Endocr Pathol (2007) 18:223–232
DOI 10.1007/s12022-007-9001-3

BASIC RESEARCH

Characterization of the Functional and Growth Properties of Cell Lines Established from Ileal and Rectal Carcinoid Tumors

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Published online: 9 October 2007

Abstract Carcinoids of the intestine are the most common gastrointestinal carcinoid tumors. Therapeutic options to treat patients with these tumors are limited. There are very few ileal carcinoid cell lines available for in vitro studies to analyze new drugs that could be effective in treating patients with metastatic tumors. A replication defective recombinant adenovirus with an SV40 early T-antigen insert was used to infect two intestinal carcinoid tumors to create carcinoid cell lines. The cell lines were studied by cell culture, reverse transcription polymerase chain reaction, Western blotting, and immunohistochemistry. Both cell lines expressed SV40 large T antigen and receptors for TGFβ1, TGFβ2, EGFR, and somatostatin receptors. Treatment with TGFβ1 led to growth inhibition and increased apoptosis in the cultured cells. Octreotide inhibited cell growth of both cell lines while stimulating apoptosis. Treatment of the HC45 cells with gefitinib also inhibited cell growth in a concentration-dependent manner. TGFβ treatment stimulated chromogranin A expression while expression of two other granins, chromogranin B and 7B2, did not change significantly. RNA profiling of cells treated with TGFβ1 showed increased expression of vitamin D3 receptor. This finding was validated by real-time quantitative polymerase chain reaction, Western blotting, and immunohistochemistry. These results indicate that these carcinoid cell lines can be used to study the proliferative and apoptotic mechanisms involved in intestinal carcinoid tumor growth regulation.

Keywords carcinoids · TGFβ receptors · EGFR receptor · vitamin D3 receptor

Introduction

Gastrointestinal carcinoids are uncommon neuroendocrine tumors [1–3]. Although surgery is the primary method of treatment for gastrointestinal carcinoids, only a limited number of treatment modalities are available for patients with unresectable tumors [1–3].

A major limiting factor in understanding the biology of these tumors is the difficulty in establishing in vitro or in vivo models for study. Although a few cell lines are available [4–9] tumor development in nude mice is uncommon [10–12], so the number of cell lines for molecular studies remain limited.

Various recent in vitro studies have shown that in addition to targeting the somatostatin receptor, which is a principal way of treating unresectable gastrointestinal carcinoids medically [13, 14], other targets such as epidermal growth factor receptor (EGFR) [15–18], platelet-derived growth factor vascular endothelial growth factor receptors, and mTOR may be useful for the medical treatment of gastrointestinal carcinoid tumors [19–21].

To analyze biological properties of metastatic carcinoid tumors in vitro, we examined tumors from liver metastases of

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ileal carcinoids and a rectal carcinoid in attempt to establish cell lines. Establishment of carcinoid cell lines was used to study cells in culture by infecting two cell lines with SV40 large T antigen [22, 23]. The cells proliferated in culture while retaining some differentiated functions. These cell lines were used for characterization of the functional and growth properties of the ileal and rectal carcinoid tumors.

Materials and Methods

Octreotide was purchased from Sigma (St. Louis, MO, USA), TGF β1 was from R and D Systems (Minneapolis, MN, USA), and gefitinib (Iressa ZD1839 was a gift from Astra Zeneca, Macclesfield, Cheshire, UK. Epidermal growth factor was purchased from Promega (Madison, WI, USA). ^{3}H thymidine (specific activity 10.1 Ci/mM) was purchased from DuPont/NEN (Boston, MA, USA) and stored at −20°C.

Cell Culture

Human carcinoid tumors metastatic to the liver were used for primary cultures. IRB permission was obtained for the study. The tissues were finely minced, dissociated with trypsin, and then rinsed with fresh growth media before plating the cells in flasks. Fibroblasts were eliminated by moving the cells to plastic surfaces for brief periods of time. Cells were grown in a humidified 37°C incubator in 5% CO_2 and cultured in medium consisting of RPMI with L-glutamine containing 10% fetal bovine serum (Media Techs, Herndon, VA, USA), 5% horse serum, 1 μg/ml insulin, and 1% antibiotics/antimycotics (Invitrogen, Carlsbad, CA, USA). Cultrex (R&D Systems) was used as an extracellular matrix.

Adenovirus Infection

A replication-defective recombinant human adenovirus with an SV40 early T-antigen insert (AD-SVR4) was used to infect four cultured carcinoid tumor cells. A recombinant adenovirus-SV40R4 used in the study was obtained as previously reported [22]. This virus is a replication deficient human adenovirus in which the adenoviral E1A and E1b genes have been deleted, and the expression of T antigen is under control of the SV40 early promoter [23]. The human embryonic kidney cell line 293 (ATCC, Rockville, MD, USA) was used as a positive control for cell infection with ADSVR4. Incubation of 293 cells for 2 to 4 days after infection led to complete cell lysis with release of abundant AD-SVR4 into the medium [22].

Carcinoid cells from two tumors were plated at 80% confluency on Cultrex-coated wells on six-well plates. Two wells were used for each of the four tumors. One well had a final virus concentration of 5×10^8 PFU. The cells were incubated in 2% FBS-RPMI medium, and AD-SVR4 was added for 1 h at 37°C. After incubation, the cells were washed with growth media and placed in fresh media.

Isolation of Cell Lines

Within 2 weeks, most of the cells had died, leaving only a few isolated colonies of human ileal carcinoid HC45 and human rectal carcinoid HC49. Cells from the other two tumors did not survive. Each colony of surviving tumor cells was placed in one well of a 12-well plate for 1 week and subsequently passed to a T25 flask. The cells reached confluency approximately 4 weeks later. One clone from HC45 was selected for further studies. The other cell line, HC49, was also used in some experiments.

Six weeks after infection of the carcinoid cells with AD-SVR4, the cells continued to proliferate. The doubling time was approximately 2 days for HC45 and 4 days for HC49. Both cell lines grew well on plastic or matrix-coated surfaces. Immunohistochemical staining with an antibody directed against the SV40-T antigen was used to detect the infected cells.

Cytogenetic Studies

The cell lines were karyotyped by G-banding. A total of 20 of each cell type was analyzed.

Immunohistochemistry

Immunohistochemical analysis was done with avidin–biotin peroxidase complex system as previously reported [24], and antigen retrieval by microwave treatment in 10 mM sodium citrate pH 6.0 was used. Antibodies used included: chromogranin A (1:500) was from Neomarker, Freemont, CA, USA), Ki-67 (1:250), serotonin (1:200), synaptophysin (1:200) were all from Dako (Carpinteria, CA, USA). Chromogranin B (1:250) and SV4-T antigen (1:2000) were purchased from Santa Cruz (Santa Cruz, CA, USA). 7B2 (1:500) was from Phoenix (Belmont, CA, USA). Caspase 3 (1:50) was from Biocare (Walnut Creek, CA, USA). VMAT1 (1:25) was from Novocastra (New Castle, Tyne, UK), and vitamin D receptor (1:50) was from Cal Biochem (San Diego, CA, USA). Somatostatin receptor 2A (1:500) and 5 (1:500) were from Gramsch Lab (Schwabhausen, Germany). Vector stain ABC kit from Vector Laboratories (Burlingame, CA, USA) was used for immunohistochemistry. Cytospin preparations were prepared on a Cytospin instrument from Shandon (Pittsburgh, PA, USA). EGFR (1:200) and phospho-EGFR (1:500) were from Cell Signaling Technology (Beverly, MA, USA).

Table 1 RT-PCR Primer Sequences

Gene	Sense	Antisense	Size (bp)	Annealing Temperature (°C)
HPRT	5′-CTTGCTCGAGATGTGATGAAG-3′	5′-GTCTGCATTGTTTTGCCAGTG-3′	290	57
SSTR2a	5′-TGACAGTCATGAGCATCGAC-3′	5′GCAAAGACAGATGATGGTGA-3′	284	58
SSTR5	5′-CGTCTTCATCATCTACACGG-3′	5′-GGCCAGGTTGACGATGTTGA-3′	222	58
TGFβR1	5′-CGGAGGCAGGAGAAGCAGCGT-3′	5′-TAGGATGTTGTCGTGTCTGAG-3′	339	66
TGFβR2	5′-TGTGTTCCTGTAGCTCTGATG-3′	5′-AGATCTTGACTGCCACTGTCTC-3′	432	57
EGFR	5′-TCTCAGCAACATGTCGATGG-3′	5′-TCGCACTTCTTACACTTGCG-3′	473	61
CGA	5′-AACCAGAGCAGCCAGGCCGAG-3′	5′-TGGCTGGAGGGTGGGTGTTGG-31	235	65
CGB	5′-CCAGTGGATAACAGGAACCAC-3′	5′-GGCTCCTGCCTCTCCCCTGCT-3′	255	60
7B2	5′-CATTTGGGTCCTTTTGGCAAC-3′	5′-CGCTTTCGTCTCTCTCCTCCC-3′	287	68

Cells from the BON cell line (A gift from Dr. JC Thompson) and a primary midgut carcinoid were used as positive controls for chromogranin A, synaptophysin, chromogranin B, Caspase 3, VMAT[1] and somatostatin receptor 2A. A renal cell carcinoma was used as a positive control for vitamin D3 receptor. The A431 cell line was used as a positive control for EGFR and P-EGFR. Negative controls consisted of substituting normal serum for the primary antibody.

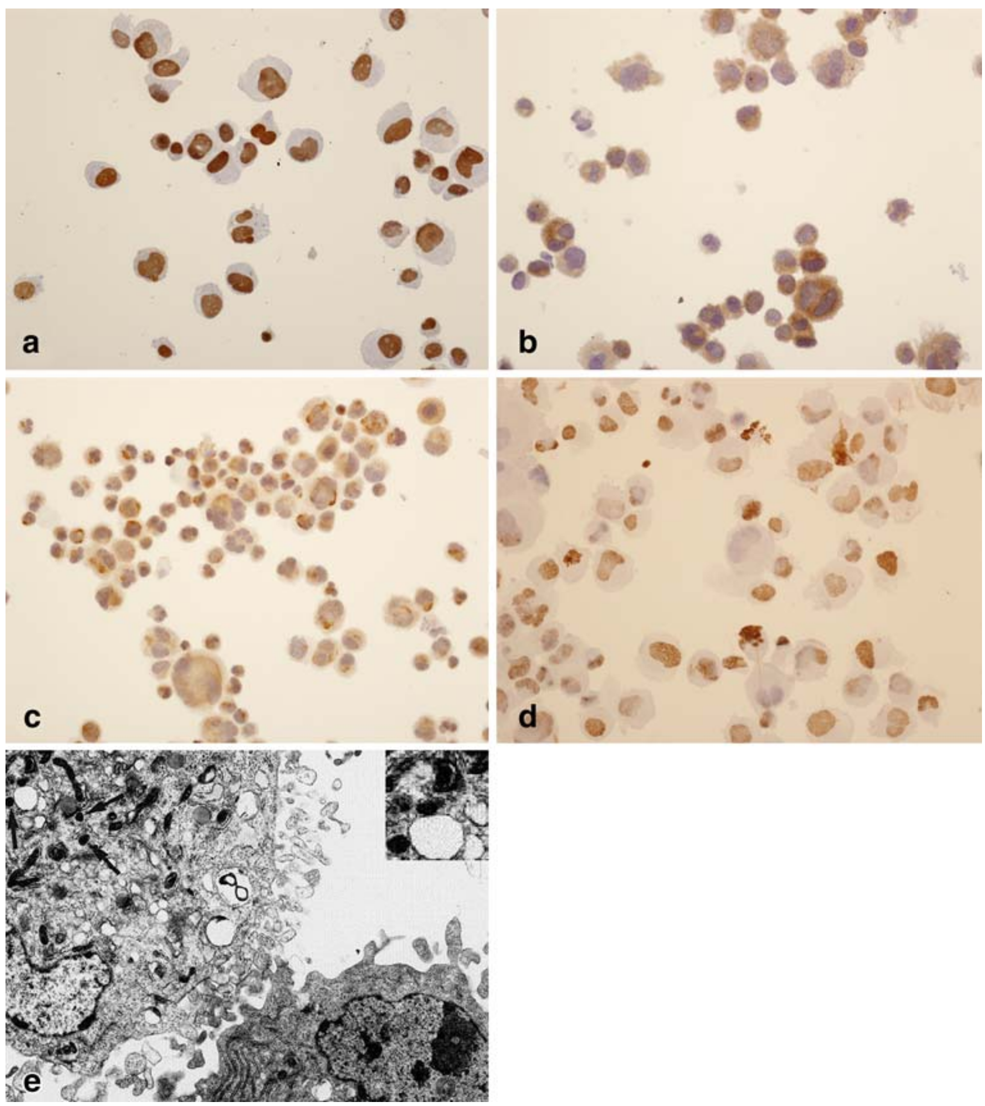

Fig. 1 **a**–**d** Immunohistochemical staining of ileal human carcinoid 45 (HC45). **a** Nuclear staining for SV40 is present in HC45. **b** Synaptophysin is present in the cytoplasm of most tumor cells. **c** Chromogranin A is present in the cytoplasm with stronger staining in the Golgi region. **d** Vitamin D receptor 3 is present in the nucleus of some HC45 tumor cells. **e** Electron micrograph of HC45 cells. The cells have few dense core secretory granules (*arrows*). Other organelles such as mitochondria and rough endoplasmic reticulum are present (×4,400). *Inset* shows a higher magnification of secretory granules (×8,000)

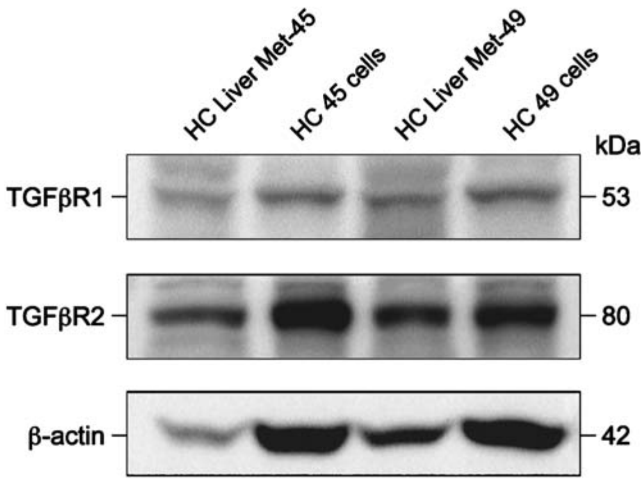

Fig. 2 Western blot showing expression of TGFβ1 and TGFβ2 in liver metastasis and cell lines for HC45 and HC49. β-actin was used to check for equal loading of the gel

Western Blotting

Western blotting was performed as previously reported. Primary and metastatic carcinoid tumors were kept frozen at −70°C until the proteins were extracted as previously reported [24, 25]. Twenty-five micrograms of protein was used in each lane. βeta-actin (Sigma) was used to check for equal loading of the gels.

RNA Extraction and Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted from primary and metastatic carcinoid tumors and from cells as previously reported [24, 25]. One microgram total RNA was first treated with 1 U DNase. Four hundred nanograms of DNase-treated total RNA was reverse transcribed using the stratascript first-strand synthesis system (Stratagene, LaJolla, CA, USA) according to the manufacturer's instructions. PCR was performed using the primers and annealing temperature listed (Table 1) for 45 cycles except for HPRT which was performed for 32 cycles. Each PCR contained 2-μl template cDNA, 0.75 U, Platinum Taq polymerase (Invitrogen), 1.5-μM magnesium chloride, and 0.2 μM each dNTP in a total volume of 25 μl, unless otherwise stated. The chromogranin A PCR was performed with 0.5 U in its Taq polymerase. PCR products were resolved on a 1.5% agarose gel and stained with ethidium bromide.

Treatment with TGFβ1, Octreotide, and Gefitinib

In experiments with octreotide (final concentration of 10^{-6} M) initial experiments used titrations of octreotide between 10^{-10} to 10^{-6} M. TGFβ was used at a final concentration of 10^{-9} M as previously reported [25]. After the preliminary experiments with dose response analyses, we selected a concentration that gave a significant difference between the control and treated cells which did not lead to rapid cell death. Octreotide (1×10^{-6} M), gefitinib (final concentration of 10^{-6} M), and the combination of octreotide and gefitinib as well as gefitinib alone and octreotide alone were used to treat cells for 4 days. All experiments and controls were run in duplicate. Cells were usually plated in 12-well plates. The following day, 2% media was used to replace complete media for the duration of the experiment.

Dose-dependent experiments were done with octreotide at concentrations of 1×10^{-10}, 1×10^{-8}, and 1×10^{-6} M. Gefitinib titrations were done at 1×10^{-6}, 5×10^{-6}, 1×10^{-5}, and 2×10^{-5} M.

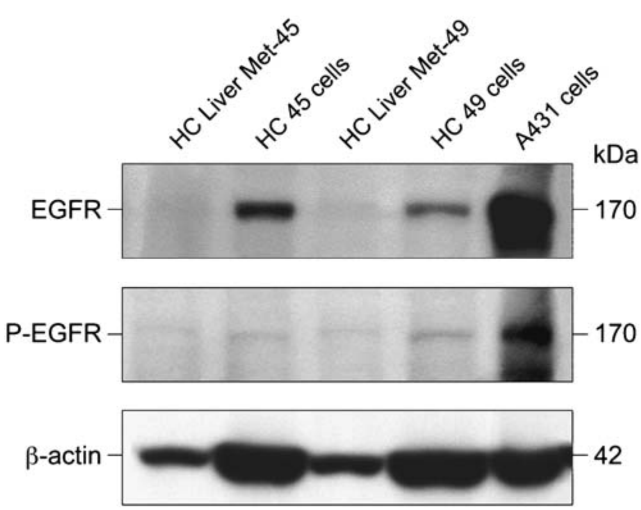

Fig. 3 Western blot showing expression of EGFR and phospho-EGFR in liver metastasis and cell lines for HC45 and HC49. The A431 cell line was used as a positive control. β-actin was used to check for equal loading of the gel

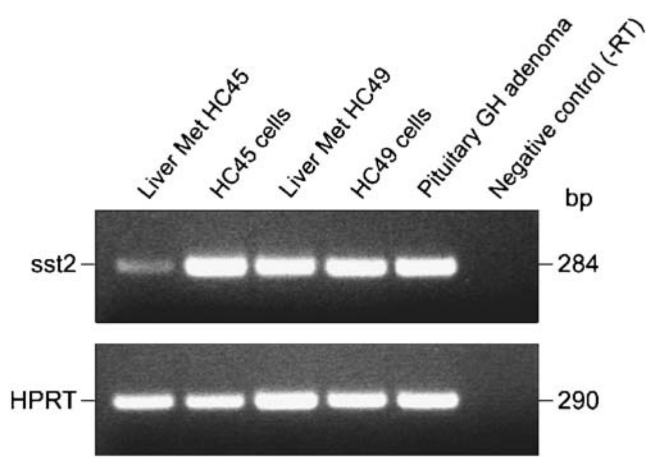

Fig. 4 RT-PCR analysis showing expression of somatostatin receptor 2a (Sst2) in liver metastasis and in HC45 and HC49 cell lines. HPRT was used as the housekeeping gene

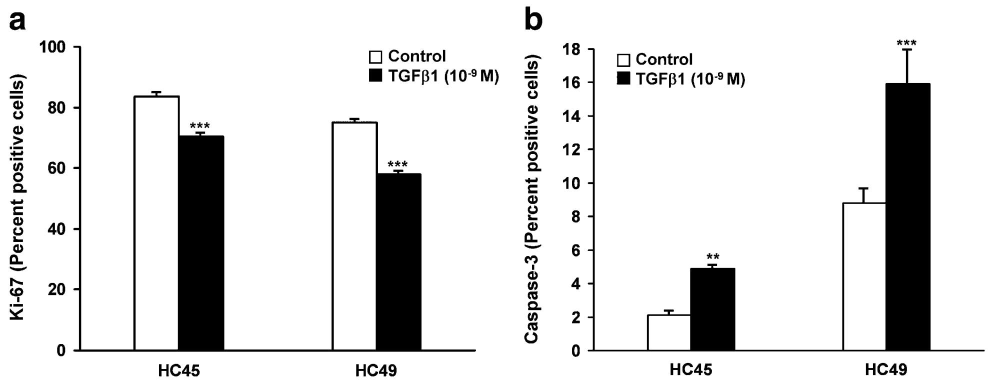

Fig. 5 Effects of TGFβ1 treatment as cell proliferation and apoptosis. **a** Analysis of proliferation by Ki-67 showed a decrease in Ki-67 index after TGFβ1 treatment in both cell lines. **b** Analysis of apoptosis by caspase-3 showed an increase in apoptosis in both cell lines. Cells were treated with TGFβ1 for 4 days. *Double asterisks* $p<0.01$; *triple asterisks* $p<0.00$

Thymidine Incorporation Experiments

Thymidine incorporation studies were done by treating HC45 cells for 4 days followed by treatment with ^{3}H thymidine for 5 h. Three sets of ^{3}H thymidine experiments for each treatment were done as previously reported [22].

HC45 cells were plated in a six-well plate.

Electron Microscopy

Ultrastructural studies were done on the cells as previously reported [22].

DNA Microarray Analysis

RNA was extracted from the cells as previously reported [24, 25]. The integrity of the RNA was verified using the Agilent A2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). A total of 8 µg of total RNA for each sample was used for the Affymetrix assay.

Gene Array Sample Preparations, Hybridization, and Scanning

The purified cDNA was used as a template for in vitro transcription for the synthesis of biotinylated complimentary RNA (cRNA) using an RNA transcript labeling reagent (Affymetrix) as previously reported [24]. Labeled cRNA was then fragmented and hybridized onto the Affymetrix GeneChip U133 plus 2.0 array with over 47,000 transcripts and variants including 38,500 genes were used. Briefly, appropriate amounts of fragmented cRNA and control oligonucleotide B2 were added along with control cRNA (Bio B, Bio C, and Bio D) herring sperm DNA and bovine serum albumin into the hybridization buffer. The hybridization mixture was heated to 99°C for 5 min followed by incubation at 45°C for 5 min before injecting the sample into the GeneChip. Hybridization was then performed at 45°C for 16 h and then mixed on a rotisserie at 60 RPM. After hybridization, the solution was removed and arrays were washed and stained with streptavidin phyco-erythrin (Molecular Probes, Portland, OR, USA).

After washes, arrays were scanned using GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA, USA). The quality of the fragmented biotin-labeled cRNA in each experiment was evaluated before hybridization onto the U133A 2.0 expression array by both obtaining electropherograms on Agilent 2100 Bioanalyzer and hybridizing a fraction of the sample onto a test-3 microarray as a measure of quality control. Once the GeneChip was scanned, the raw data (CEL files) was uploaded into GeneSpring 7.2 software (Agilent Technologies, Palo Alto, CA, USA) was used for further data analysis and visualization.

Gene Array Data Analysis

The raw data (.CEL files) was preprocessed using GCRMA algorithm included in the GeneSpring software. GeneSpring built-in tools were used to calculate fold change and significance between the groups of samples. The significance was calculated by Welch *t* test with Benjamin and Hochberg false discovery rate (FDR) multiple testing correction. A gene was

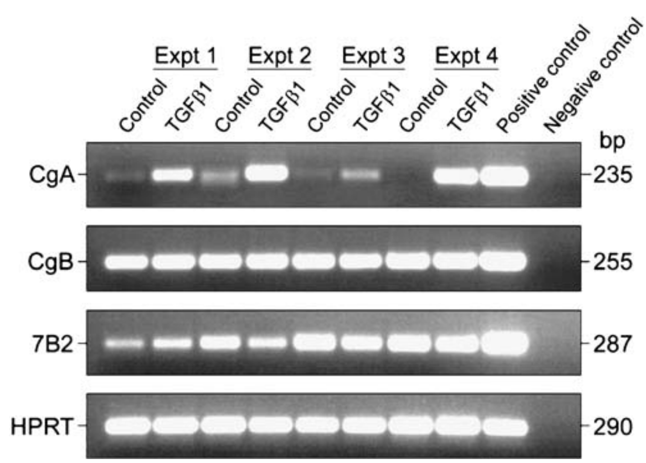

Fig. 6 Effects of TGFβ1 (10^{-9} M) treatment on granin expression in HC45 cells. There was an increase in chromogranin A mRNA while chromogranin B and 7B2 remained relatively constant

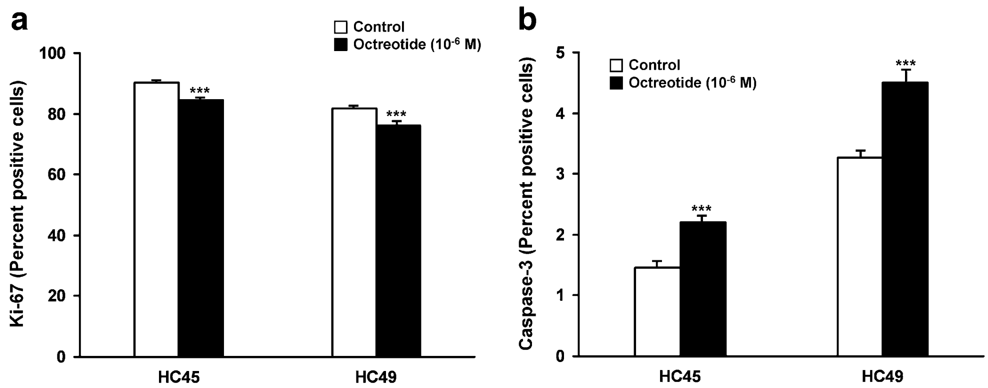

Fig. 7 Effects of octreotide on cell proliferation and apoptosis in HC45 and HC49 cell lines. **a** TGFβ1 caused a decrease in Ki-67 positive cells in both cell lines. **b** Apoptosis was increased in both cell lines after treatment with 10^{-6} M octreotide in HC45 and HC49 cell lines. Cells were treated with octreotide for 3 days. *Triple asterisks* $p<0.001$

identified as differentially expressed in groups being compared if: (1) fold change was equal or greater than the cutoff value (twofold) and (2) FDR value was 0.09 or less.

Gene Array Validation

Validation was done by Western blotting and real-time PCR. As previously reported [24], reverse transcriptase real-time quantitative PCR (RT-qPCR) was performed by the LightCycler system (Roche) using the FastStart DNA Master SYBR Green 1 kit (Roche) as previously reported [24].

Statistics

For immunohistochemical evaluation of staining 500 cells were counted and the percentage of positive cells determined. A lmm^2 grid was used in the ocular of the microscope and cells in the grid counted moving up and down the slides sequentially. Experiments were run in duplicates and repeated three to four times. The Student's *t* test with the two-tail analysis was used for statistical comparison.

Results

Characterization of Cell Lines

Cell lines from the ileal carcinoid HC45 and rectal carcinoid HC49 were obtained 6 weeks after infection of the cells with SV40R4. Immunohistochemical staining for SV40 showed nuclear localization of the protein (Fig. 1a). Both cell lines expressed synaptophysin (Fig. 1b) and chromogranin A (Fig. 1c). Immunohistochemical staining of the cells showed somatostatin receptor 2 in both cell lines and HC45 also stained for somatostatin receptor 5 (not shown). The cell lines also expressed chromogranin A, chromogranin B, and 7B2. The tumor cells were also positive for vitamin D3 receptor (Fig. 1d) and rare tumor cells from both groups were positive for VMAT1 (data not shown). Serotonin staining was negative in both cell lines. The parental cell lines in the liver metastases both expressed chromogranin A, chromogranin B, 7B2, VMAT1, and serotonin (data not shown).

Ultrastructural studies showed rare secretory granules ranging in size from 100 to 300 nm in diameter in the

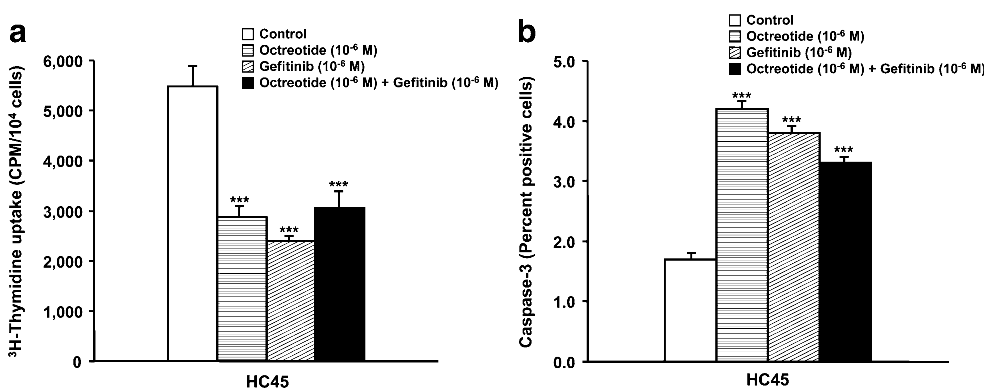

Fig. 8 Analysis of octreotide and gefitinib on cell proliferation and apoptosis in HC49 cells. **a** Both octreotide (10^{-6} M) and gefitinib (10^{-6} M) inhibited proliferation of HC45 cells in cell lines. The combined use of both drugs was not additive. **b** Both octreotide (10^{-6} M) and gefitimib (10^{-6} M) stimulated apoptosis in HC45 cells. The combined effects of both drugs were not additive. Cells were treated with octreotide and gefitinib for 4 days alone or in combination. *Triple asterisks* $p<0.001$

cytoplasm of the tumor cells. The cells contained well-developed rough endoplasmic reticulum and Golgi complexes (Fig. 1e).

Western blot analysis of the metastatic tumor in the liver and the cell line showed expression of TGFβ receptor 1 and 2 (Fig. 2) as well as EGFR and activated EGFR had the predicted molecular sizes (Fig. 3). The metastatic tumors in the liver had high TGFβ but low amounts of EGFR compared to the cell lines (Fig. 3).

Reverse transcription polymerase chain reaction (RT-PCR) analysis showed a 284-base pair product for somatostatin receptor 2a in both cell lines (Fig. 4). The metastatic tumors to the liver also expressed somatostatin receptor 2a.

Cytogenetic analysis by G-banding showed the following karyotype for HC45: a85-90,XXXX,add(1)(q21),-17add(19)(q13.3),der(20;22)(q13.3;q13)ins(20:?)(q13.3;?),dic(20;?)(q13.3),-22,+0-2mar[cp15]/b43-47,XX,abnormal nonclonal[4]/n46,XX[1] and for HC49:a40-43,X,X,der(X)t(X;5)(p22.1;q11.2),add(5)(p13),add(6)(p23),der(6;22)(p23;q13)ins(6;?)(p23;?),add(7)(q11.2),add(8)(q24.3),add(10)(p13),add(12)(q24.1),+0-3mar[cp12]/2a76-90,idemx2[cp8].

TGFβ Treatment

TGFβ treatment (10^{-9} M) inhibited proliferation of the HC45 and HC49 cell lines as measured by Ki-67 staining (Fig. 5a). All experiments were performed with cells between passages 4 to 8. There was also an increase in apoptosis as measured by caspase 3 in the cells (Fig. 5b).

When the HC45 cell line was treated with 10^{-9} M TGFβ for 4 days and the RNA extracted and analyzed for chromogranin A, chromogranin B, and 7B2, there was an increase in the expression of chromogranin A mRNA with TGFβ treatment. However, there was no significant change in expression of chromogranin B or 7B2 in the cell line (Fig. 6).

Octreotide and Gefitinib Treatment

Analysis of HC45 and HC49 cell lines with octreotide showed a dose-dependent decrease in proliferation 1×10^{-10} to 10^{-6} M octreotide (data not shown). Treatment of HC45 cells with 10^{-6} M octreotide resulted in a decrease in cell proliferation as determined by Ki-67 expression (Fig. 7a). The cells showed a concomitant increase in apoptosis as evaluated by caspase 3 (Fig. 7b).

Treatment of HC45 cells with gefitinib showed an inhibition of proliferation as judged by Ki-67 labeling index between 1 to 20×10^{-6} M (data not shown). Analysis of apoptosis with caspase 3 showed inhibition of apoptosis with 1 μM, but not by 10 or 20×10^{-6} M gefitinib (data not shown). Treatment with 1×10^{-6} M gefitinib led to decreased proliferation and increased apoptosis of HC45 cells in vitro

Table 2 RNA Profiling Showing Genes Significantly Upregulated and Downregulated in HC45 Cell Line After TGFβ1 (10^{-9} M) Treatment

Gene Symbol	Gene Name	Change	Common	Chromosome	Description
NEDL2	232080 at	13.08	NEDL2	2q33.1	NEDD4-related E3 ubiquitin ligase
VDR	204254 s	7.149	VDR	12q12-q14	Vitamin D3 receptor (1,25-dihydroxyvitamin)
ADAM19	209765 at	6.488	ADAM19	5q32-q33	A disintegrin and metalloproteinase
TPM1	206117 at	6.29	TPM1	15q22.1	Tropomyosin 1 (alpha)
6ARK5	204589 at	5.905	ARK5	12q24.11	
CSPG2	204620 s	5.812	CAPG2	5q14.3	Chondroitin sulfate proteoglycan 2
FOXC2	239058 at	5.359	FOXC2	16q22-16q	Forkhead box C2 (MFH-1, mesenc)
–	222802 at	4.422	–	–	Homo sapiens endothelin-1 (EDN)
COL5A1	203325 s	4.268	COL5A1	9q34.2-q34	Collagen, type V, alpha 1
–	210987 x	3.832	–	–	Tropomyosin; human tropomyosin
SRPUL	205499 at	2.509	SRPUL	Xq21.33-q2	Sushi-repeat protein
MT1H	206461 x	2.412	MT1H	16q13	Metallothionein 1H
TIMP1	201666 at	2.057	TIMP1	Xp11.3-p1	Tissue inhibitor of metalloproteinase
–	226806 s	−2.65252	–	–	MRNA, cDNA DKFZp686J23256
NFIB	209289 at	−2.793296	NFIB	9p24.1	Nuclear factor I/B
CLU	208792 s	−3.164557	CLU	8p21-p12	Clusterin (complement lysis inhibitor)
TM4SF1	215034 s	−3.663004	TM4SF1	3q21-q25	Transmembrane 4 superfamily mem)
SLC39A8	209267 s	−6.289308	SLC39A8	4q22-q24	Solute carrier family 39 (zinc trans)
CAMK2D	228555 at	−7.246377	CAMK2D	4q26	Calcium/calmodulin-dependent pro)
PPAP2B	209355 s	−8.196721	PPAP2B	1pter-p22	Phosphatidic acid phosphatase type
–	229004 at	−8.403361	–	–	CDNA FLJ26557 fis, clone LNF01
CLECSF2	209732 at	−8.474576	CLECSF2	12p13-p12	C-type (calcium dependent, carbo)
CYP1B1	202436 s	−8.849558	CYP1B1	2p21	Cytochrome P450, family 1, subfar
C4A	208451 s	−30.67485	C4A	6p21.3	Complement component 4A

Only genes which showed a twofold or greater change with an FDR value ≤0.09 are shown.

(Fig. 8a,b), so this concentration of gefitinib was used in all experiments.

When the HC45 cells were treated with combined octreotide (10^{-6} M) and gefitinib (10^{-6} M), there was an inhibition of cell proliferation as judged by Ki-67 expression and ^{3}H thymidine incorporation. The combined treatment of octreotide and gefitinib also led to an increase in apoptosis in the treated cells compared to the controls (Fig. 8a). However, the combined treatment was not any more effective in inhibiting proliferation or stimulating apoptosis than the two individual drug treatment (Fig. 8b).

Expression Profiling Analysis

When the HC45 cells were analyzed by DNA microarray after treatment with TGFβ (10^{-9} M) for 4 days, there was upregulation or downregulation of several genes from which the fold change was greater than the cutoff value (twofold) and the FDR was ≤0.09 (Table 2). Vitamin D3 receptor was upregulated sixfold. This gene was further analyzed by real-time PCR (Fig. 9a), Western blotting (Fig. 9b), and immunohistochemistry (Fig. 1d). Real-time PCR (Western blotting) showed an increase in the RNA and protein expression after TGFβ treatment.

A series of primary and metastatic carcinoids (n=8 cases) were analyzed to determine if the primary and metastatic tumors also expressed vitamin D3 receptor. There was expression of vitamin D3 receptor in both primary and metastatic carcinoid tumors by Western blotting (Fig. 9c).

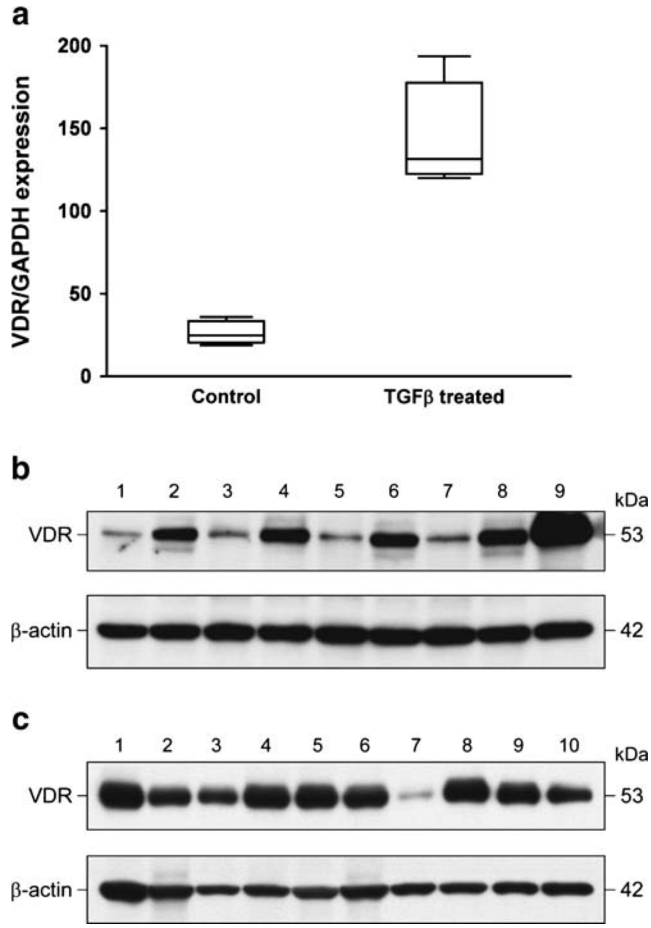

Fig. 9 Real time RT-qPCR and Western blot analysis showing the effects of TGFβ1 (10^{-9} M) treatment on vitamin D3 receptor expression in HC45 cells. **a** In the RT-qPCR analysis, the *whiskers* represent the maximum and minimum values. The *boxes* represent the 25th and 75th percentile ranges of values and the *bar* represents the median value. There was more than a threefold higher level of expression of vitamin D3 receptor mRNA after TGFβ1 treatment. **b** There was a three- to fourfold increase in vitamin D3 receptor after TGFβ1 treatment. Lanes 1, 3, 5 and 7 are untreated controls while lanes 2, 4, 6 and 8 are TGFβ1-treated cells. Lane 9 is a positive control. **c** Analysis of primary ileal carcinoid and metastatic ileal carcinoid tumors to the liver for vitamin D3 receptors showing expression in all of the tumors. Lanes 1, 3, 5, 7 and 9 are primary carcinoids while lanes 2, 4, 6, 8 and 10 are matching metastatic carcinoids

Discussion

Carcinoid tumors from the ileum are difficult to grow in culture, so there are relatively few of these cell lines available for in vitro studies [4, 5, 7, 8]. In this study, we generated new ileal and rectal carcinoid cell lines which were used for in vitro studies. The cell lines both showed a complex karyotype which is not uncommon in malignant cells. Our studies showed that both cell lines expressed TGFβ receptors and responded to TGFβ treatment by inhibition of proliferation and increased apoptosis as measured by caspase 3. The HC49 cell line had significantly more apoptosis after treatment with TGF beta compared to the HC45 cell line and suggests differences in susceptibility to programmed cell death between the two cell lines. The cell lines also expressed somatostatin receptors.

Analysis of EGFR receptors in the HC45 ileal carcinoid cell line showed that the cultured tumor cells expressed EGFR and only a weak band for activated EGFR was detected. EGFR has a major effect on many tumors because activation of EGFR stimulates tumor growth, invasion, and metastasis [15, 26, 27]. Recent studies by Hopfner et al. [15] showed that gefitinib could inhibit the growth of slowly as well as rapidly proliferating neuroendocrine tumor cell lines by cell cycle arrest in the more rapidly growing cells to a predominantly apoptotic effect in the more slowly growing cell lines [15]. A phase II trial of gefitinib for metastatic carcinoid tumor has been reported with 51% of 55 patients with previously progressing tumors showing prolonged stabilization of disease beyond 6 months [28]. Our studies showed that gefitinib at $\times 10^{-6}$ M inhibited both cell growth and stimulated apoptosis in the HC45 cell line. However, higher concentrations of getifinib (10×10^{-6}

and 20×10^{-6} M) inhibited proliferation, but did not stimulate apoptosis in the HC45 cells. There was a decrease in apoptosis as measured by caspase 3 with 10 and 20×10^{-6} M gefitinib. The mechanism regulating these differences is currently being investigated. It is interesting to note that a combination of octreotide (1×10^{-6} M) and gefitinib (1×10^{-6} M) on cell growth inhibition were not additives which would suggest that similar intermediate pathways may be involved with growth inhibition and apoptosis with these two drugs.

We used Ki67 labeling and ^{3}H-thymidine incorporation to study proliferation in the HC45 cells. Based on these experiments, the ^{3}H-thymidine method appears to be more sensitive as a measure of proliferation because of the in vitro incorporation of labeled thymidine.

We examined the effect of TGFβ expression on the neuroendocrine markers chromogranin A, chromogranin B, and 7B2 [29, 30]. We observed that while chromogranin B and 7B2 were constant after TGFβ treatment, chromogranin A messenger RNA was increased two- to fourfold after TGFβ treatment for 4 days. This finding was associated with inhibition of cell growth and increased apoptosis. This observation of differences in the response of these neuroendocrine cell granins to TGFβ stimulation might suggest that chromogranin A might be the principle regulatory granin in neuroendocrine cells [31]. However, other workers have found that chromogranin B was also important for granule function [32]. A recent report from our laboratory also showed that transfection of chromogranin A into nonneuroendocrine anterior pituitary cells induced neuroendocrine differentiation in these cells and concomitantly inhibited cell growth [33] which would be similar to the increased expression of chromogranin A in HC45 cells with inhibition of cell growth in the present study. Another recent study also reported on the influence of chromogranin A in differentiation in nonneuroendocrine cells [34].

RNA profiling studies with HC45 cells before and after treatment with TGFβ1 revealed a small number of genes whose expressions were significantly increased or decreased in the present study. We observed for the first time, expression of vitamin D3 receptor in intestinal carcinoid tumors. The presence of vitamin D3 receptor and the increased expression of this receptor after TGFβ treatment were validated by RT-PCR, Western blotting, and immunohistochemical studies. These findings suggest that vitamin D3 receptor probably has an important role in differentiation of midgut carcinoid tumors. The role of vitamin D3 and its receptor in regulating tumor growth has been well characterized in knock-out mouse models and have been studied in some human tumors [35, 36]. We examined vitamin D3 receptor in tissues from primary and metastatic ileal carcinoids and found expression of this steroid receptor in all tumors adding support to our hypothesis that vitamin D3 receptor may have an important role in ileal carcinoid cell growth because vitamin D is actively absorbed in the intestine.

The use of carcinoid cell lines induced by replication defective recombinant adenovirus expressing an SV40 early T-antigen insert has been useful for short-term experiments. The utility of these cell lines for long-term experiments is uncertain. However, a human pituitary cell line (HP75) induced with an SV40 early T-antigen insert has been very useful for research studies after more than 10 years in the laboratory [22, 37]. Both the HC45 and HC49 cell lines have been maintained in our laboratory for the past 2 years. The proliferative activity of the cells has been maintained after up to 20 passages in vitro.

In summary, we have developed carcinoid cell lines from the ileum and rectum by adenovirus infection and have shown that the cells express receptors for several growth factors and respond to drugs targeting the TGFβ receptor, EGFR receptor, and somatostatin receptors. These results indicate that in vitro studies with these cell lines should help to elucidate the mechanisms regulating proliferation and apoptosis in ileal and rectal carcinoid tumors.

Acknowledgments This work was supported in part by a grant from Dr. and Mrs. Raymond R. and Beverly Sackler. The authors thank AstraZeneca, Great Britain for providing gefitinib and Dr. J.C. Thompson, University of Texas Medical Branch, Galveston, Texas for BON cell line.

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