Early Invasiveness Characterizes Metastatic Carcinoid Tumors in Transgenic Mice

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The conversion of a normal cell into a metastatic tumor is thought to occur in a stepwise progression of genetic changes that affect both growth control and interactions with the extracellular environment. The development of invasiveness allows tumor cells to escape from their primary site. We have investigated transgenic mice that develop both invasive intestinal neuroendocrine tumors and noninvasive tumors of the pancreatic β-cells. Visual inspection and gene expression studies indicate that the β-cell tumors rarely metastasize. In contrast, intestinal tumors that first appear in submucosal areas metastasize with high frequency to the lymph nodes and liver. No evidence of preneoplastic mucosal lesions was seen in the intestine, indicating that invasiveness is acquired early in the tumorigenic progression of these cells. Comparison of intestinal and pancreatic neuroendocrine tumors in transgenic mice suggests that an early requirement for invasiveness may contribute to metastatic potential.

INTRODUCTION

Genetic changes that allow a normal cell to develop into an invasive and metastatic tumor appear to be fundamental to the process of tumor progression. It is increasingly evident that tumor phenotypes develop by way of irreversible qualitative changes in one or more characters, such as proliferation, invasiveness, and the ability to disseminate (1-4). The expression of viral and cellular oncogenes and the inactivation of tumor suppressors have been clearly implicated in the control of tumor cell proliferation (5-11). In many cases, there are either historical or genetic indications that additional changes underlie the development of invasiveness and the ability to metastasize (12-15). Moreover, the local extracellular environment may be important in selecting tumor cell subpopulations that have acquired the capacity to invade and disseminate (16, 17).

Analysis of transgenic mice that express oncogenes in different tissues has revealed that both cell type and oncogene type influence the tumor phenotype (for reviews, see Refs. 18 and 19). It is notable that metastasis is infrequent in most transgenic mouse tumorigenesis models (20-22), possibly because oncogene expression selectively promotes proliferative steps in tumor progression. Tumors in transgenic mice expressing oncogenes often progress from benign hyperplasia to development of a vascular tumor, while both invasiveness and metastasis are later rare events (18). This pattern of tumor progression is characteristic of some human tumors such as mammary tumors and intestinal adenocarcinomas. Carcinoids of the gut, however, are highly metastatic and are often not presaged by detectable preneoplastic lesions (23).

We have used transgenic mice harboring a hybrid gene construct linking the rat insulin promoter, which drives expression in pancreatic β-cells, to the SV40 early region encoding the potent oncogene large Tag. Three different lineages of mice carrying the same transgene integrated at different chromosomal sites develop tumors of the β-cells (24). We recently observed that mice of one of these lineages (RIP1Tag2) also developed metastatic intestinal tumors at a low but reproducible frequency. The incidence of the intestinal tumors in RIP1Tag2 mice could be increased by breeding these mice to transgenic mice of one lineage carrying a polyoma small T antigen gene linked to the rat insulin promoter (RIP2PyST lineage). These mice have been analyzed to document two tumorigenesis pathways in transgenic mice expressing SV40 Tag that contrast in metastatic characteristics.

MATERIALS AND METHODS

Gene Constructs and Generation of Transgenic Mice. The insulin promoter Py small T antigen gene (RIP2PyST) was made by replacing the SV40 Tag gene of RIP2Tag with a Clal-BamHI restriction fragment from Pxl3ST containing the PyST cDNA sequences. A Clal linker was used to change the XbaI site of RIP2Tag to a Clal site, and the Clal-BamHI fragment containing 458 base pairs (−450 to +8) of the rat insulin II promoter and plasmid backbone was linked to the PyST cDNA Clal-BamHI restriction fragment (Py numbering +152 to +4632). Transgenic mice were generated by standard protocols as described previously (25, 26).

Histology and Immunocytochemistry. Organs were collected for histology by fixation in 10% formaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. For immunocytochemistry, mice were perfused with fresh 4% paraformaldehyde. Organs were excised and placed in 4% paraformaldehyde for 1 h and then mounted in embedding medium (M-1; Lipshaw). Ten-μm sections were cut using a cryostat-microtome (Hacker Instruments 5030) and processed by a modification of the method of Alpert et al. (27). Sections were permeabilized in 0.25% Triton X-100 and blocked with 5% fetal calf serum, 3% goat serum, 1% bovine serum albumin, 0.3% H2O2, and 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.2) in Dulbecco’s modified Eagle’s medium. Sections were reacted with rabbit polyclonal antisera to SV40 Tag (the gift of S. Alpert) and a goat anti-rabbit horseradish peroxidase-conjugated second antibody (Accurate, Westbury, NY). All antibody incubations were in the blocking solution without bovine serum albumin and H2O2. Visualization was accomplished by reaction with diaminobenzidine as described (28).

RNA Analysis. Total cellular RNA was prepared from frozen tissues or cells using RNAzolTM (Cinna/Biotex). Total RNA (10 μg/lane) was loaded onto a horizontal formaldehyde-1.2% agarose gel. Transfer to nitrocellulose by capillary blotting, prehybridization, hybridization, preparation of 32P-labeled nick-translated DNA probes, filter washing, and autoradiography was as described previously (29, 30). The probes were inserts derived from the following plasmids: the mouse insulin I cDNA; BamHI-EcoRI of pMINIC/pKS (31); and the chicken β-actin 2-kilobase HindIII fragment of pA1 (32).

Cell Lines and Transplants. Intestinal and pancreatic tumors excised from RIP1Tag2/RIP2PyST1 mice were washed with saline, minced

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4 The abbreviations used are: Tag, tumor antigen; Py, polyoma; ST, small tumor antigen; RIP2, rat insulin promoter II; cDNA, complementary DNA.
5 D. Hanahan, unpublished observations.
with scissors, and injected s.c. and i.p. as small clumps into B6D2F1 mice. The method of establishing β-cell lines from transgenic mice is described in detail by Efrat et al. (33) and was used to derive both pancreatic and intestinal neuroendocrine cell lines. The following established cell lines were made: pancreatic β-cell; BTC-3 (RIP1Tag2), BTC-4, and BTC-5 (RIP1Tag2/RIP2PyST1); and intestinal cell line STC-1 (RIP1Tag2/RIP2PyST1, same mouse as BTC-5). All cell lines grow as epithelioid-like clusters adherent to plastic, with a doubling time of approximately 2–3 days.

RESULTS

Description of Phenotypes and Time Course. Mice of the RIP1Tag2 lineage, carrying the insulin-promoted SV40 Tag gene, develop pancreatic tumors at a faster rate than do other lines carrying the same gene (24). In the present study intestinal tumors were found in a small proportion of the RIP1Tag2 mice (Fig. 1B) and never in mice of other lineages (data not shown). These observations suggest that intestinal tumors in RIP1Tag2 mice are specific to this lineage and may relate to the chromosomal location of transgene integration.

As part of an analysis of the effects of murine Py virus genes on β-cells, one of us (V. L. B.) generated transgenic mice containing the insulin promoter linked to the Py virus ST cDNA (designated RIP2PyST). PyST is a protein of unknown function encoded by the Py early region (34), and we hypothesized that targeted expression of the gene in mice would help elucidate its function (35). Three lineages of RIP2PyST transgenic mice with the transgene integrated at different chromosomal positions were analyzed, and none developed any detectable pathology. We bred mice from these lineages with RIP1Tag2 lineage mice to generate doubly transgenic mice, and one of these combinations, RIP1Tag2/RIP2PyST1, developed intestinal tumors at a much higher incidence and sooner than did siblings that carried only the RIP1Tag2 transgene (Fig. 1B).

Intestinal tumors were first visible macroscopically (Fig. 2B) at 10 weeks of age in RIP1Tag2/RIP2PyST1 double transgenic mice and at 13 weeks of age in RIP1Tag2 mice (Fig. 1B). The incidence of intestinal tumors in RIP1Tag2/RIP2PyST1 double transgenic mice increased with age, and by 14 weeks, every mouse had intestinal tumors (Fig. 1B). Coincident with the increased incidence of intestinal tumors was an increase in mesenteric lymph node and liver tumors (Fig. 1, C and D; Fig. 2, B and C). One explanation for the correlation in incidence of intestinal, mesenteric, and liver tumors is that they were related by metastatic spread of primary intestinal tumors to mesenteric lymph nodes and liver. Pancreatic tumors were visible (Fig. 2A) at 8 weeks of age in both RIP1Tag2 and RIP1Tag2/RIP2PyST1 transgenic mice, and there was no difference in the incidence of pancreatic tumors between these mice (Fig. 1A). Both the pancreatic tumors and the intestinal

A. PANCREAS

B. INTESTINE

C. MESENTERY

D. LIVER

Fig. 1. Histogram of tumor frequency in different organs. Transgenic mice of the RIP1Tag2 lineage (a) and RIP1Tag2/RIP2PyST1 double transgenics (b) were sacrificed at weekly intervals and examined for the presence of macroscopic tumors. Ordinate, total number of mice examined; □, number of mice with visible tumors. Male RIP1Tag2/RIP2PyST1 double transgenic mice were mated with female B6D2F1 nontransgenic mice, and the offspring were analyzed by Southern blot analysis of genomic DNA (29). Litters contained the expected numbers of RIP1Tag2/RIP2PyST1 double transgenic mice, RIP1Tag2 mice, RIP2PyST mice, and nontransgenic mice. Where possible, siblings were compared for tumor formation to minimize genetic background effects.

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tumors were transplantable to s.c. sites in histocompatible mice (data not shown).

Genetic and biochemical experiments have failed to document expression of the PyST transgene in extrapancreatic tumors, although low levels of PyST RNA were detected in pancreatic tumors of RIP1Tag2/RIP2PyST1 mice (data not shown). The failure to detect PyST expression in the extrapancreatic tumors by sensitive RNA protection analysis is consistent with the finding that two independent RIP2PyST lineages failed to influence intestinal tumor incidence. Visual examination at necropsy of two mice each of the genotypes RIP1Tag2/RIP2PyST4 or RIP1Tag2/RIP2PyST5 showed no evidence of extrapancreatic pathology. These data suggest that the increased incidence of intestinal tumors was not strictly dependent upon expression of the transgene in RIP2PyST1 mice, and the phenomenon may be mediated by the chromosomal integration site of the transgene in the RIP2PyST1 lineage.

Macroscopic and Microscopic Pathology. To understand the origins of the extrapancreatic tumors and their relationship to the pancreatic tumors, we examined tumors from all sites both macroscopically and microscopically at different ages. The first abnormality detected by histological examination of the intestine was seen at 6 weeks of age in RIP1Tag2/RIP2PyST1 double transgenic mice. Collections of normal appearing tissue were observed within the lamina propria of intestinal villi, beneath an apparently intact intestinal epithelium (Fig. 1C). These collections of cells could expand to fill the villus and invade the submucosal structures of the intestine wall by 12 weeks of age (Fig. 2B). The large vascularized masses protruding into the peritoneal cavity (Fig. 2B) resulted from these lesions as well as infiltration of the Peyer’s patches by tumor cells. The lymphatic channels that drain the intestine contained tumor cells (Fig. 2C), and the mesenteric lymph node typically became filled with tumor cells by 12 weeks of age (Figs. 1C and 2B).

Livers of older RIP1Tag2/RIP2PyST1 double transgenic mice frequently contained tumors. Macroscopically, these tumors appeared as large vascularized lesions randomly dispersed throughout the organ. More typically, the liver contained numerous small (a few millimeters in diameter) white foci (Fig. 2C). Histologically both types of lesion were collections of tumor cells (Fig. 3D). The smallest liver tumors were localized within the venules that receive blood from the intestine via the portal vein. This pattern implies that tumor cells have metastasized to the liver by hematogenous spread from the intestinal tumors. Tumors in other organs have not been detected.

The histology of pancreatic, intestinal, lymph node, and liver tumors revealed that each has a morphology characteristic of neuroendocrine carcinomas (Fig. 3, E and F). The intestinal tumor cells, however, had a more malignant appearance and more mitotic figures than did the pancreatic tumor cells (Fig. 3, E and F). There was no obvious difference in morphology between tumors from the same site in RIP1Tag2/RIP2PyST1 double transgenic and RIP1Tag2 transgenic mice, even though the incidence of the extrapancreatic tumors was increased in RIP1Tag2/RIP2PyST1 double transgenic mice.

In contrast to the marked effects on the temporal development of the intestinal tumors, the pattern of tumorigenesis in the pancreas appeared equivalent in RIP1Tag2/RIP2PyST1 double and RIP1Tag2 single transgenic mice. The ß-cell tumors of RIP1Tag2 mice arise from a hyperplastic state, are noninvasive, and infrequently metastasize (24, 27, 28, 36).Because RIP1Tag2 mice develop highly metastatic intestinal tumors with low frequency, it was difficult to determine the precise
Fig. 3. Microscopic pathology of tissues from 12-week-old RIP1Tag2/RIP2PyST1 double transgenic mice. A, cluster of intestinal neuroendocrine tumor cells in the core of a villus. x 100. B, intestinal neuroendocrine carcinoma. Top, lumen of the intestine; bottom, tumor mass with invasion through the muscularis. x 20. C, nest of neuroendocrine carcinoma cells within the lumen of a mesenteric lymphatic vessel. x 100. D, metastatic neuroendocrine carcinoma (small dark staining cells) in liver. x 50. E and F, higher magnification (x 300) of the cells populating an islet tumor in the pancreas (E) and an intestinal neuroendocrine carcinoma that shows extensive mitotic figures (F).

frequency of β-cell metastases. Several other RIPTag lineages that do not develop intestinal tumors, however, had a β-cell tumor metastasis rate of less than 5% (percentage of mice with metastases at death).7

Gene Expression. The relationship of the intestinal tumors to the pancreatic tumors was further investigated by examining gene expression in tumors and tumor-derived cell lines. Cell lines were generated from both pancreatic and intestinal tumors of RIP1Tag2/RIP2PyST1 mice. Although the pancreatic and intestinal tumors have different metastatic behavior in vivo,

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Fig. 4. Evaluation of insulin mRNA expression. Total cellular RNA (10 μg/ lane) was analyzed by Northern blot analysis. Top, 0.66-kilobase bands that hybridize with a mouse insulin I cDNA probe; bottom, 2.2-kilobase bands that hybridize with a chicken β-actin cDNA probe. Lane 1, RIP1Tag2 pancreatic tumor; Lane 2, RIP1Tag2[RIP2PyST1 pancreatic tumor; Lane 3, RIP1Tag2/RIP2PyST1 intestinal tumor; Lane 4, RIP1Tag2/RIP2PyST1 mesenteric lymph node tumor; Lane 5, RIP1Tag2 pancreatic tumor cell line (BTC-3); Lane 6, RIP1Tag2/RIP2PyST1 pancreatic tumor cell line (BTC-5); Lane 7, RIP1Tag2/RIP2PyST1 intestinal tumor cell line (STC-1).

their in vitro cell morphology was similar. The intestinal and the pancreatic cell lines differed in their pattern of gene expression (see below), and they maintained the expression of neuropeptide markers seen in their respective in vivo tumors (37).

RNA was isolated from pancreatic, intestinal, and mesenteric tumors as well as from tumor-derived cell lines. High levels of insulin mRNA were detected by Northern blot analysis in every pancreatic tumor and at lower levels in the pancreatic tumor cell lines of RIP1Tag2 mice and RIP1Tag2/RIP2PyST1 double transgenic mice (Fig. 4). In contrast to the pancreatic tumor material, insulin mRNA was not detectable in intestinal tumors, mesenteric lymph node tumors, or in an intestinal tumor cell line (STC-1). Immunohistochemical analysis with anti-insulin antiserum showed that the extrapancreatic tumors do not express insulin protein (data not shown).

The observation that the endogenous insulin genes were expressed in β-cell tumors but not in intestinal, mesenteric, or liver tumors supports the conclusion that the extrapancreatic tumors arose independently from the pancreatic tumors. The lack of expression of the endogenous insulin genes in cells expressing an insulin-promoter-driven transgene was unexpected but not unprecedented, since RIP1Tag2 lineage mice express SV40 Tag in both embryonic neurons and endocrine progenitor cells, neither of which express detectable insulin (27).

To follow the ontogeny of intestinal tumors, the expression of SV40 Tag was documented. Immunohistochemistry, using antisera specific for SV40 Tag, revealed that the intestinal

Fig. 5. Immunohistochemical analysis for SV40 T-antigen of tissues from 14.5-week-old RIP1Tag2/RIP2PyST1 double transgenic mice. A, cross-section of intestine, with a stained cluster of cells occupying the core of a villus. x 50. B, single stained intestinal epithelial cell. x 250. C, stained tumor cells in the liver. x 50. Expression of SV40 Tag was confirmed by both protein and mRNA analysis of pancreatic, intestine, and mesenteric lymph node tumors. The protein analysis showed that both SV40 large Tag and SV40 small Tag were present (data not shown).
epithelium contained SV40 Tag-immunoreactive cells that were usually found as single cells or pairs of staining cells (Fig. 5A, inset). The abnormal collections of cells within the villus were also immunoreactive for SV40 Tag (Fig. 5A), as were the large invasive intestinal tumors (Fig. 5B), mesenteric lymph node tumors, and liver tumors (Fig. 5C). The only SV40 Tag-positive cells that were considered histologically normal were found in the intestinal epithelium, which suggests that the intestinal tumors originated from these epithelial cells.

DISCUSSION

The characterization of these transgenic mice has revealed evidence for two distinct tumorigenesis pathways. The first results in the development of encapsulated β-cell tumors of the pancreas that metastasize infrequently. The second pathway involves neuroendocrine cells of the intestine and is characterized by the development of invasive carcinomas that metastasize with high frequency to lymph nodes and liver. The events of the second pathway are accelerated in transgenic mice carrying two transgenic loci. The lineage-specific nature of the interaction in crosses with other insulin promoter-SV40 Tag and insulin promoter-PyST lineages carrying the same gene indicates that the chromosomal position of the locus is important in both cases. Since expression of SV40 Tag is completely correlated with tumorigenesis, the chromosomal position of the RIP2Tag2 locus may result in the aberrant expression of the transgene in intestinal neuroendocrine cells. This hypothesis is supported by the finding that endogenous insulin gene expression is not detectable in the intestinal cells or tumors.

In contrast, the lack of detectable PyST expression in the intestinal tumors suggests that the influence of the RIP2PyST1 locus is independent of PyST expression, although a role for PyST antigen is not excluded. The RIP2PyST1 locus accelerates intestinal tumor formation in a dominant manner at the level of the phenotype. The genetic lesion probably affects a cellular gene and may either be dominant, such as insertional activation of a gene, or recessive, such as insertional inactivation with predisposition to loss of the second allele of a gene. Although transgenic mice heterozygous for the RIP2PyST1 locus show no detectable phenotype, preliminary evidence indicates that RIP2PyST1 homozygotes are inviable and supports the model of insertional inactivation. The molecular basis for the observed synergism in tumor development is not known, but the relevant area of mouse genomic DNA is amenable to cloning because it is “tagged” by the RIP2PyST transgene (38).

The distinct nature of the two pathways is indicated by both histological analysis and gene expression studies. The intestinal tumors appear to be more aggressive and invasive than the β-cell tumors, and the latter lesions are the only tumors to express detectable levels of endogenous insulin. The hypothesis that Tag-positive histologically normal cells of the intestinal mucosa give rise to the intestinal tumors is supported by a neuroendocrine marker analysis (37) showing that these epithelial SV40 Tag-immunoreactive cells express chromogranin A and secretin (intestinal neuroendocrine cell markers), as do the intestinal, mesenteric, and liver tumor cells. The fact that both neuroendocrine tumors express the SV40 large T oncoprotein indicates that other factors determine the specific characteristics of each tumorigenesis pathway. One clear difference between the two tumor phenotypes is invasiveness, which is a property of every intestinal tumor. The apparent origin of the intestinal tumors from neuroendocrine cells within the intestinal epithelium suggests that invasion across the basement membrane is essential to the development of these tumors. The intestinal epithelium is a layer of absorptive cells with a few interspersed neuroendocrine cells (39). Some of these neuroendocrine cells express SV40 Tag in the transgenic mice and were seen as single cells or pairs of cells. Larger clusters of Tag-positive neuroendocrine cells, however, were detected only in the intestinal villus on the other side of the basement membrane. Since neuroendocrine cells are not normally observed within the core of the villus, it would appear that a transformed epithelial neuroendocrine cell cannot give rise to a proliferating tumor mass unless it has invaded through the basement membrane. Thus the rate-limiting step in this pathway of tumor progression may be the acquisition of invasiveness by Tag-positive neuroendocrine cells. In contrast, invasion is apparently not required for pancreatic β-cell tumorigenesis which occurs in a different cellular environment.

An early requirement for invasiveness in the intestinal tumorigenesis pathway may contribute to the metastatic potential of intestinal tumors. Invasion of tumor cells into lymphatic channels and blood vessels provides an opportunity for tumor cells to travel to local lymph nodes and liver. The rapid appearance of lymph node and liver metastases subsequent to intestinal tumor development indicates that these tumor cells have acquired invasive and metastatic potential coincidentally. However, invasiveness is also a feature of fibrosarcoma development in transgenic mice carrying the bovine papillomavirus genome (40), yet these tumors do not metastasize with discernible frequency. Comparison of these observations implies that invasiveness is not the only requirement for metastasis and that additional components of the metastatic phenotype exist.

The neuroendocrine tumors of these transgenic mice bear a clear resemblance to neuroendocrine tumors observed in humans. Carcinoid tumors are among the most common tumors of the small intestine. The tumors arise from the epithelium of the small intestine and lack a preneoplastic intraepithelial stage, but they invade the villus and metastasize to local lymph nodes and liver (23). Like the tumors in the transgenic mice, human carcinoid tumors have a predilection for metastasis to the liver, with minimal metastatic disease elsewhere. A wide range of hormones are synthesized by both human carcinoid tumors and transgenic mouse intestinal tumors (37). Like their counterparts in transgenic mice, the majority of human β-cell tumors are noninvasive and metastasize infrequently (23).

Transgenic mice that develop invasive and metastatic carcinomas present a new model to study the genetic and epigenetic mechanisms of invasion and metastasis. A variety of cellular changes have been implicated in invasion and metastasis, including changes in expression of collagens, proteases, growth factors, oncogenes, antioncogenes, histocompatibility antigens, and cell-type-specific adhesion molecules (11–15). Our studies indicate that invasiveness can be a discrete property that appears at different stages in multistep tumorigenesis pathways and that the point at which invasiveness is elaborated may dramatically influence the subsequent characteristics of the cancer.

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