Site-Specific Epithelial-Mesenchymal Interactions in Digestive Neuroendocrine Tumors

An Experimental in Vivo and in Vitro Study

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Little is known about the functional interactions between digestive neuroendocrine tumor cells and their stromal microenvironment. The focus of our study is whether mesenchymal cells modulate peptide expression, cell proliferation, and invasiveness in digestive neuroendocrine tumor cells. We designed an experimental *in vivo* and *in vitro* study using the mouse enteroendocrine cell line STC-1. *In vivo*, STC-1 cells were injected subcutaneously in 18 immunosuppressed newborn rats. At day 21, all animals presented poorly differentiated neuroendocrine tumors with lung metastases. Subcutaneous tumors were usually limited by a capsule containing basement membrane components and myofibroblasts that presented a low mitotic index. Lung tumors were devoid of capsule and poor in myofibroblasts, and their mitotic index was high. The profile of peptide expression in STC-1 tumors was different from that of cultured STC-1 cells. *In vitro*, STC-1 cells were cultured with fibroblasts of different origins, including dermis, lung, digestive tract, and liver. Based on their origin, myofibroblasts differentially modulated hormone synthesis, proliferation, spreading, and adhesion of STC-1 cells. In conclusion, our results show that site-specific functional interactions between mesenchymal and neuroendocrine cells may contribute to modulating the behavior of digestive neuroendocrine tumors, depending on their growth site. (*Am J Pathol* 2000, 156:671–683)

work of capillary vessels embedded in variable amounts of extracellular matrix.^{1,2,3} In many instances, the stroma of neuroendocrine tumors is formed by a delicate connective matrix, intimately associated with tumor cells. However, in some tumors, such as midgut carcinoids, the amount of extracellular matrix may be very high, resulting in a so-called desmoplastic appearance. Histopathological studies have shown that neuroendocrine tumor cells are able to synthesize several growth factors known to promote mesenchymal cell migration and proliferation and to stimulate extracellular-matrix synthesis and deposition.^{4–11} *In vitro* studies have confirmed that some neuroendocrine cell lines, such as the BON cell line, may induce cell proliferation and extracellular matrix protein synthesis in fibroblastic cells.^{12,13} It is, therefore, likely that neuroendocrine tumor cells regulate stromal cell proliferation and activity through paracrine interactions.

The possible reciprocal influence of stromal cells on the growth and differentiation of neuroendocrine tumor cells has received little attention. Clinical and experimental data show that, in various types of cancer, mesenchymal cells are able to modulate the proliferative activity, the invasive and metastatic properties, and the differentiation state of neoplastic cells. It can therefore be hypothesized that, as for other types of tumors, the biological characteristics of digestive neuroendocrine tumors are modulated by mesenchymally derived factors. This hypothesis is supported by embryological¹⁴ and experimental^{15,16} data, showing the role of extracellular matrix proteins and mesenchymal factors in the normal differentiation process of digestive neuroendocrine cells. Moreover, tissue-specific mesenchymal influences may help to explain the differences in hormone content, stromal characteristics, and local behavior observed between primary neuroendocrine tumors originating from the different segments of the digestive tract (foregut,

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Gastrointestinal neuroendocrine tumors contain a distinctive fibrovascular stroma, characterized by a dense net-

Table 1. Antibodies Used in the Study

Reactivity	mAb or pAb*	Origin	Application (working dilution)
Chromogranin A	Rabbit pAb	Incstar BioRad, Ivrysur Seine (France)	IH (1:100)
Vimentin	Mouse mAb clone V9	Dako, Glostrup (Denmark)	IC (1:25)
α -Smooth muscle actin	Mouse mAb	Sigma, St Louis, MO	IH, IC (1:400)
Rat PECAM-1	Mouse mAb	R&D Systems, Oxon (UK)	IH (1:500)
Laminin-1	Rabbit pAb 1219	JC Lissitzky, INSERM U387, Marseille (France)	IH, IC (1:100)
Type IV collagen	Rabbit pAb	Chemicon, Temecula, CA (USA)	IH, IC (1:100)
Cholecystokinin	Rabbit pAb	Novocastra, Nottingham (UK)	IH (1:100)
Glucagon	Rabbit pAb	Novocastra, Nottingham (UK)	IH (1:1000)
Somatostatin	Rabbit pAb (56D)	Our laboratory (23)	IH (1:500) RIA (1:250000)
Cholecystokinin	Rabbit pAb (39A)	Our laboratory (24)	RIA (1:300000)
Truncated glucagon-like peptide	Rabbit pAb (199D)	Our laboratory (25)	RIA (1:250000)

Antibodies used for immunohistochemistry (IH), immunocytochemistry (IC) and radioimmunoassay (RIA).

*mAb, monoclonal antibody; pAb, polyclonal antibody.

midgut, hindgut), and between the primary and secondary lesions of the same tumors. Recent experimental evidence has underlined the marked functional differences existing between fibroblasts originating from the various segments of the digestive tract.¹⁷ In turn, gut-associated fibroblasts are functionally different from the various organ-specific mesenchymal cell subsets identified so far, such as those of the liver¹⁸ and the lung,¹⁹ which represent the most frequent metastatic sites of human digestive neuroendocrine tumors. Such site-specific differences in their mesenchymal environment may contribute to modulating the behavior of neuroendocrine tumor cells.

To test these hypotheses, we 1) evaluated whether mesenchymal cells may modulate the hormone content, cell proliferative activity, and invasive capacities of digestive neuroendocrine tumor cells and 2) searched for site-specific differences in mesenchymal interactions with digestive neuroendocrine tumor cells. To address our questions, we designed an experimental *in vivo* and *in vitro* study using the enteroendocrine mouse cell line STC-1.²⁰

Materials and Methods

Cell Lines

The intestinal STC-1 plurihormonal cell line, a gift from Dr. D. Hanahan through the courtesy of Dr. A. Leiter (New England Medical Center, Boston, MA), is derived from an endocrine tumor that developed in the small intestine of a double transgenic mouse expressing the rat insulin promoter linked to the simian virus 40 large-T antigen and to the polyomavirus small-t antigen, respectively.²⁰ The standard culture medium consisted of Dulbecco's modified Eagle's Medium (DMEM) supplemented with 5% fetal calf serum (FCS), 2 mmol/L glutamine, and antibiotics (100 U/ml penicillin plus 50 mmol/L streptomycin).

Mesenchyme-derived intestinal cell lines (MICs), a gift from M Plateroti, Institut National de la Santé et de la

Recherche Médicale (INSERM) U381, Strasbourg, France, were isolated from 8-day postnatal rats. Clonal cell lines that were derived from mixed subepithelial fibroblast parental cell lines were characterized as myofibroblasts: MIC 101-1, MIC-219, and MIC-316, respectively, from jejunum, ileum, and colon.¹⁷ All of these cell lines were maintained in DMEM supplemented with 10% FCS, 2 mmol/L glutamine, antibiotics, and 0.25 U/ml insulin. All cultures were done in a humidified 8% CO₂/92% air incubator at 37°C.

Antibodies

The antibodies used in this study are listed in Table 1.

In Vivo Experimental Study

Xenografting

STC-1 cells in exponential growth were detached by trypsinization. They were suspended in culture medium, counted, centrifuged (10 minutes, 1500 rpm), and resuspended in phosphate buffered saline (PBS). Eighteen Wistar newborn rats received a subcutaneous injection in the abdominal region of 1.2×10^6 cells suspended in 100 μ l of PBS. All of the rats were subsequently immunosuppressed by dorsal subcutaneous injections of antithymocyte serum²¹ on days 0, 2, 7, and 14 and maintained in a specific pathogen-free environment throughout the experiment. Three weeks after cell inoculation, subcutaneous tumors and lung metastases were counted, excised, and processed for morphological, immunohistochemical, and biochemical analyses.

Morphological Analysis

Tissue Processing: Tissue samples were divided into three parts. The first part was processed for light-microscopical examination. Tissue samples were fixed in formalin and embedded in paraffin. Three- μ m-thick sections were stained with hematoxylin and eosin.

Another part of the tissue samples was processed for ultrastructural examination. Tissue samples were fixed with 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer for 60 minutes. These samples were subsequently rinsed overnight in the same buffer, then postfixed with 1% osmium tetroxide. After dehydration in graded ethanols and propylene oxide, they were embedded in epoxy resin. Ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and examined with a Jeol 100 CX 2 electron microscope (JEOL, Tokyo, Japan). The remaining tissue samples were immediately snap-frozen in Freon that had been prechilled in liquid nitrogen.

Immunohistochemistry: For detection of cell markers and extracellular-matrix proteins, an indirect immunofluorescence technique was applied to cryostat sections of frozen tissue. Briefly, acetone-fixed 5- μ m-thick cryostat sections were incubated sequentially, with the primary antibody diluted in PBS, then with polyclonal fluorescein isothiocyanate-conjugated species-specific anti-mouse or -rabbit immunoglobulin antibodies (Immunotech, Marseille-Luminy, France).

For detection of endogenous peptides, a streptavidin-biotin-peroxidase technique was applied to sections of formalin-fixed, paraffin-embedded tissue. As a brief description of the procedure, 3- μ m-thick sections were allowed to dry on silane-coated slides. After rehydration, they were incubated for 60 minutes with the primary antibody at the appropriate dilution. Antigen-antibody complexes were revealed by the streptavidin-biotin technique (Dako, Glostrup, Denmark), using diaminobenzidine as chromagen.

In all techniques, negative controls were included and obtained by either omission of the primary antibody, substituted for by PBS alone, or incubation with isotypic immunoglobulins.

Quantitation of Morphological Data: The following data were quantitated: mitotic index, apoptotic index, extent of basement membrane deposition at the periphery of tumor nodules, intratumoral vascular density, and number of peptide-containing cells. For quantitation of the mitotic index and of the apoptotic index, the respective numbers of mitotic and apoptotic bodies within tumor nodules were counted in 10 consecutive fields with a $\times 40$ objective lens, as previously described.²² Results were expressed as the mean number of mitoses or apoptotic bodies per field.

For quantitation of basement membrane deposition around tumor nodules, a semiautomated image analysis technique was used to evaluate 1) the total length of the perimeter of tumor nodules and b) the fraction of the perimeter length occupied by immunodetectable deposits of laminin 1. The final result was expressed as a percentage of the total perimeter length.

For quantitation of intratumoral vascular density, the structures expressing PECAM-1, a specific endothelial cell marker, were counted in five consecutive fields with a $\times 40$ objective. Results were expressed as the mean number of vessels per field.

For quantitation of peptide-containing cells, the percentages of cells labeled by the antibodies used for detection of glucagon, somatostatin (STS), and cholecys-

tokinin (CCK) were evaluated in 10 consecutive fields with a $\times 40$ objective.

Radioimmunoassay

Radioimmunoassay (RIA) for the detection of endogenous peptides was performed only on homogenates of subcutaneous tumors. After peptide extraction, RIAs were performed using the rabbit polyclonal antibodies 56D for STS, 39A for CCK, and 199D for truncated glucagon-like peptide 1 (TGLP-1), as previously described.²³⁻²⁵ Peptide concentrations were calculated as nanograms per milligram wet weight. Cellular CCK, TGLP-1, and STS concentrations were calculated as ng/ 10^6 cells.

In Vitro Experimental Study

Reagents

DMEM, additive, and FCS were obtained from Life Technologies, Inc. (Cergy-Pontoise, France). α -³²P-labeled dCTP, ³⁵S-methionine, and ³H-thymidine were purchased from Amersham (Les Ulis, France). All other reagents were of analytical grade.

Culture of Rat Organ-Specific Fibroblasts

Dermal, lung, and liver fragments of Wistar newborn rats were taken and washed once with a 30% sodium hypochloride solution and twice with sterile PBS. Each fragment was dissociated first mechanically by using scissors and then enzymatically with collagenase I (Sigma Chemical Co., St. Louis, MO) in culture medium. After incubation at 37°C (15–60 minutes), the tissue fragments were filtrated and centrifuged (15 minutes, 1500 rpm). The cell pellet was resuspended with 5 ml of culture medium supplemented with 10% FCS and was plated in culture flasks. Primary cultured fibroblasts were respectively obtained from subcutis, lung, and liver and were used at passages 1–5.

Immunofluorescence

Organ-specific fibroblasts were cultured for 3 days in Lab-Tek tissue culture chambers/slides (Miles, Elkhart, IN). After PBS rinsing, they were fixed and permeabilized with 100% cold methanol for 5 minutes. Cells were incubated for 1 hour with specific antibodies diluted from 1:25 to 1:400. Controls were incubated without specific antibodies. After abundant washing with 10% FCS in PBS, slides were incubated with fluorescein isothiocyanate (1/50)- or rhodamin (1/100)-labeled, species-specific antibodies for 30 minutes. After rinsing, coverslips were mounted with Fluoprep (Biomérieux, Lyon, France), and the slides were examined using an epifluorescence microscope.

Direct Cocultures

STC-1 cells were cultured on confluent fibroblastic layers. To describe the procedure briefly, 10×10^4 STC-1 cells that had been suspended in medium supplemented with 5% FCS were seeded onto fibroblast monolayers in 24-well plates (Costar, Cambridge, MA) in medium supplemented with 5% FCS. Direct cocultures were maintained for 5 days. All experiments were performed in triplicate.

Ultrastructural analysis of cellular interactions was performed on 3- and 5-day direct cocultures in 60-mm petri dishes (Costar). Cocultures were fixed with 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer for 15 minutes and 60 minutes, respectively. They were subsequently rinsed overnight in the same buffer and postfixed with 1% osmium tetroxide. After dehydration in graded ethanols and propylene oxide, they were embedded in epoxy resin. Ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and examined with a Jeol 100 CX 2 electron microscope (JEOL).

Attachment Assays

To study the adhesion of endocrine tumor cells onto fibroblast monolayers, fibroblasts were seeded in 24-well plates (Costar) and grown to reach confluence. The wells were then washed twice with PBS, and nonspecific sites were saturated with 0.1% bovine serum albumin (BSA). After washing with PBS, STC-1 cells (4×10^4 cells/ml) prelabeled with ^{35}S -methionine (Amersham) were added onto the fibroblast monolayer in serum-free medium. Control experiments were made in plastic wells after BSA saturation. At different times (30 minutes, 1 hour) after seeding, the unattached cells were washed away. Adherent cells were removed in 100 ml of 1% sodium dodecyl sulfate (SDS), and the radioactivity was counted in a scintillation counter. Similarly, the adhesion of endocrine tumor cells onto extracellular-matrix proteins was performed. The wells were coated with laminin-1 (5 $\mu\text{g}/\text{ml}$), type IV collagen (10 $\mu\text{g}/\text{ml}$), or type I collagen (10 $\mu\text{g}/\text{ml}$) (Becton Dickinson, Mountain View, CA) and washed twice with PBS, and nonspecific sites were saturated with 0.1% BSA.

To study the cell spreading on fibroblasts, cultures were initiated as described above and daily were observed under phase microscopy and photographed. The spreading and adhesion of endocrine tumor cells were also studied on extracellular-matrix proteins in 24-well plates (Costar) in a similar manner. The wells were coated with laminin-1, type IV collagen, or type I collagen as described for attachment assays. All experiments were performed in triplicate.

Indirect Cocultures

In vitro indirect coculture assays were performed using modified Boyden chambers (Falcon). STC-1 cells (10^5) suspended in medium supplemented with 5% FCS were seeded into the upper wells on polycarbonate filters

(0.45- μm porosity). Fibroblast monolayers were in the lower wells in medium supplemented with 5% FCS. Indirect cocultures were maintained for 3 days.

Fibroblast-Conditioned Media

Myofibroblast-conditioned media (FCM) were prepared by the method of Cornil et al.²⁶ Fibroblastic cells were seeded into 150- cm^2 culture flasks in 40 ml of culture medium and grown to confluence, at which time the medium was removed, and 40 ml of fresh DMEM containing 5% FCS, glutamine, and antibiotics were added to each flask. After a 48-hour incubation, FCM were collected, centrifuged at $1000 \times g$ for 5 minutes to remove cellular debris, pooled, and stored at -20°C . They were thawed and centrifuged immediately before use. Conditioned media were diluted 1:1 (v/v) with fresh DMEM containing 5% FCS, glutamine, and antibiotics. STC-1 cells were cultured in diluted FCM for 3 days. All experiments were performed in triplicate.

Assessment of Cell Proliferation

Cell proliferation was assessed by measuring the rate of DNA synthesis. This last parameter was determined through ^3H -thymidine incorporation into the trichloroacetic acid-insoluble cellular fraction. After a 2-hour incubation with 1 mCi/ml of ^3H -thymidine, cells were washed twice in ice-cold PBS and precipitated with ice-cold 5% trichloroacetic acid. Then cells were washed once with ice-cold 95% ethanol and solubilized with 1 N NaOH. An aliquot was neutralized, and the radioactivity was determined in a liquid scintillation counter. All experiments were performed in triplicate.

RNA Preparation and Northern Blot Hybridization

Culture medium was removed and cells were harvested in guanidine thiocyanate. Total RNA was extracted by the method of Chomczynski and Sacchi.²⁷ Northern blot analysis of RNA was performed as previously described,²⁸ using complementary DNA (cDNA) probes. Probes for CCK (575-bp cDNA fragment), STS (570-bp cDNA fragment), and glucagon (408-bp cDNA fragment) were kindly provided by J Philippe (Geneva, Switzerland).

All membranes were prehybridized in 1 mol/L NaCl, 1% SDS, and 10% dextran for 3 hours at 60°C . cDNA probes were labeled with α - ^{32}P -dCTP, using a random primer labeling kit (Amersham). Membranes were first hybridized with CCK and glucagon radiolabeled cDNA probe overnight in hybridization buffer at 60°C , washed for 20 minutes in $2\times$ standard saline citrate (SSC) and 2% SDS at 60°C , then twice in $0.2\times$ SSC and 0.2% SDS for 20 minutes each at 60°C , and exposed to Hyperfilm (Amersham) at -70°C with intensifying screens. Membranes were then immersed in a boiling solution of $0.01\times$ SSC and 0.01% SDS maintained at 70°C for 30 minutes. Membranes were hybridized with the STS-radiolabeled cDNA probe, in the hybridization buffer supplemented

Figure 1. Morphological features of STC-1 xenografts in the immunosuppressed newborn rat. **a:** The histological appearance of a subcutaneous tumor obtained 21 days after the xenografting of STC-1 cells in an immunosuppressed newborn rat. The tumor is composed of well-limited, partially encapsulated nodules of various sizes. **b:** At higher magnification, tumor cells resemble poorly differentiated neuroendocrine cells. They are monomorphic and present numerous mitotic figures. Apoptotic bodies are frequent. **c:** A lung tumor obtained from the same animal. It appears as a small, unencapsulated nodule, formed by neuroendocrine cells as shown by the presence of neurosecretory granules at ultrastructural examination (**d; arrows**). **a** and **c:** H&E staining (original magnifications: **a**, $\times 120$; **c**, $\times 300$; **b:** semithin section, toluidine blue staining (original magnification, $\times 600$); **d:** ultrastructural examination (original magnification, $\times 28,000$).

with salmon sperm DNA (100 mg/ml) to avoid nonspecific staining.

The equal loading of the lanes with total RNA was checked by staining 28S and 18S ribosomal RNA with ethidium bromide. Each dehybridization step was verified by exposing the membranes to Hyperfilm.

Protein Extraction and Western Blotting

STC-1 cells from indirect cocultures were lysed in lysis buffer. An aliquot of each lysate, containing 300 mg of protein as determined by the Bradford method was resolved on a 10% SDS-polyacrylamide gel. The fraction-

Table 2. Comparison of Morphological Parameters in Subcutaneous and Lung Tumors

Parameter	Subcutaneous tumors	Lung tumors	
Mitotic index (mean number of mitoses per field)	4.2 ± 2.3	6.5 ± 2.0	$P < 0.01$
Apoptotic index (mean number of apoptotic bodies per field)	7.9 ± 2.2	2.2 ± 1.1	$P < 0.01$
Basement membrane deposition (% of the circumference)	84.9 ± 8.7	23.7 ± 11.5	$P < 0.001$
Intratumoral vascular density (mean number of vessels per field)	17.3 ± 0.9	3.3 ± 1.8	$P < 0.001$

Results are given as mean $\pm$ SD. Differences were considered as significant when $P < 0.05$ (Student's *t*-test).

ated proteins were transferred to Protran nitrocellulose transfer membranes (Schleicher & Schüll, Ecqueville, France). The filter was blocked and incubated with a mouse monoclonal antibody against Ets-1 (Transduction Laboratories, Lexington, KY). Antibody-antigen complexes were visualized by enhanced chemiluminescence (Amersham), according to the manufacturer's instructions.

Results

In Vivo Study

Subcutaneous tumors located at the site of injection were obtained in all 18 animals in the study group. At sacrifice 21 days after injection, tumor sizes ranged from 4 to 7 mm (mean $\pm$ SD, 5.2 ± 1.5). At gross examination, there were no significant necrotic-hemorrhagic lesions. In all of the animals included in the study group, lung metastases were detected. They were usually less than 1 mm in diameter.

Morphological Characteristics of STC-1-Induced Subcutaneous and Lung Tumors

The histological appearance of STC-1-induced subcutaneous tumors was comparable in all of the animals of the study group. STC-1-induced tumors presented the aspect of poorly differentiated neuroendocrine tumors (Figure 1a). The tumor population was composed of monomorphic, small- to medium-sized cells, characterized by a centrally located, often irregular nucleus and by an abundant, basophilic cytoplasm (Figure 1b). The architecture of the neoplastic population was usually compact. However, characteristic trabecular architecture could be found in all tumors examined.

Lung tumors developing in STC-1-injected immunosuppressed newborn rats presented as tiny nodules (Figure 1c) formed by medium- to large-sized neuroendocrine cells. Cells were densely packed, and no evidence of trabecular architecture was observed.

In both lung and subcutis, tumor cells retained a neuroendocrine differentiation, as assessed by their constant expression of chromogranin A (data not shown) and the presence of neurosecretory granules at ultrastructural examination (Figure 1d).

Proliferative Activity in STC-1-Induced Subcutaneous and Lung Tumors

In subcutaneous tumors, the mitotic index of the endocrine neoplastic population was high (Table 2). It varied by case from 1 to 8 mitoses/10 high-magnification fields. In lung tumors, the mitotic index ranged from 4 to 10 mitoses/10 high-magnification fields (Table 2). The mitotic index was significantly higher in pulmonary lesions than in subcutaneous tumors (Table 2). Comparison between subcutaneous and lung tumors in the same animal confirmed this result (Figure 2).

Numerous apoptotic bodies were present in subcutaneous STC-1 tumors. They were rarely observed in lung

Figure 2. Mitotic index in STC-1 xenografts. Comparison of the mitotic index in subcutaneous tumors (solid bars) and lung tumors (hatched bars) in six representative animals.

tumors (Table 2). The difference in the apoptotic index was statistically significant (Table 2).

Stromal Component and Capsule Formation in STC-1-Induced Subcutaneous and Lung Tumors

Subcutaneous STC-1 tumor nodules were encapsulated. The fibrous capsule was thin and contained numerous capillary vessels. In 5 animals, limited capsular effraction with invasion of the subcutaneous fat and/or superficial muscle layers was observed. Immunohistochemical analysis showed the presence of large amounts of basement membrane proteins laminin-1 and type IV collagen (Figure 3a) at the interface between the neoplastic proliferation and the adjacent connective tissue (Table 2). In subcutaneous tumors, the intratumoral stroma was constituted of thin fibrous septa, interspersed within the neoplastic proliferation and containing numerous capillary vessels. The high density of intratumoral capillary vessels was readily highlighted by an antibody directed to the endothelial cell marker PECAM-1 (Figure 3b). The intratumoral vascular density was high (Table 2). Mesenchymal cells expressing α -smooth muscle actin were detected in intratumoral fibrovascular septa and at the periphery of the neoplastic nodules (Figure 3c). Ultrastructural examination confirmed the presence of nu-

Figure 3. Composition of tumor stroma. **a:** Basement membrane type IV collagen is easily detected at the interface between a subcutaneous tumor and the adjacent connective tissue. **b:** Tumor cells are intermingled with a high number of small capillary vessels, highlighted by their expression of the endothelial cell marker PECAM-1. **c:** Myofibroblasts expressing α -smooth muscle actin are detected in intratumoral fibrovascular septa and at the periphery of the neoplastic nodules. Immunofluorescence technique (original magnifications: **a**, $\times 100$; **b**, $\times 250$; **c**, $\times 180$).

merous myofibroblastic cells at the periphery of tumor nodules (data not shown).

In contrast to subcutaneous tumors, lung tumors were poorly limited. No evidence for capsule formation was found at histological and ultrastructural examinations. Peripheral deposits of basement membrane proteins were inconsistently observed (Table 2). The stromal component of STC-1-induced lung tumors was minimal. Only a few capillary vessels were present (Table 2). Very few myofibroblastic cells were detected at immunohistological and ultrastructural examinations (data not shown).

The comparison between subcutaneous and lung tumors showed that the deposition of basement membrane proteins and the intratumoral vascular density were significantly higher in subcutaneous tumors than in lung tumors (Table 2).

Peptide and Hormone Content in STC-1-Induced Tumors

The hormonal profile of xenografted STC-1 cells, as determined by RIA, was different from that of STC-1 cells maintained in culture. In contrast to cultured STC-1 cells, subcutaneous STC-1 tumor cells synthesized larger

amounts of CCK and TGLP-1 than STS (Table 3). No RIA could be performed on lung tumors, because their small size precluded obtaining homogenates.

CCK, STS, and glucagon were detectable by immunohistochemistry in subcutaneous tumor nodules (Figure 4). CCK was detected in small clusters of neuroendocrine cells, amounting to about 10% of the tumor cell population. STS was detected in a few tumor cells scattered among the neoplastic population and representing usually less than 5% of the tumor cell population. Glucagon was detected in clusters of tumor cells, ranging from about 10% to 20% of the tumor cell population depending on the case. In lung tumors, only a few cells that were positive for CCK, STS, and glucagon were observed (data not shown).

Table 3. Peptide and Hormone Content in STC-1-Induced Tumors (ng/mg) and STC-1 Cell Line (ng/ 10^6 cells) as Determined by RIA

	CCK	STS	TGLP-1
STC-1 tumor number			
4	5.5	3.0	9.5
6	7.6	2.9	13.9
7	4.9	6.5	22.6
12	5.9	5.5	21.9
15	3.9	3.0	4.0
17	3.9	8.4	8.0
STC-1 cell line	1.8	47.6	6.7

Figure 4. Peptide detection in STC-1-induced tumors. CCK (**a**) and glucagon (**b**) were detected in small clusters (arrows) of neuroendocrine cells in subcutaneous tumor nodules. Immunoperoxidase technique (original magnifications: **a**, $\times 250$; **b**, $\times 140$).

Figure 5. Extracellular matrix proteins deposited by organ-specific fibroblasts. After 3 days of culture, organ-specific fibroblasts are associated with distinctive basement membrane products. Cultures of MIC-316 (**a**) contain sporadic deposits of type IV collagen, whereas cultures of MIC-219 (**b**) contain large amounts of type IV collagen forming an organized basement membrane-like structure. Immunofluorescence technique (original magnifications, $\times 400$).

In Vitro Analysis of Influence of Organ-Specific Fibroblasts on STC-1 Cells

Characterization of Organ-Specific Fibroblasts

After isolation, dermal, lung, and liver rat fibroblasts were characterized by a panel of antibodies to cytoskeletal proteins. All cells expressed vimentin and α -smooth muscle actin and were negative for keratin. Dermis-, lung-, and liver-derived cells therefore presented the features of myofibroblasts.

Immunodetection of laminin-1 and type IV collagen was performed after 3 days of culture of organ-specific

Figure 6. Effects of fibroblast-soluble factors on peptide gene expression in STC-1 cells. Cells were incubated for 72 hours with the indicated FCM. FCM obtained from primary cultures of both dermal (D) and lung (Lu) fibroblasts drastically enhanced cholecystokinin and glucagon mRNA levels in STC-1 cells; FCM obtained from liver (Li) fibroblasts drastically enhanced glucagon, but not cholecystokinin mRNA levels; and FCM from dermal (D) and liver (Li) fibroblasts, but not from lung (Lu) fibroblasts, reduced somatostatin mRNA levels.

fibroblasts. In cultures composed of dermal and colon (MIC-316) myofibroblasts, only small and widely scattered deposits of basement membrane proteins could be detected (Figure 5a). In contrast, cultures of lung, liver, jejunal (MIC-101.1), and ileal (MIC-219) myofibroblasts contained large amounts of laminin-1 and type IV collagen, which formed an organized basement membrane-like structure (Figure 5b).

Influence of Dermal, Lung, Liver, and Intestinal Organ-Specific Fibroblasts on Hormone Gene Expression

CCK, proglucagon, and STS gene expressions were studied on the same cell preparations (Figure 6). Conditioned media obtained from primary cultures of both dermal and lung fibroblasts drastically enhanced CCK and proglucagon messenger RNA (mRNA) levels in STC-1 cells. Conditioned media from liver fibroblasts enhanced proglucagon mRNA levels, but not CCK mRNA levels, in STC-1 cells. Finally, STS mRNA levels were reduced by conditioned media from dermal and liver fibroblasts, but

Figure 7. Effects of fibroblast-soluble factors on proliferation rate of STC-1 cells. Cells were incubated for 72 hours together with the indicated fibroblasts. When compared with control, STC-1 3 H-thymidine incorporation decreased to 27% in the presence of dermal fibroblasts (DFibro) and to 35% in the presence of liver fibroblasts (LiFibro), but was not affected by lung fibroblasts (LuFibro). * $P < 0.05$ versus control (Student's *t*-test).

Figure 8. Effects of fibroblast monolayer on spreading of STC-1 cells. STC-1 cells were cocultivated for 3 days onto MIC-101-1 (**a**), MIC-219 (**b**), MIC-316 (**c**), dermal fibroblasts (**d**), lung fibroblasts (**e**), and liver fibroblasts (**f**). STC-1 cells readily spread over MIC-101-1, MIC-219, lung, and liver fibroblasts but not over MIC-316 and dermal fibroblasts. Phase contrast microscopy (original magnifications, $\times 450$).

not from lung fibroblasts. Similar results were obtained in indirect cocultures (data not shown). We verified that, in accordance with our previous results, myofibroblastic subsets specific for different segments of the intestine (jejunum, ileum, colon) modulated hormone expression in STC-1 cells¹⁶ (data not shown).

Influence of Dermal, Lung, Liver, and Intestinal Organ-Specific Fibroblasts on STC-1 Proliferation Rate

The growth of STC-1 cells maintained in coculture with fibroblasts of different origins was inhibited to various degrees. When compared with controls, STC-1 ³H-thymi-

Figure 9. Direct interactions between STC-1 cells and fibroblasts. Sagittal semithin sections confirm that STC-1 cells grow as clusters onto dermal fibroblasts without spreading (**a**), whereas STC-1 spread onto MIC-219 (**b**) with formation of intercellular contacts (**arrowheads**) and basement membrane deposition (**arrows**) at ultrastructural examination (**c**). Original magnifications: **a** and **b**, $\times 800$; **c**, $\times 20,000$.

Figure 10. STC-1 cell adhesion onto fibroblast monolayer at 1 hour. Results are expressed as percent ($\pm$ SEM) of total radioactivity initially seeded by well. The adhesion of STC-1 cells was found to be higher on lung (LuFibro), liver (LiFibro), jejunal (MIC-101.1), and ileal (MIC-219) fibroblasts than on dermal (DFibro) or colon (MIC-316) fibroblasts.

dine incorporation decreased by 27% in the presence of dermal fibroblasts and by 35% in the presence of liver fibroblasts, but was not affected by lung fibroblasts (Figure 7). Myofibroblast-conditioned media had similar effects (data not shown). We verified that, in accordance with our previous results, myofibroblastic subsets specific for different segments of the intestine (jejunum, ileum, colon) reduced proliferative activity in STC-1 cells¹⁶ (data not shown).

Influence of Dermal, Lung, Liver, and Intestinal Organ-Specific Fibroblasts on STC-1 Spreading and Adhesion

The pattern of spreading of STC-1 cells depended on the origin of fibroblasts (Figure 8). On dermal and colon (MIC-316) fibroblasts, after 3 days of co-culture, STC-1

Figure 11. STC-1 cell spreading and adhesion onto extracellular-matrix proteins. **a:** Results of adhesion experiments on laminin-1 (LAM 1), type IV collagen (COLL IV), and type I collagen (COLL I), expressed as percent ($\pm$ SEM) of total radioactivity initially seeded by well (* $P < 0.05$ versus control). **b, c,** and **d:** phase contrast microscopy of representative experiments. The adhesion and spreading of STC-1 cells is higher on laminin-1 (**b**) and type IV collagen (**d**) than on type I collagen (**c**). Original magnifications, $\times 400$.

cells exhibited very limited spreading. In contrast, on ileal, jejunal, liver, and lung fibroblasts, STC-1 cells readily spread and formed organized trabecular layers.

Sagittal semithin sections confirmed the absence of spreading of STC-1 cells onto dermal (Figure 9a) or colon (MIC-316) fibroblasts. In contrast, STC-1 cells readily spread onto lung, liver, ileal (MIC-101-1), and jejunal (MIC-219; Figure 9b) fibroblasts associated with formation of intercellular contacts and basement membrane

Figure 12. Effects of fibroblast-soluble factors on Ets-1 protein level in STC-1 cells. STC-1 cells were incubated for 72 hours together with the indicated fibroblasts, and Ets-1 protein was detected by Western blotting. As compared with STC-1 alone (C), FCM from dermal (D), liver (Li), ileal (MIC 101-1), and jejunal (MIC 219) fibroblasts drastically reduce Ets-1 levels in STC-1 cells, whereas lung (Lu) and colon (MIC 316) fibroblasts have no significant effect.

deposition at the interface at the ultrastructural examination (Figure 9c).

In a second step, we quantified the adhesion of STC-1 cells to fibroblastic layers after prelabeling with ^{35}S -methionine (Figure 10). There was a significant difference in the attachment of STC-1 cells based on the origin of myofibroblasts. At 30 minutes (data not shown) and 1 hour, the attachments were higher on lung, liver, jejunal, (MIC-101-1) and ileal (MIC-219) fibroblasts than on dermal or colon (MIC-316) fibroblasts.

To evaluate the role played by the extracellular matrix proteins synthesized and secreted by organ-specific fibroblasts, we verified that STC-1 cells showed a marked ability to spread and adhere onto laminin-1 and type IV collagen but not onto type I collagen (Figure 11).

Modulation of Ets-1 Protein Levels in STC-1 Cells by Fibroblasts

The protein level of the Ets-1 transcription factor in STC-1 cells was evaluated by Western blotting (Figure 12) after indirect coculture with organ-specific fibroblasts. As compared with controls, the amount of Ets-1 protein was markedly decreased after coculture with dermal and intestinal fibroblasts, but was not affected after coculture with lung and liver fibroblasts.

Discussion

In this work, we show that 1) xenografting mouse enteroendocrine STC-1 cells to the immunosuppressed newborn rat makes it possible to constantly obtain primary subcutaneous neuroendocrine tumors and secondary lung tumors in the same animal; 2) the subcutaneous and lung tumors obtained in the same animal show differences in stromal reaction, capsule formation, and proliferative activity; 3) correlatively, *in vitro* studies show that rat dermal and lung myofibroblasts selectively alter hormone content, cell proliferation, spreading, and adhesion of STC-1 cells; and 4) these observations can be extended to fibroblasts originating from different segments of the intestine (jejunum, ileum, colon) and to liver-derived myofibroblasts.

Most experimental models currently available for the study of endocrine cells have been devised for the study of endocrine cell differentiation or for the analysis of the transition from hyperplasia to neoplasia.²⁹ Very few models make it possible to regularly obtain neuroendocrine metastatic tumors.^{30,31} The experimental model used in our study allows the development of comparatively large primary tumors at the site of injection in a short period of time and makes it possible to regularly obtain secondary lung tumors in the same animals, as previously reported.^{21,32,33} It is not possible to definitely exclude that the secondary lung tumors observed in our model are caused by the intravascular release of tumor cells at the time of injection and not by direct metastasis from subcutaneous primary tumors. However, for our purposes, this experimental model was useful to test *in vivo* the behavior of STC-1 cells growing in different tissue micro-environments.

The general appearance of STC-1 xenografted tumors was very reminiscent of that of the poorly differentiated neuroendocrine tumors observed in human pathology,^{2,3} with which they share a high mitotic index.^{22,34} However, STC-1 xenografted tumors presented significant morphological differences depending on their growth site. Subcutaneous tumors were well-limited, usually encapsulated, and always surrounded by deposits of basement membrane proteins, including laminin 1 and collagen IV. In contrast, lung tumors were poorly limited, devoid of peripheral capsule, and usually lacked organized basement membrane protein deposition. Differences in cell proliferative activity were also noted. Cell proliferation was higher in lung tumors than in subcutaneous nodules. In contrast, the apoptotic index was higher in subcutaneous than in lung tumors. It seems unlikely that only different size and probable different length of evolution may be sufficient to explain all of the morphological and proliferative differences observed between dermal and lung tumors.

The hormonal profile of xenografted STC-1 cells growing in subcutaneous nodules was markedly different from that of STC-1 cells maintained in culture. The relative amounts of CCK and glucagon were higher in xenografted cells, and the expression of STS was lower. In agreement with RIA results, immunohistochemically reactive glucagon-containing cells were more frequent than STS-containing cells in xenografted subcutaneous tumors. Accurate comparisons between the hormone and peptide content of subcutaneous and lung tumors could not be performed, because the very small size of pulmonary lesions precluded any reliable attempt at peptide quantitation by RIA. However, our immunohistochemical investigation showed that, in lung tumors, hormone-containing cells were rarely detected and suggested that their numbers were much lower than in subcutaneous lesions.

The differences observed between xenografted STC-1 subcutaneous and lung tumors might be related either to the selection of a more aggressive clone during the metastatic process³⁵ or to the existence of site-specific differences in the local reaction to tumor implantation and growth. To test the second hypothesis, we analyzed *in*

vitro the functional interactions between STC-1 cells and either dermal or lung rat myofibroblasts. Using direct and indirect cocultures, we were able to show that the hormone content, proliferative capacity, and spreading pattern of STC-1 cells were modulated in different ways by dermal or lung fibroblasts. Both dermal and lung myofibroblasts enhanced CCK and proglucagon mRNA levels in cocultured STC-1 cells, as compared with basal levels. However, dermal but not lung fibroblasts induced a decrease in STS mRNA levels. These results confirm the capacity for mesenchymal cells to modulate hormone synthesis and secretion in neuroendocrine cells. In our *in vitro* model system, mesenchymal cells were also able to modulate the proliferative activity of STC-1 cells; dermal but not lung fibroblasts inhibited the growth of cocultured STC-1 cells. This recalls our *in vivo* results, showing that the mitotic index was higher in lung tumors than in subcutaneous ones. Finally, distinct patterns of spreading and adherence were observed on either dermal or lung myofibroblasts. STC-1 readily spread and adhered to lung myofibroblast monolayers, but not to dermal myofibroblast monolayers. This may be due in part to the different composition of the extracellular matrix secreted by these two cell subsets; dermal fibroblasts mainly secrete collagen I, whereas lung fibroblasts secrete large amounts of basement membrane proteins.

We finally tested the possible effects of mesenchymal cells on *ets-1* expression in STC-1 cells. *ets-1* encodes a transcription factor controlling the expression of various target proteins, including certain metalloproteases.^{36–40} In tumors, Ets-1 up-regulation is associated with the acquisition of invasive capacities.⁴¹ Ets-1 induction is frequent in stromal cells associated with invasive tumors.^{42,43} Ets-1 gene product has also been detected in the epithelial component of certain digestive tumors, including invasive gastric carcinomas,⁴⁴ and in human neuroectodermal cells.⁴⁵ Our findings show that *ets-1* is constitutively expressed in STC-1 cells maintained in culture and that this expression is markedly reduced by dermal but not lung fibroblasts. This reinforces the hypothesis that the lung microenvironment may favor STC-1 cell growth.

Finally, we verified that myofibroblasts derived from the usual sites of primary growth of digestive neuroendocrine tumors, such as the jejunum, the ileum, and the colon, as well as myofibroblasts derived from the liver—the main site of metastatic neuroendocrine tumors, are also able to modulate, in a site-specific manner, hormone synthesis, proliferation, spreading, adhesion, and *ets-1* expression in STC-1 cells. In particular, liver myofibroblasts enhanced glucagon expression, reduced STS synthesis, markedly reduced cell proliferation, and favored the adhesion and spreading of STC-1 cells. This reinforces the concept that site-specific epithelial-mesenchymal interactions may contribute to some of the differences observed between neuroendocrine tumors originating from the different segments of the gastrointestinal tract or between primary tumors and their metastatic liver lesions.

Most of the effects of mesenchymal cells on STC-1 cells are likely to be mediated through soluble factors, because they can be reproduced by conditioned media.

Mesenchymal cells are known to secrete various growth factors that can modulate the differentiation and growth of tumor cells, such as transforming growth factor- β , basic fibroblast growth factor, keratinocyte growth factor, insulin-like growth factor-I, and hepatocyte growth factor. Recent studies have pointed out that organ-specific fibroblast subsets differ in the nature and relative amounts of their secreted products.^{17–19,46} Such functional differences may be one of the factors responsible for the site-specific effects observed in our study. Differences in the nature and relative amounts of extracellular matrix proteins secreted by these various mesenchymal subsets may also contribute to the modulation of STC-1 functions. Further studies are necessary to test these various hypotheses.

In conclusion, our experimental *in vivo* and *in vitro* results suggest that mesenchymal cells may modulate the differentiation state and the growth pattern of digestive neuroendocrine tumor cells in a site-specific manner, which suggests that a close reciprocal interplay between local mesenchymal cells and tumor cells contributes to the acquisition of the characteristic histopathological features and biological behavior of the various types of digestive neuroendocrine tumors.

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