera against secretin and the different parts of the proglucagon molecule (GLP I, GLPII, glicentin and glucagon)[31] were found (Figure 6a and b). In addition, scattered rare cells reacted to neurotensin and PP antisera. No immunoreactivity was detected for insulin antibodies in the STC-1 cells.

Radioimmunoassays of culture medium and cell extracts (estimated number of cells per 10 cm Petri dish = 10^6) indicated that the BTC-5 cell line synthesizes large amounts of insulinlike immunoreactant, and low but detectable amounts of secretinlike and pancreatic glucagonlike immunoreactants (Table 4). Total glucagonlike im-

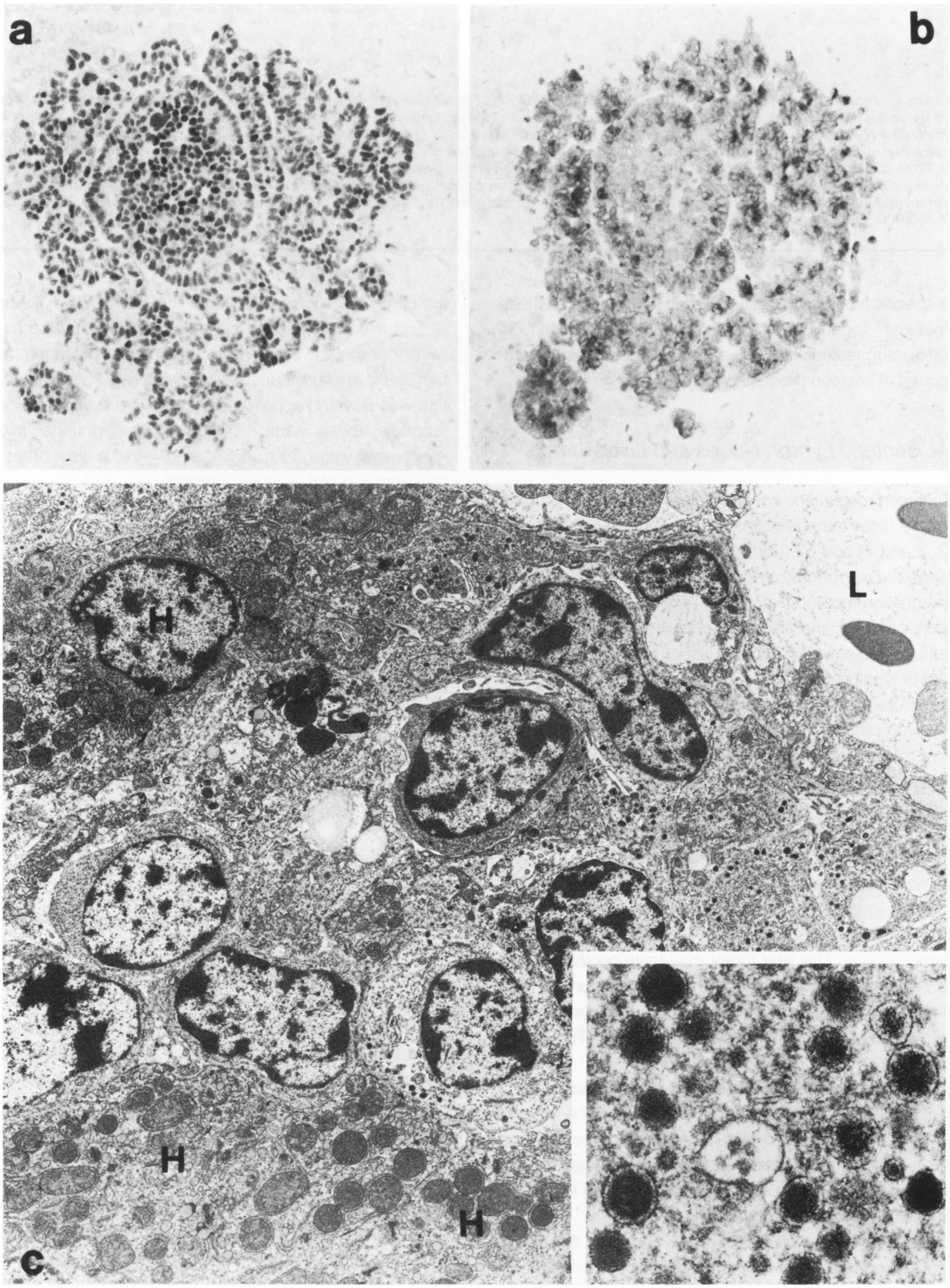

Figure 5. *Liver metastases.* a *and* b: *RIP1Tag2/RIP2PyST1 #4. Nuclear large T antigen* (a) *and cytoplasmic secretin immunoreactivity* (b) *in a liver metastasis. Note that all the neoplastic nuclei appear positive for T antigen, while the cytoplasmic immunoreaction for secretin shows a variable distribution. No immunoreactivity is detectable in any surrounding hepatocyte for either sera. ABC method, hematoxylin counterstain, ×400.* c: *RIP1Tag2/RIP2PyST1 #3. Electron micrograph of a liver metastasis. A group of cells showing electron-dense endocrine granules in the cytoplasm infiltrate the space between some hepatocytes (H) and a sinusoid (L, lumen). The morphology of the granules appear to be the same as in the small intestinal tumors (insert; compare with Figure 4b). Note the variability in the number of the granules per cell. Osmium postfixed sample, uranyl acetate, lead citrate counterstain, ×4300; insert ×40,000.*

Table 4. *Radioimmunoassay Data*

Sample	PT	IT		INS	SEC	PG
6 to 7-Week-old mice						
RIP1Tag2/RIP2PyST1 #5	3	1	Plasma	130	<8	60
RIP1Tag2/RIP2PyST1 #6	5	0	Plasma	970	<8	110
RIP1Tag2/RIP2PyST1 #8	1	0	Plasma	30	<8	110
			Mean	380	<8	90
10 to 14-Week-old mice						
RIP1Tag2/RIP2PyST1 #2	4	14	Plasma	890	140	150
RIP1Tag2/RIP2PyST1 #3	5	21	Plasma	430	1003	160
RIP1Tag2/RIP2PyST1 #4	10	12	Plasma	320	<8	180
RIP1Tag2/RIP2PyST1 #7	9	16	Plasma	700	400	140
			Mean	580	510	160
Controls						
C57B1/6J #1	0	0	Plasma	30	<8	110
C57B1/6J #2	0	0	Plasma	30	<8	150
C57B1/6J #3	0	0	Plasma	90	<8	120
C57B1/6J #4	0	0	Plasma	20	<8	170
C57B1/6J #5	0	0	Plasma	390	<8	90
			Mean	110	<8	130
Cell lines						
BTC-5 cells	NA	NA	Extract	>1000	600	300
BTC-5 cells	NA	NA	Medium	>1000	<8	40
STC-1 cells	NA	NA	Extract	30	3700	1980
STC-1 cells	NA	NA	Medium	30	600	400

All RIA values expressed in pmol/l except for cell lines (fmol/ml, media; fmol/10^6 cells, cell extracts). PT, number of pancreatic tumors; IT, number of intestinal tumors; INS, insulinlike immunoreactivity; SEC, secretinlike immunoreactivity; PG, pancreatic glucagonlike immunoreactivity; NA, not applicable. Cell lines samples were from one 10-cm Petri dish at 90% confidence.

munoreactant was tested, revealing low values (cell media, 100 fmol/ml; cell extracts, 350 fmol/10^6 cells). In contrast, high levels of secretinlike, total glucagonlike (cell media, more than 5000 fmol/ml; cell extracts, more than 5000 fmol/10^6 cells), and no insulinlike immunoreactants were detected (Table 4) in STC-1 cell line.

The above data, taken as a whole, suggest that both BTC-5 and STC-1 cells retained the hormone expression properties of the tumors from which they derived.

Discussion

We have studied neuroendocrine tumors, plasma samples, and tumor-derived cell lines from transgenic mice carrying insulin-promoted oncogenes[11] to further characterize the ontogeny and endocrine properties of the tumors. The tumors of the pancreas in RIP1Tag2/RIP2-PyST1 transgenic mice show features previously reported for transgenic mice carrying the insulin promoter linked to

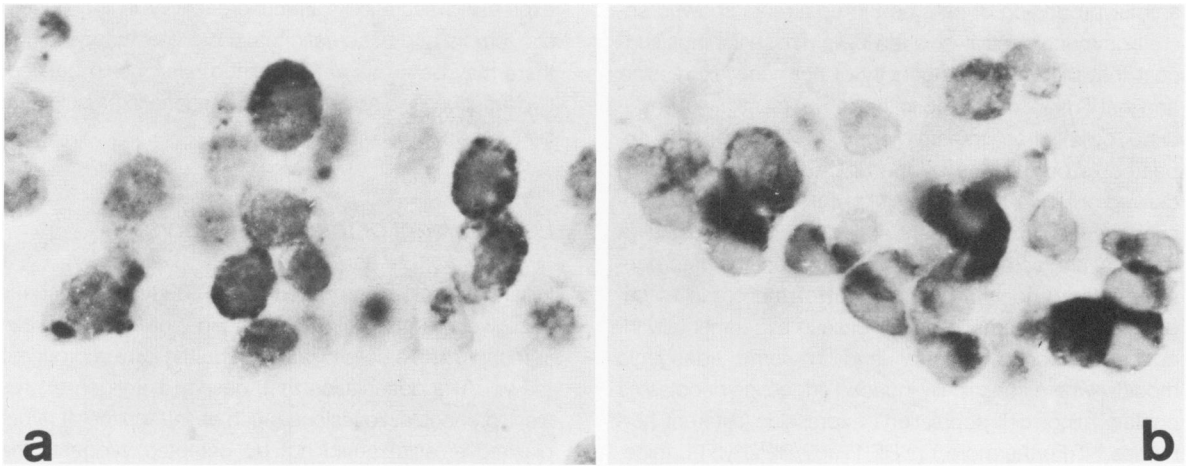

Figure 6. *Tumor cell lines immunohistochemical findings.* **a** *and* **b**: *STC-1 intestinal tumor-derived cell line (see text): epithelioid clusters of secretin-*(**a**) *and glicentin-*(**b**) *immunoreactive cells. ABC method, hematoxylin counterstain, ×1100.*

the SV40 early region genes.[4-6] In fact, pancreatic tumors from both transgenic lineages arise from insulin-producing B cells expressing the SV40 oncoprotein large T antigen and are the source of nonphysiologic serum insulin levels. Our data corroborate the observation that the tumors of the small intestine do not express insulin and provide evidence to confirm the suggestion that they are derived from an intestine mucosal neuroendocrine cell.[10] In fact, secretin was found to be the major hormone expressed by the intestinal tumor cells, which contained secretin-storing S-type granules.[32,33] Large amounts of secretinlike immunoreactant were also detected in plasma samples of three of four older transgenic mice having many intestinal tumors. In addition, a few apparently normal mucosal cells, in young as well as in older mice, were immunoreactive for large T antigen. Some of them contained chromogranin A (a general endocrine marker),[16,27-30] with secretin as their specific product. None of the other peptides tested for with the adopted panel of antisera were detected. This may suggest that a well-differentiated endocrine cell producing secretin (S-type cell) is the original target of transgene expression in the gut of RIP1Tag2/RIP2PyST1 mice. It also indirectly explains the fact that the neoplasms were restricted to the small intestine, the only part of the rodent gastrointestinal tract that contains secretin cells.[34,35] Together these data furnish evidence proving that the intestinal tumors are a separate entity from the pancreatic tumors.

Heterogeneity of Hormone Expression

In the intestinal tumors, we have identified six more endocrine cell types (Table 2), occasionally representing the majority of the neoplastic population, in addition to secretin-producing S cells. Similarly, in the pancreas 20 of 43 investigated tumors expressed pancreatic-polypeptide in a small proportion of cells and three tumors showed secretin immunoreactivity in a few cells. These findings suggest that there is heterogeneity of hormone expression and that it might be related to the known plasticity of endocrine cells when transformed.[36-38] This property appeared to be retained by the BTC-5 and STC-1 tumor-derived cell lines. Heterogeneity of cells composing endocrine tumors is a frequent feature, if not the rule in humans, and sometimes appears to reflect the heterogeneity of the endocrine component of the organ of origin.[2,39,40] This seems to be the case in experimentally induced endocrine tumors[41] and in some transgenic models, where specifically induced endocrine neoplasms contain minor cell populations expressing different hormones.[4-8] Furthermore, in RIP1Tag2/RIP2PyST1 mice, the panel of antisera we used showed that all intestinal tumors had a variable proportion of nonimmunoreactive cells and in one third of them most neoplastic elements could not be characterized. Although the presence of possible other or unknown hormonal products cannot dismissed, these findings can be interpreted as expression of two possible phenomena: 1) progressive loss of differentiation due to the transforming events inside the cell (consistent with the observation that the total percentage of cells immunoreactive to all endocrine markers diminishes with tumor growth); 2) and/or increased release of the endocrine product due to imbalance of physiologic control inside the transformed cells. We have observed that the T-antigen–positive mucosal neuroendocrine cells only express secretin (and not other tested hormones), and also that the spectrum of hormones expressed apparently increases with tumor progression. This suggests that the proliferating intestinal neuroendocrine cell is able to switch to multiple alternative differentiated states, a quality ascribed to stem cells of the intestinal epithelium.[42-45]

It is surprising that in RIP1Tag2/RIP2PyST1 the regulatory sequences of rat insulin II gene induce transformation in the secretin cell, an epithelial, endocrine element anatomically and functionally quite distinct from the insulin-producing B cell. Nevertheless, the pancreas and upper gut have a common embryologic origin[46,47] and share some endocrine cells in adulthood (eg, somatostatin cells).[48] Graft experiments in chick embryos[49] also showed that during embryologic development, the pancreas and upper gut express a largely overlapping hormone profile of enteroendocrine cells, with insulin elements in gut structures and secretin cells in pancreas. Furthermore, in humans, endocrine tumors of pancreas and gut share several types of hormones: it has been reported that insulin, glucagon, and PP can be produced by intestinal tumors,[15,50-52] while gut-type hormones like gastrin, neurotensin, and serotonin have been reported in pancreatic growths.[39,53,54] Our finding that there are cells expressing secretinlike immunoreactivity in the pancreatic tumors and pancreatic tumor cell lines suggests that there may be an as yet undefined relationship between two functionally distinct sites of transgene expression, the beta cell and the S cell.

Development of Intestinal Tumors

Initial intestinal lesions in RIP1Tag2/RIP2PyST1 transgenic mice were nests of large T-antigen–positive,[11] secretin-immunoreactive cells located inside the lamina propria of the villi. The observation that, despite T-antigen expression, preneoplastic lesions (such as intraepithelial hyperplasia-dysplasia) could not be detected suggests that proliferating enteroendocrine cells move away from their epithelial context, revealing notable intramucosal mobility,

which is a quality peculiar to endocrine cells during embryologic development.[55] Similarly, in humans, preneoplastic lesions of intestinal endocrine cells have not been described as an independent pathologic entity nor have they been associated with endocrine tumors.[56]

Tumors in Lymph Nodes and Liver

On the basis of morphology, time course, and insulin gene expression, Grant et al[11] propose that the mesenteric lymph node and liver tumors are metastases from the intestinal neoplasms. In this study, 17 of 18 lymph nodes and 57 of 58 liver tumors were immunostained with anti-secretin serum and showed the same ultrastructural features described for the intestinal tumors (Figure 5). This supports the evidence that these lesions are metastases from the intestinal neoplasms. Insulin expression was found in no lymph nodes and in only 1 of 58 liver tumors, and therefore the latter tumor is probably a metastasis of pancreatic origin. This apparent low frequency of insulin expression in liver tumors has been observed in other insulin-SV40 transgenic mice that develop beta cell tumors (S. Grant and D. Hanahan, unpublished data).

This paper provides evidence that the neuroendocrine tumors of the RIP1Tag2/RIP2PyST1 transgenic mice arise independently of pancreatic beta cells and intestinal S cells. The intestinal tumors are the first recorded examples of functioning secretin-producing tumors, secretinomas, and, together with the secretin-producing cell line (STC-1), provide novel tools for studying this hormone. Furthermore, RIP1Tag2/RIP2PyST1 transgenic mice represent the first animal model available for studying oncogenesis in the neuroendocrine system of the mammalian intestine. The heterogeneity of gut hormone expression with tumor progression indicates a common origin for gut neuroendocrine cells and a possible relationship with pancreatic islets.

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