Further delineation of the continuous human neoplastic enterochromaffin cell line, KRJ-I, and the inhibitory effects of lanreotide and rapamycin

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Abstract

Small intestinal carcinoids (SICs) are the most prevalent gastrointestinal carcinoid and characterized by local invasion metastasis and protean symptomatology. The proliferative and secretory regulation of the cell of origin, the enterochromaffin (EC) cell has not been characterized. The absence of either a pure preparation of normal EC cells or human EC carcinoid cell lines has hindered the development of therapeutic agents. We therefore further characterized the neoplastic SIC cell line, KRJ-I by assessing its secretory (serotonin (5-HT)) and proliferative responses and defining its log growth phase transcriptome. Electron microscopy demonstrated oval, lobulated nuclei and substance P, and 5-HT-positive cytoplasmic vesicles. RT-PCR detected transcripts for chromogranin A (*CHGA*), *VMAT1* (SLC18A1), tryptophan hydroxylase (*TPH1*), substance P (*TAC1*), guanylin (*GUCA2A*), and SERT (*SLC6A4*). By immunohistochemistry, all cells were positive for CHGA, SERT, VMAT1, and TPH1. Transcriptome analysis (Affymetrix U133 Plus chips) identified somatostatin SSTR2/3, adrenergic α 1C and β 1, dopamine D2, nicotinic-type cholinergic A5, A6, B1, muscarinic acetylcholine M4, and 5-HT-2A receptors. The presence of transcripts for SSTR1, SSTR2, and SSTR3 receptors was confirmed by RT-PCR and sequencing. Isoproterenol (ISO) resulted in a dose-dependent increase in intracellular cAMP (EC_{50} =340 nM) and 5-HT (EC_{50} =81 nM) which was completely inhibited by the cAMP antagonist 2',5'-dideoxyadenosine (10 μ M). Preincubation with a SSTR agonist, lanreotide, inhibited Ip-stimulated 5-HT secretion (IC_{50} =420 nM). Both lanreotide (10 nM) and rapamycin (50 nM) inhibited proliferation (20 ± 12 and $35 \pm 5\%$ respectively) in serum-free medium whereas gefitinib (1 nM–10 μ M) inhibited proliferation at micromolar concentrations. KRJ-I is a neoplastic EC cell line that can be used as an *in vitro* model of SICs as it will allow elucidation and clarification of the secretory and proliferative mechanism(s) of neoplastic EC cells and the molecular signatures that characterize each of these responses.

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Introduction

Neuroendocrine or 'carcinoid' tumors of the gut are generally misperceived as a rare, indolent form of neoplasia, have not rigorously been studied, are poorly understood and usually misdiagnosed. However, the perception that these tumors are exceedingly rare has been altered by the introduction of diagnostic strategies, including endoscopy, the measurement of plasma biochemical markers such as chromogranin, and nuclear medicine techniques, including somatostatin receptor scintigraphy (SRS; Boushey & Dackiw 2002). Thus, a review of the current Surveillance Epidemiology and End Results (SEER) database indicated that small intestinal carcinoids (SICs) are not only the most prevalent gastrointestinal (GI) carcinoid but have also increased in incidence by 740% over the last three decades (Modlin *et al.* 2003). Similarly, the concept that

SICs are benign and indolent is meaningless given the recognition that these lesions exhibit locally aggressive invasion, substantial lymph node and hepatic metastasis with an overall 5-year survival rate of <60% (Modlin *et al.* 2003, 2005). Additionally, they often secrete bioactive agents, including connective tissue growth factor (*CCN2/CTGF*), that result in both local (peritoneal adhesions and bowel obstruction) and distant (cardiac and pulmonary) fibrosis reflecting the intrinsically neoplastic and biologically malignant phenotype of this neuroendocrine neoplasia (Modlin *et al.* 2004, Kidd *et al.* 2006b).

Although the cell of origin of SIC has been identified as the enterochromaffin (EC) cell, its secretory and proliferative regulation is unknown and, as a result, the progress in the development of effective therapeutic strategies has been limited. The characterization of the receptor profile, transcriptome, and mechanistic basis

of neoplastic EC cell function is critical to defining the molecular basis of SIC disease. The availability of such information is necessary to identify appropriate secretory and proliferative regulatory targets and facilitate clinical management of this disease. To date, three human cell lines of variable applicability, namely the BON pancreatic tumor cell line (Evers *et al.* 1991), the COLO320DM cell line (Quinn *et al.* 1979) and the GOT1 cell line (Kolby *et al.* 2001) have been utilized as *in vitro* models considered to be representative of human GI carcinoids. The BON cell line, however, is a more accurate model of a pancreatic endocrine tumor (adenocarcinoid) rather than a GI carcinoid tumor (Zikusoka *et al.* 2005), while the COLO320DM cell line was derived from a neuroendocrine-differentiated human colorectal cancer rather than an EC cell-derived carcinoid (Park *et al.* 1987). The GOT1 cell line was established from a liver metastasis (of an ileal carcinoid tumor) rather than the primary GI tumor, and is characterized by a long (>18 days) doubling time (Kolby *et al.* 2001). In this manuscript, we further characterize the rapidly growing, long-term, primary tumor-derived, human ileal EC carcinoid cell line, KRJ-I.

KRJ-I is a continuous cell line (Pfragner *et al.* 1996) with a doubling time of approximately 2 days that displays classical morphological and immunocytochemical features of a carcinoid (Pfragner *et al.* 1996). It was established in 1992 from a multifocal ileal carcinoid tumor with an insular histological appearance (type I) from a 75-year-old Caucasian male. To define whether KRJ-I could be utilized as an appropriate model system for the investigation of small intestinal EC carcinoids, our aims were to: (1) confirm the neuroendocrine, EC cell lineage of KRJ-I cells; (2) delineate their morphology; (3) define the KRJ-I transcriptome; (4) identify receptors potentially involved in secretory and proliferative regulation; (5) characterize serotonin secretion; and (6) investigate whether compounds with anti-proliferative effects in other neuroendocrine tumors, namely lanreotide (Imam *et al.* 1997), rapamycin (von Wichert *et al.* 2000), and gefitinib (Hopfner *et al.* 2003), inhibited KRJ-I proliferation.

Materials and methods

Cell culture

KRJ-I cells grow as a suspension culture and were cultured in Ham's F12 medium (Gibco) supplemented with 10% fetal calf serum (Sigma-Aldrich) and antibiotics (100 U penicillin/ml + 100 µg streptomycin/ml, Sigma-Aldrich). Cells were incubated at 37 °C with 5% CO₂. Cell counts were undertaken using a Coulter ZM particle counter. The population doubling level (PDL)

was calculated using the formula: $n = 3.32 (\log X_2 - \log X_1)$, where X_2 is the cell count at the end of a given subculture and X_1 is the cell count of the original subculture inoculum (Hayflick 1973). PDL 20–25 was used for experiments.

Confirmation of neuroendocrine, enterochromaffin cell origin of KRJ-I

RT-PCR, immunocytochemical, and electron microscopy studies were undertaken for confirmation and characterization using established neuroendocrine and EC cell markers.

Real-time RT-PCR

RNA was extracted from 5×10^6 KRJ-I cells in log phase growth ($n=3$) using TRIZOL (Invitrogen), and then cleaned using the Qiagen RNeasy kit in conjunction with the DNeasy Tissue kit (Qiagen Inc.) to ensure the absence of any contaminating genomic DNA. The clean RNA was then converted to cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems).

RT-PCR was performed as described by Kidd *et al.* (2005) using the set of SSTR-specific primers designed by Jung *et al.* (2006). Transcripts for *ADRB1*, *CHGA*, *SLC18A1* (VMAT1), *TPH1*, *TAC1* (substance P), *GUCA2A*, *CHRM4*, and *SLC6A4* (serotonin transporter) were amplified using primers and PCR conditions detailed in Table 1. Purified PCR products were sequenced and analyzed by the W M Keck Biotechnology Resource Laboratory at Yale University using Applied Biosystems Dye terminator chemistry and an automated Applied Biosystems 373A Stretch DNA sequencer (Perkin-Elmer, Norwalk, CT, USA).

Immunohistochemistry

KRJ-I cells (5×10^4 cells/antibody) were fixed in methanol and pipetted onto frosted microscope slides stained with a series of antibodies: (neuroendocrine specific: CHGA antibody (1:1000, DakoCytomation), or VMAT1 (1:100; Chemicon, Temecula, CA, USA); EC cell-specific: TPH (1 µg/ml, Calbiochem, San Diego, CA, USA), serotonin (DAKO, 1:20), or substance P (1:200, GeneTex, San Antonio, TX, USA) and 4',6'-diamidino-2-phenylindole (DAPI) (20 mg/ml) overnight at 4 °C. After washing (0.1% Tween/phosphate buffered saline (PBS)), cells were stained with secondary antibody (fluorescein isothiocyanate (FITC) anti-mouse/rabbit, 1:100, Promega; 1 h/room temperature). Fluorescence microscopy (Leica, Mannheim, Germany) was used to visualize and count the number of positive cells, with 1×10^3 cells counted for each antibody. As a control for non-specific staining, primary antibodies were excluded in matched groups.

Table 1 Primers sequences used for RT-PCR amplification of neuroendocrine and EC cell markers from KRJ-I

Marker	Primer sequence (5' → 3')	Reference
β-adrenergic receptor type I (ADRB1)	F: CCGCTGCTACCACGA CCCCAG R: AGCCAGTTGAAGAAGAGCAAGAGGCG	Chernogubova <i>et al.</i> (2005)
Muscarinic type 4 receptor (CHRM4)	F: TCCTCAAGAGCCCACTAATGAAGC R: TTCTTGCGCACCTGGTTGCGAGC	Buchli <i>et al.</i> (2001)
Chromogranin A (CHGA)	F: GAGTGGGAGGACTCCAAACG R: CCACTTTCTCCAGCTCTGCC	Begueret <i>et al.</i> (2002)
VMAT1 (SLC18A1)	F: TGACATGGAGTTCAAAGAAGTCAAC R: GAGAGCGAGGGCATGTG	Huynh <i>et al.</i> (2005)
Tryptophan hydroxylase (TPH1)	F: CCCTTTGATCCCAAGATTAC R: CATTGATGGCACTGGTTATG	Gustafsson <i>et al.</i> (2006)
Substance P (TAC1)	F: ACTGTCCGTGCGAAAATCC R: ACTGCTGAGGCTTGGGTCTC	Harmar <i>et al.</i> (1986)
Guanylin (GUCA2A)	F: TCAGAGGCTGGAGGAAATCG R: GCTGGGAGTGGAGTATCATG	Lee <i>et al.</i> (2004)
SERT (SLC6A4)	F: GGGTACTCAGCAGTTCCAAG R: ATGTAAGAGCGGGATTCT	Maurer <i>et al.</i> (2004)

F, forward; R, reverse.

Confocal microscopy

For confocal microscopy, micrographs were recorded using a confocal microscopy system equipped with three Ar488, Kr568, and HeNe633 lasers (TCS-SP; Leica, Mannheim, Germany) 400× magnification.

Morphological characterization

Transmission electron microscopy (TEM) and immunogold staining were undertaken in conjunction with the Center for Cell and Molecular Imaging at Yale Medical School. For TEM, 2×10^6 KRJ-I cells, either unstimulated or treated with 10^{-7} M lanreotide for 10 min, were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4) for 1 h, washed, and postfixed in 1% osmium tetroxide in the same buffer. Fixed samples were processed for Epon embedding and thin-sectioned for TEM. Samples were viewed at 80 kV using a Tecnai 12 Biotwin electron microscope (Hillsboro, OR, USA). For immunogold labeling of KRJ-I cells, cells were fixed in 4% paraformaldehyde and thereafter embedded in gelatin. The cells were infused with 2.3 M sucrose overnight at 4 °C and pieces of pellet transferred to liquid nitrogen. Following the initial preparation of ultrathin cryosections, unreacted aldehyde groups were first quenched with 0.1 M NH_4Cl in PBS for 10 min at room temperature (RT), and then non-specific-binding sites blocked by the addition of 1% fish skin gelatin in PBS for 20 min at RT. The primary antibody (TPH: 0.1 µg/ml) diluted in PBS/1% fish skin gelatin (FSG) was added for 30 min at RT and after washing four times in PBS, grids were labeled with Protein A Gold for 30 min at room temperature. For double labeling, sections were exposed to mouse monoclonal serotonin (1:5, DAKO) or mouse monoclonal chromogranin A (1:50, DAKO) for 2 h followed by polyclonal rabbit substance P (1:50,

GeneTex) for a further 2 h. Specimens were washed thrice in PBS followed by 1-h incubation in a mixture of goat anti-rabbit 10 nm gold and goat anti-mouse 5 nm gold antibodies. Thorough rinsing (five PBS washes) was followed by a 3-min postfixation in 1% glutaraldehyde. The labeled replicas were floated on distilled water and picked up on copper 460 grids for electron microscopic examination. All sections were mounted using Citifluor mounting medium (Agar Scientific, Stansted, Essex, UK). The following controls were run in parallel: (1) omission of primary antibody; (2) switching of detection systems in single labeling experiments (e.g. using mouse monoclonal followed by anti-rabbit secondary); (3) reversing the order of primary antibodies in the double labeling procedure. Grids were air-dried at room temperature and examined by electron microscopy (Tecnai 12 Biotwin electron microscope) at 80 kV.

KRJ-I transcriptome

An Affymetrix GeneChip approach (Human U133 Plus 2 Array) was used to acquire the KRJ-I transcriptome as follows. RNA was extracted from two replicates of 5×10^6 KRJ-I cells in log phase (described previously). For each hybridization experiment ($n=2$), 10 µg high-quality (ratio > 1.9) total RNA was provided to the Keck Affymetrix facility (Santa Clara, CA, USA) to perform cRNA labeling, hybridization, and data analysis (<http://keck.med.yale.edu/affymetrix>). The hybridized arrays were scanned using an Affymetrix GeneChip 3000 Scanner. Arrays were scaled to an average intensity of 500 and hybridization intensity data converted into presence/absence calls for each gene using GeneChip Operating Software (Affymetrix Inc.). The signal intensity data were then statistically analyzed using S-plus 2000 software (Mathsoft) to obtain

correlation coefficients and scatter plots for evaluation of the reproducibility and quality of the array analysis. All microarray data have been deposited in the minimum information about microarray experiment (MIAME)-compliant gene expression omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>) GEO accession number GSM105630. The GeneChip data were examined for the presence of neuroendocrine transcripts *CHGA* and *SLC18A1* as well as the EC cell-specific transcripts *TPH1*, *SLC6A4*, *TAC1*, and *GUCA2A* and the cell cycle marker *Ki-67*.

In addition, the presence of RNA encoding somatostatin receptor (SSTR) transcripts, adrenergic, dopaminergic, cholinergic, and muscarinic receptor transcripts was assessed, since we and other researchers (Modlin *et al.* 1994, Sandor *et al.* 1996, Lindstrom *et al.* 1997) have demonstrated the relationship of these receptors in the proliferative and secretory regulation of the enterochromaffin-like cells (ECL), the gastric neuroendocrine equivalent of the small intestinal EC cell. The presence of transcripts for putative regulatory receptors in KRJ-I cells was confirmed using reverse transcription (RT)-PCR as described previously.

Physiological characterization

Serotonin secretion

Isoproterenol and a somatostatin analog (BIM-23014C/lanreotide), previously identified as secretory regulators in a variety of neuroendocrine cell types, were used to evaluate serotonin (5-HT) secretion (Danila *et al.* 2001). Isoproterenol activates adenylate cyclase via β -adrenergic receptors and is a secretory agonist, whereas lanreotide is a somatostatin analog with high affinity ($IC_{50} < 1$ nM) for SSTR2 and exhibits inhibitory activity. Isoproterenol was dissolved in water, and BIM-23014C was dissolved in 0.01 M acetic acid containing 0.1% BSA (buffer A). Treatment of the cultures with solvent alone was used as the baseline control. Twenty-four hours prior to the addition of the individual compounds, cells were transferred to serum-free medium and seeded in 96-well plates at a density of 5×10^4 cells/well. To identify the optimal time frame for the dose–response experiments, cells were initially stimulated over 60 min with 10 μ M isoproterenol (Tran *et al.* 2004). Media were then collected from three wells for each time-point (1, 3, 10, 15, 20, 30, 45, and 60 min) and serotonin measured using a commercially available serotonin EIA (Labor Diagnostika Nord, GmbH & Co., Nordhorn, Germany) according to manufacturer's instructions for serum samples. Serum-free growth medium was used as a control. Experiments were repeated in duplicate. Once the optimal time course for 5-HT stimulation had been determined for isoproterenol, dose–response curves were generated by stimulating KRJ-I cells (5×10^4 cells/well, each concentration in

triplicate) with a range of isoproterenol concentrations (10^{-4} – 10^{-9} M). The EC_{50} of isoproterenol for serotonin secretion was calculated from nonlinear regression analysis using Prism 4 (Graphpad Software). In order to establish whether serotonin release was due to cell lysis: (1) cell viability was evaluated by Trypan Blue exclusion after the addition of isoproterenol or BIM-23014C and compared with the count taken prior to addition of the analogue; and (2) lactate dehydrogenase (LDH) release was measured using a commercially available LDH assay (Promega) according to the manufacturer's instructions for cultured cells to provide an indication of membrane integrity. The effect of a 10-min preincubation of KRJ-I cells with a range of BIM-23014C concentrations (10^{-6} – 12×10^{-12} M) prior to evaluating the effect of isoproterenol (EC_{50}) on serotonin secretion was also studied.

β -adrenergic receptor activation

To test whether KRJ-I cells were physiologically responsive to the selective β -adrenergic receptor agonist isoproterenol, intracellular cAMP generation in response to isoproterenol was assayed using a cAMP ELISA assay (R&D Research). Cells (5×10^4 cells/well, in triplicate) were stimulated with isoproterenol (10^{-4} – 10^{-8} M) after which 0.1 M HCl was added to each well to lyse the cells and the content then transferred to 1.5 ml Eppendorf tubes and centrifuged at 600 g for 10 min. The supernatant was transferred to clean 1.5 ml tubes and dried using a Savant SpeedVac. Pellets were resuspended in 200 μ l Assay buffer ED2. All samples and controls were acetylated prior to performing the ELISA (R&D cAMP ELISA handbook). Absorbance readings were measured at 405 nm on a microplate reader (Bio-Rad 3500).

To evaluate whether serotonin secretion is specifically mediated by cAMP in neoplastic EC cells, KRJ-I cells, 5×10^4 cells/well (in triplicate) were incubated for 30 min with the cell-permeable adenylyl cyclase inhibitor 2',5'-dideoxyadenosine (2',5',-dd-Ado, 10^{-5} M; Sigma-Aldrich) followed by isoproterenol stimulation (10^{-6} M) for 45 min. Isoproterenol-stimulated serotonin secretion in the absence of 2',5',-dd-Ado was assayed in parallel cells (5×10^4 cells/well in triplicate). Serotonin content was assayed as detailed previously.

KRJ-I proliferation: effect of BIM-23014C (lanreotide), rapamycin, and gefitinib

It has previously been reported that proliferation of the BON cell line can be inhibited by lanreotide, rapamycin, and gefitinib (Imam *et al.* 1997, von Wichert *et al.* 2000, Hopfner *et al.* 2003). To evaluate whether these compounds inhibited the growth of the KRJ-I cell line, cells were seeded in 96-well plates at 5×10^4 cells/well in Ham's F12 and 100 U penicillin/ml + 100 μ g streptomycin/ml. BIM-23014C (dissolved in buffer A),

rapamycin, and gefitinib (both dissolved in dimethylsulfoxide (DMSO)) were added at concentration ranging from 1 nM to 1 μ M ($n=8$ wells for each substance; *Zatelli et al. 2001*). The final maximal concentration of buffer A or DMSO in experimental and control media was 0.1%. Cells were incubated for 72 h after the addition of each compound, after which MTT was added to a final concentration of 0.5 mg/ml per well. Following an additional 3-h incubation period at 37 °C, 5% CO₂, the reaction was stopped by adding acid-isopropanol and the formazan dye solubilized by adding an equal volume of acid-isopropanol as described previously. The optical density was read at 595 nm using a microplate reader (Bio-Rad 3500).

Results

Confirmation of neuroendocrine, enterochromaffin origin of KRJ-I cells

RT-PCR and immunohistochemistry

Transcript for *CHGA*, *SLC18A1* (VMAT1), *TPH1*, *TAC1*, *GUCA2A* (guanylin), and *SLC6A4* (SERT) were identified in KRJ-I cells (Fig. 1A) and all were positive for

CgA and TPH proteins (Fig. 1B and C) as well as SERT and VMAT1, confirming their neuroendocrine, EC cell origin. Using confocal immunofluorescence microscopy, 100% of cells were both substance P- and serotonin-positive (Fig. 2).

KRJ-I morphology

KRJ-I cell ultrastructure demonstrated oval or irregular and lobulated nuclei, with most containing only one nucleolus. Well-developed Golgi and numerous mitochondria were present. Dense vacuolated granules were rarely found in the cytoplasm of unstimulated KRJ-I cells (data not shown). Of interest was the presence of numerous vesicles in cells preincubated with the somatostatin receptor agonist, lanreotide. TPH immunogold staining demonstrated association with the endoplasmic reticulum (ER) and Golgi (Fig. 3A). Double immunogold labeling showed colocalization of serotonin and substance P on the endoplasmic reticulum (Fig. 3B), Golgi, multivesicular bodies as well as exocytic endosomal vesicles. Substance P and chromogranin A were similarly colocalized (data not shown). Of interest was the abundance of substance

Figure 1 RT-PCR and immunohistochemical confirmation of the neuroendocrine enterochromaffin origin of KRJ-I cells. (A) RT-PCR products run on a 3% agarose gel and later excised for sequence analysis. Lane 1, *SLC6A4* (SERT, 270 bp); lane 2, *TAC1* (212 bp); lane 3, *SLC18A1* (VMAT1, 76 bp); lane 4, *ADRB1* (441 bp); lane 5, *TPH1* (211 bp); lane 6, *CHRM4* (430 bp); lane 7, *CHGA* (336 bp); lane 8, *GUCA2A* (guanylin, 158 bp). The size and the identity of these PCR products were confirmed by sequence analysis. A size bar (base pairs) is on the left. (B) KRJ-I cells (Cy-5 anti-chromogranin A, DAPI for nuclei, 400 $\times$ magnification) demonstrating uniform staining for this protein. (C) KRJ-I cells (FITC anti-TPH, DAPI for nuclei 400 $\times$ magnification) demonstrating uniform staining. Scale bars for each of the pictograms are included.

Figure 2 Confocal immunofluorescence microscopic picture of triple-stained KRJ-I cells. (A) Nuclei (blue, DAPI). (B) Substance P (green, FITC). (C) Serotonin (red, Cy5). (D) Co-localization (yellow) of substance P and serotonin can clearly be identified within the cytoplasm.

P in the cells as well as identification of non-vesicular cytoplasmic substance P and serotonin in KRJ-I cells. The presence of substance P-positive exosomal bodies was also noted (Fig. 3C).

Transcriptome and receptor profiling

The two KRJ-I GeneChip hybridizations showed high correlation coefficients ($r^2=0.97$) and thus were highly reproducible. Each sample had low 3'/5' ratios of *GAPDH* <1.0 demonstrating consistent amplification not skewed to the 3' end. Of the >47 000 transcripts represented on the U133 Plus 2.0 chip, >43.0% were scored as present in KRJ-I. Transcripts for *CHGA*, *SLC18A1*, and *SLC6A4* as well as *TPH1*, *TAC1*, and *GUCA2A* were present, corroborating the RT-PCR and immunocytochemical results.

Candidate secretory and proliferative regulators initially identified by transcriptome analysis included SST receptor subtypes 2 and 3 (*SSTR2* and *SSTR3*), the $\alpha 1C$ (*ADRA1C*) and $\beta 1$ (*ADRB1*) adrenergic receptors, the dopamine D2 receptor (*DRD2*), the nicotinic-type cholinergic receptors A5 (*CHRNA5*), A6 (*CHRNA6*), B1 (*CHRNBI*), and the

muscarinic acetylcholine M4 receptor (*CHRM4*). The serotonin 2A receptor (*CHRNA5*) was also present on KRJ-I based on GeneChip data.

The presence of putative regulatory receptors in KRJ-I cells, including *SSTR2*, *SSTR3*, the *ADRB1* adrenergic receptor and the muscarinic acetylcholine M4 receptor (*CHRM4*) was confirmed using RT-PCR and transcripts were detected in all KRJ-I samples ($n=3$). Additionally, RT-PCR detected the presence of transcript for *SSTR1* and this result was confirmed by sequence analysis. This false-negative call on the GeneChip may be due to a combination of levels of transcript below the threshold of Affymetrix GeneChip detection and the presence of only two probes on the U133A chip with which to detect this transcript (Hill *et al.* 2000).

Transcript for the EGF receptor (target of gefitinib) was detected for 3/14 EGFR probes on the U133 Plus array. To confirm that transcripts for the EGFR protein were expressed, the EGFR was amplified using real-time PCR (Assays-on-Demand probe: Hs00193306_m1) and transcript was detected in all KRJ-I samples ($n=3$). Additionally, KRJ-I cells were immunostained with anti-EGFR (5F18 – Cell Signaling Technology, Danvers,

Figure 3 Electron micrographs showing (A) association of tryptophan hydroxylase with the endoplasmic reticulum (ER) and Golgi network (G), (B) colocalization of substance P (10 nm gold particles) and serotonin (5 nm gold particles) on the ER, and (C) substance P-positive exosome in extracellular space. Note the absence of background labeling in the mitochondria.

Figure 4 Isoproterenol-stimulated serotonin secretion (A) Isoproterenol-stimulated serotonin release over 1 h. Maximal serotonin secretion occurred 45 min after stimulation with 10 μ M isoproterenol. (B) Secretion of serotonin by KRJ-I cells after stimulation with isoproterenol for 45 min. The EC_{50} of isoproterenol for serotonin secretion was 81 nM. (C) Preincubation of cells with BIM-23014C/lanreotide inhibited serotonin secretion in response to 81 nM isoproterenol with an IC_{50} of 4.2×10^{-7} . Values are presented as the mean $\pm$ s.d. for $n=3$ independent experiments. Some standard errors were too small ($<4\%$) to be graphically represented.

MA, USA). Cells were uniformly immunopositive (data not shown) confirming the presence of a gefitinib-responsive receptor.

Physiological characterization

Serotonin secretion

Isoproterenol (10 μ M) stimulated maximal KRJ-I serotonin secretion at 45 min (Fig. 4A); the dose-response study demonstrated cells with an EC_{50} of 81 nM for isoproterenol (Fig. 4B). Preincubation with lanreotide inhibited isoproterenol- (EC_{50}) stimulated serotonin secretion with an IC_{50} of 0.42 μ M (Fig. 4C).

Serotonin secretion versus serotonin release. Trypan Blue counts of cells prior to stimulation and after

stimulation at different concentrations of compounds were $>95\%$ in all cases, indicating that the serotonin measured was due to active secretion rather than passive release due to cell lysis or membrane damage. Similarly, LDH measurements supported this conclusion, with no greater than a 0.1% maximal difference in LDH levels observed when comparing basal LDH levels with those of stimulated cells (data not shown).

Isoproterenol-induced cAMP accumulation. Isoproterenol induced a concentration-dependent increase of cAMP (Fig. 5A) with an EC_{50} of 340 nM.

Serotonin-secretion by KRJ-I inhibited by 2',5'-dideoxyadenosine. Preincubation of KRJ-I cells with 10 μ M cAMP antagonist 2',5'-dideoxyadenosine completely inhibited isoproterenol-stimulated serotonin secretion,

Figure 5 β -Adrenergic activation stimulates cAMP production and serotonin secretion in KRJ-I cells. (A) KRJ-I cells incubated for 45 min with isoproterenol generated increasing amounts of cAMP in a concentration-dependent manner. The concentration of isoproterenol that generated a half-maximal response (EC_{50}) was calculated using nonlinear regression analysis. The EC_{50} value of isoproterenol to increase cAMP in KRJ-I cells was calculated to be 3.4×10^{-7} M. Mean $\pm$ s.e. for $n=3$ experiments. (B) The membrane-permeable cAMP inhibitor 2',5' dideoxyadenosine (2', 5', dd-Ado, 10 μ M) completely inhibited isoproterenol (ISO, 1 μ M). stimulated serotonin secretion (measured over 45 min), with a reduction of serotonin levels to 73% of basal. Values are presented as the mean $\pm$ s.d. from three independent experiments. * $P<0.0001$ versus ISO. Some standard errors were too small ($<4\%$) to be graphically represented.

with a reduction of serotonin levels to 73% of basal (Fig. 5B), indicating that serotonin secretion by KRJ-I cells is, at least in part, cAMP mediated.

Inhibition of KRJ-I proliferation by lanreotide and rapamycin

Both lanreotide and rapamycin inhibited proliferation (Fig. 6). Lanreotide at 10 nM ($20 \pm 12\%$; Fig. 6A) and rapamycin (target of rapamycin (TOR) signaling) at 50 nM ($35 \pm 5\%$; Fig. 6B) maximally inhibited proliferation, whereas gefitinib (tyrosine kinase inhibitor, 1 nM–10 μ M) inhibited proliferation only at micromolar concentrations (Fig. 6C).

Discussion

KRJ-I is the only rapidly dividing, continuous human EC carcinoid cell line currently established. The paucity of other available carcinoid cell lines is the consequence of a number of factors including: (1) the limited amount of neoplastic tissue available for establishment of the cell line; (2) the generally slow proliferation times of carcinoid primary cell cultures; (3) substantial problems with bacterial and fungal infection given the bowel origin of the material; and (4) the low long-term survival rate of primary carcinoid cultures (Debons-Guillemain *et al.* 1982, Ahlman *et al.* 1989, Nilsson *et al.* 1992, Pfragner *et al.* 1996, Westberg *et al.* 1997).

The KRJ-I cell line was established from tissue obtained from the primary tumor of an ileal carcinoid tumor metastatic to the liver and the lymph nodes (Pfragner *et al.* 1996). The presence of markers of both neuroendocrine (CgA and VMAT1) and EC cells (TPH, SERT, and substance P) substantiate that KRJ-I is an appropriate model for *in vitro* studies of EC cell SICs.

KRJ-I also displayed morphological features characteristic of secretory cells, for example, the presence of well-developed Golgi apparatus and numerous mitochondria and the presence of substance P- and serotonin-containing vesicles consistent with secretory function. The identification of tryptophan hydroxylase localized to the ER and Golgi, and serotonin and substance P in endosomal and exosomal vesicles as well as the ER and Golgi provides further validation that this cell line is of EC origin. Transcriptome analysis revealed the presence of transcripts for a variety of known secretory and proliferative regulators of neuroendocrine cells. The presence of catecholaminergic and cholinergic pathways is consistent with current concepts of the physiology of enterochromaffin cells given that EC cells, when stimulated via β -adrenergic receptor activation – noradrenaline (Modlin *et al.* 2006, Kidd *et al.* 2006a), vagal cholinergic activation (Kuemmerle *et al.* 1987), or via luminal activation (pressure; Fujimiya *et al.* 1997) secrete 5-HT.

The basal secretion of serotonin by these neoplastic EC cells supports the clinical observation that such tumors continuously produce and release the amine into the circulation as evidenced by elevated plasma levels of serotonin and increased urinary 5-hydroxy indole acetic acid levels (Modlin *et al.* 2005). The low values of media serotonin noted over the first 15–30-min culture potentially reflects the rapid uptake of serotonin by SERT (Gershon 2003) and is supported by studies in the GOT1 cell line that demonstrate high levels of the main serotonin metabolite, 5-HIAA, in culture media (Kolby *et al.* 2001). In these studies, a high turnover of serotonin was noted due to the complete intracellular metabolism with rapid release of the metabolite into the culture medium. As KRJ-I is also derived from a neuroendocrine carcinoid, and these tumors are known to demonstrate high serotonin

Figure 6 Effect of pharmacological agents on KRJ-I proliferation. (A) Proliferation was measured by MTT uptake and is represented as percentage of difference to control (unstimulated cells). Lanreotide inhibited KRJ-I proliferation with a maximal effect of $20 \pm 12\%$ at 10×10^{-9} M. (B) Rapamycin inhibited proliferation with a maximal effect of $35 \pm 15\%$ at 5×10^{-9} M. An inhibitory effect $>20\%$ was identified at concentrations as low as 1×10^{-9} M. (C) Gefitinib had no effect on KRJ-I proliferation except when used in micromolar (2×10^{-6} M) concentrations. The maximal effect was noted at 5×10^{-6} M ($20 \pm 8\%$ inhibition). Values are presented as the mean $\pm$ s.d. from three independent experiments.

catabolism (Westberg *et al.* 1997), the results in Fig. 4 are biologically consistent with this cell type. Of note was the observation that isoproterenol-stimulated serotonin secretion 60% above basal levels, consistent with information derived from enteroglucagon and neurotensin cells and the presence of adrenergic receptors identified in the transcriptome (Barber *et al.* 1986a, b, Buchan *et al.* 1987). This major release in association with exposure to an adrenergic agent is consistent with clinical reports of sudden paroxysmal crises associated with trauma or anesthetic induction and the major release of catecholamines that occur under such conditions (Bissonnette *et al.* 1990, Koopmans *et al.* 2005). In contradistinction, the BON cell line when stimulated by isoproterenol released little serotonin (~4% above basal) further reinforcing the fact that it is not of EC origin and does not reflect the clinical behavior of a SIC (Wallukat 2002, Tran *et al.* 2004).

Also, of note was the observation that isoproterenol-induced serotonin secretion was completely inhibited by the cAMP analog 2'5'-dideoxyadenosine. This would suggest that KRJ-I serotonin secretion is a cAMP-mediated event and is consistent with cAMP-mediated serotonin secretion noted in rat Leydig cells and the neuroectodermal cell line MTC (Tamir *et al.* 1990, Tinajero *et al.* 1993).

As predicted, based on the presence of the SSTR2 receptor on this cell line, the SST agonist BIM-23014C inhibited the isoproterenol-stimulated secretion of serotonin by KRJ-I cells in a dose-dependent manner with an IC_{50} in the sub-micromolar range (0.42 μ M). The cell system therefore provides evidence of having both a functional secretory mechanism and of being appropriately responsive to an agent known to activate the SSTR2 receptor and effectively decrease serotonin secretion and hence ameliorate clinical symptomatology.

The anti-proliferative effect of lanreotide on KRJ-I proliferation is of interest; although some clinical studies have proposed that somatostatin analogues inhibit tumor growth (stabilization), the evidence has largely been equivocal and there appears to be inconsistent data when comparing *in vitro* studies, animal experiments, and clinical experience (Schally 1988, Patel *et al.* 1995, Arnold *et al.* 1996, Rohaizak & Farndon 2002, Modlin *et al.* 2005, Plockinger *et al.* 2005). It is likely that many of the apparent differences in tumor inhibitory data reflect the variety of models used as well as the numerous alternative regulatory pathways responsible for neoplastic cell proliferation and especially neuroendocrine neoplasia itself (Sippel & Chen 2002, Sippel *et al.* 2003, Goke *et al.* 2004, Van Gompel & Chen 2004, Hofslie *et al.* 2005, Van Gompel *et al.* 2005). Rapamycin targets the mTOR protein which controls cell growth and has been shown to exert anti-proliferative effects on a number of human tumor

cell lines (Jacinto & Hall 2003, Rowinsky 2004, Rao *et al.* 2005), including the BON cell line (von Wichert *et al.* 2000) and the KRJ-I cell line (this study) indicating that it may represent a novel therapeutic target for pancreatic and carcinoid tumors. Gefitinib inhibited KRJ-I proliferation at micromolar concentrations, similar to its effect in the BON cell line (Hopfner *et al.* 2003). The effectiveness of gefitinib only at high concentrations may indicate that the EGFR signaling pathway is not a primary growth activation pathway for KRJ-I cells. An alternative possibility is that KRJ-I cells may be characterized by a constitutively active K-Ras and/or SRC phenotype. Both these products activate the same downstream targets as the EGFR, namely Akt and Erk, and resistance to gefitinib due to activation of these products has been demonstrated in human gallbladder adenocarcinoma cell lines (Qin *et al.* 2006). We have sequenced codons 12 and 13 (exon 1) of the *K-RAS* gene which represent common mutational hotspots within this gene (Kahn *et al.* 1987) but have detected no mutations (M Kidd, unpublished data). Although low concentrations of gefitinib appeared to stimulate KRJ-I proliferation, these results were not significantly different from the unstimulated control. Further studies are required to investigate the contribution of the EGFR signaling pathway to KRJ-I proliferation and the activation status of the downstream signaling components.

In these studies, we confirm the neuroendocrine, EC cell lineage of KRJ-I cells, define the KRJ-I transcriptome and identify receptors that may potentially be involved in secretory and proliferative regulation, characterize serotonin secretion, and demonstrate that lanreotide, rapamycin, and gefitinib, compounds with anti-proliferative effects in other neuroendocrine tumors (von Wichert *et al.* 2000, Hopfner *et al.* 2004), only have a moderate effect in this tumor cell line.

We have recently produced and characterized a 99% pure homogenous human ileal EC cell preparation (Modlin *et al.* 2006), and expect that the identification of upregulated receptors linked to cell proliferation in KRJ-I relative to normal EC cells will provide areas of important regulatory information that define the differences between normal and neoplastic EC cells. The opportunity to dissect out the mechanistic basis of EC cell neoplasia, as well as identify appropriate molecular targets, has obvious scientific and clinical relevance and application.

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