Methods in the Development of Human Carcinoid Cell Lines

Eric Sceusi[1], Asif Rashid[3], Yunfei Zhou[2], Shaija Samuel[2], Fan Fan[2], Ling Xia[2], Xiang-Cang Ye[2], Jia Lu[2], Federico Tozzi[1], Puja Gaur[1], Patrick Zweidler-McKay[6], Raymond Meyn[5], James Yao[4] and Lee M. Ellis[1, 2]

Departments of [1]Surgical Oncology, [2]Cancer Biology, [3]Pathology, [4]Medical Oncology, [5]Experimental Radiation Oncology and [6]Pediatrics The University of Texas M. D. Anderson Cancer Center, Houston, Texas

ABSTRACT

Background: Research in carcinoid and neuroendocrine tumors (NETs) is limited by the lack of midgut derived cell lines. The existing foregut NET lines, BON and H727, are phenotypically distinct from midgut NETs; highlighting the importance of establishing midgut derived NET cell lines. NETs are difficult to culture and we are developing methods to improve primary NET cell culture.

Methods: Sterile human NET surgical specimens were digested to a single cell suspension and fibroblast depleted using magnetic beads. The NET cells were maintained on collagen I coated plates for 1) culture and 2) immortalization. FACS was performed with a portion of cells using the Aldefluor assay to isolate potential cancer stem cells (CSCs). Sorted cells were grown in serum-free media on low attachment plates to assess sphere formation. Subcutaneous xenografts in nude and NOD/SCID gamma mice using fresh tumor implants and NET cell suspensions were performed.

Results: 1% serum improves primary cultured cell survival compared with 10% serum media. The effect of low serum reverses after approximately one week. Subcutaneous xenografts formed tumors in 5/59 mice over 4-10 months. FACS sorting has identified an ALDH+ population of NET cells, which can form spheres (a characteristic of CSCs) more frequently than ALDH(-) negative cells, which did not proliferate in sphere forming media. NET markers, somatostatin receptor 2 and chromogranin A, were detectable in cultures from a midgut carcinoid liver metastasis at passage 3 and 5, as well as in one mouse xenograft tumor; however both lines failed to grow beyond this point. We are now studying tumor xenografts in more immunodeficient NOD/SCID gamma mice.

Conclusions: We have developed a system allowing for NET primary culture up to 4 weeks. These techniques are the first step in obtaining cells for in vitro studies. Supported by The Raymond and Beverly Sackler Foundation.

PURPOSE

To develop human carcinoid cell lines, particularly of midgut origin and to develop a NET primary culture system to apply to further *in vitro* studies.

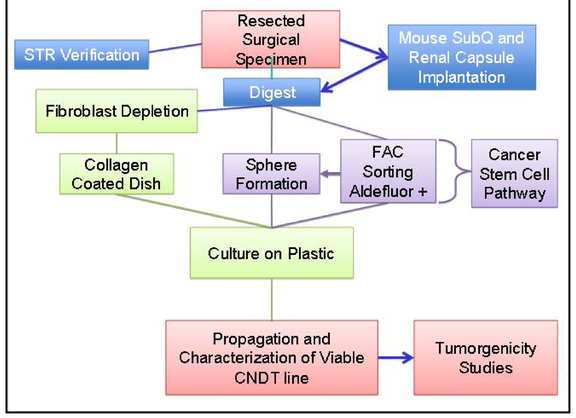
METHODS

Patient samples and tissue. Patients with metastatic carcinoid tumors were identified at our bimonthly NET meeting (chaired by Dr. James Yao, GI Medical Oncology, MDACC). Residual tissue following surgery was harvested and placed in 10% FBS DMEM/F12 media on ice, with care to avoid inclusion of contaminating liver parenchyma.

Tumor Cell Culture. A small piece of tumor was frozen for DNA extraction and subsequent STR profiling. Xenografts, using fragments of tumor (2 mm^3) were implanted subcutaneously and under the renal capsule of nude mice. A GentleMacs dissociator was used to disperse tumor cells from the specimen followed by one hour of treatment with collagenase II and another cycle of dissociation. The cells were then washed once with PBS and treated with RBC lysis buffer to remove RBCs. The majority of the cell suspension was plated in 2-D culture systems for establishing a new cell line. Cells designated for 2-D culture were processed using anti-fibroblast magnetic beads according to the manufacturer's protocol (Miltenyi Biotec), and the re-suspended in 1% or 10%DMEM/F12 + BDNF and IGF for 2-D culture on collagen I coated plates. The fibroblast-enriched portion was eluted separately and was cultivated and frozen for potential future studies. A portion of the cell suspension was filtered through sterile 40 µM membranes and subjected to the Aldefluor assay in order to FAC-sort for ALDH+ cells (putative cancer stem cells). A portion of cells was resuspended in Trevigen Cultrex® 3D Culture Matrix™ and injected subcutaneously into NOD-SCID gamma mice. FAC-sorted ALDH+ cells were cultured in 2-D and 3-D systems for establishing new cell lines. Beyond initial cell isolation, the cultures were observed and media was supplemented by 72 hours. Non-adherent cells in the media were removed and plated on a new plate rather than discarded, as they potentially may attach with increased time in culture. Mice with xenografts were observed and when the tumor is palpable (over 500mm), tumor was removed and processed following the protocols for clinical patient samples. In parallel, small tumor chunks were implanted back to nude mice for a second generation of tumor xenograft growth.

METHODS (continued)

Figure 1. Current Carcinoid Tissue Processing Algorithm



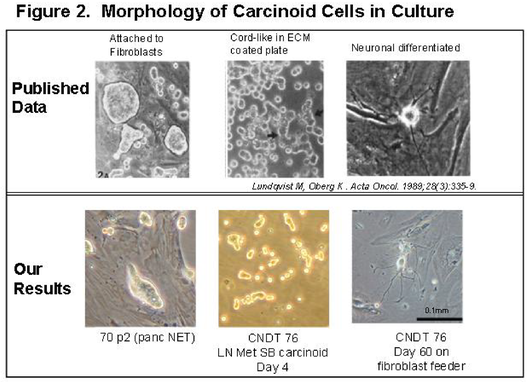
FACS Sorting for ALDH. After isolation of tumor cells as described above, a portion of the cells were treated with the Aldefluor kit (StemCell Tchnologies) to identify a population of cells with high ALDH activity. Briefly, the cells were washed in PBS solution then filtered through a 40um membrane to make a single cell suspension. An aliquot of the sample was treated with the Aldefluor assay according to manufacturer protocol for 30 minutes. A negative control sample was treated with the ALDH inhibitor, diethyl-aminobenzaldehyde (DEAB). The FITC/FL1 channel was used to analyze the sample with the flow cytometer. Gating was performed using the DEAB control to select a population of 0.1-0.3% bright cells in the negative control. This gate was then applied to the ALDH sample population. Resulting cells were resuspended and cultured in serum free media in ultralow attachment plates for sphere forming assay/

In vivo tumor xenograft studies. After subcutaneous implantation of 2mm^3 fresh tumor samples into nude mice, the growth of the tumors is monitored on a weekly basis. A successful tumor implantation is defined as visible, firm masses >3X larger than the original volume of implant, with histological confirmation. Upon successful primary xenograft growth from renal or subcutaneous implants, a second generation of tumors was established by processing the tumor as described for our human specimens to continue xenograft propagation and develop the cells in culture.

Western Blotting. Whole cell protein was isolated from BON, H727, CNDT 65.1 (from mouse xenograft) and CNDT 84 (passage 3 and 5 after primary culture) and run on standard Western blot. The membranes were probed for carcinoid specific primary antibodies: Chromogrannin A(CgA - Abcam), Somatostatin receptor 2a (SSTR2a – Affinity Bioreagents) and Vinculin as a control.

RESULTS

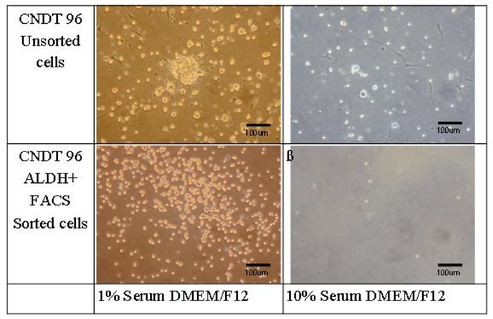
Figure 2. Morphology of Carcinoid Cells in Culture



Result: Carcinoid tumor cells in culture display a morphology consistent with previously published results. In addition to a terminally differentiated neuronal phenotype, there are tumor cells that are closely associated with fibroblasts that are capable of proliferation.

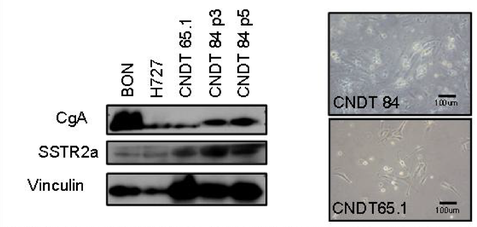
RESULTS (continued)

Figure 3. Carcinoid Primary Culture in Low Serum Media



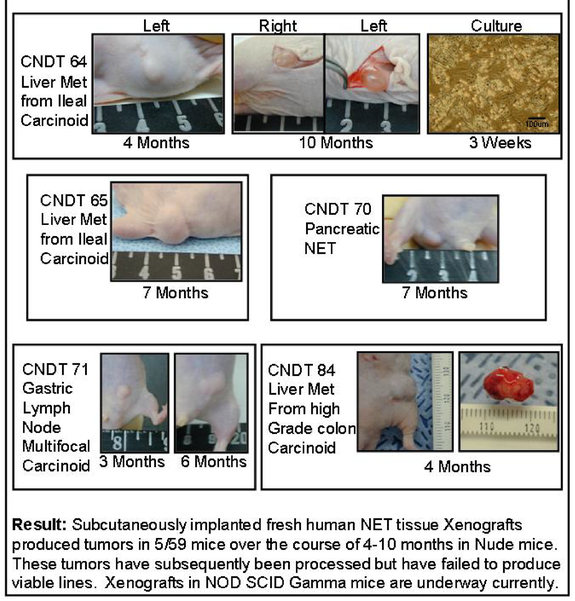
Result: Primary culture of NET tumors shows improved viability, attachment and reduction in contaminating fibroblast overgrowth in 1% compared to 10% serum medium. FACS sorted ALDH+ cells failed to attach or survive in 10% serum medium, but readily attached in 1% serum. This improved viability diminishes after about 1 week in culture, at which point 10% media improves survival.

Figure 4. Carcinoid Markers in Cultured Patient Samples



Result: Cultured cells from CNDT 84, a high grade colon NET liver metastasis, retained protein expression of the NET markers, chromogranin A (CgA) and somatostatin receptor 2a (SSTR2a) up to 5 passages in culture, however they failed to grow further. CNDT 65.1, a secondary culture from a mouse xenograft from a human ileal carcinoid liver met, also exhibited CgA and SSTR2a, however they did not continue to grow in culture.

Figure 5. Human NET Xenografts

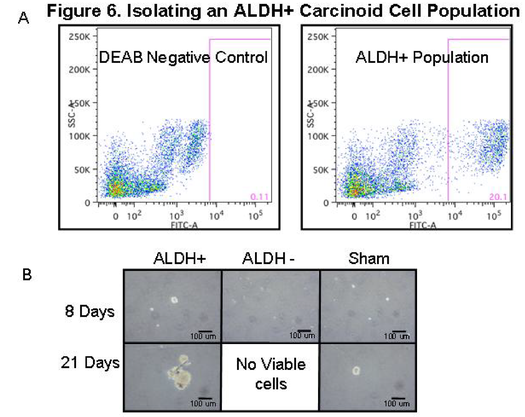


Result: Subcutaneously implanted fresh human NET tissue Xenografts produced tumors in 5/59 mice over the course of 4-10 months in Nude mice. These tumors have subsequently been processed but have failed to produce viable lines. Xenografts in NOD SCID Gamma mice are underway currently.

Supported by The Raymond and Beverly Sackler Foundation.

RESULTS (continued)

Figure 6. Isolating an ALDH+ Carcinoid Cell Population



Result: A. NET Specimens were sorted for ALDH+ cells by gating for negative control cells treated with DEAB inhibitor at a threshold of 0.1% DEAB cells, (CNDT 96 representative sorting shown).
B. The CNDT 96 cells (colonic carcinoid) ALDH+ population grew larger and more frequent spheres in sphere forming media when 10,000 cells per well in each group were grown after sorting. The ALDH- cells did not survive in sphere forming media and failed to form spheres.

Figure 7. ALDH+ Population in Human NET Specimens

ALDH+ Population in Neuroendocrine Tumor Specimens				
		n	*Mean %ALDH+*	*Range*
All Samples		19	7.5	0.2 - 37.7
Midgut Carcinoid	Total	14	9.3*	0.8 - 37.7
	Primary	7	12.1	0.5 - 37.7
	Liver Met	4	10.4	0.8 - 8.5
	Lymph Node	3	3.8	3.9 - 19.8
Pancreatic NET	Total	5	2.3*	0.2 - 5.9
	Primary	1	3.2	3.2
	Liver Met	4	2.0	0.2 - 5.9

Result: Human NET specimens have ALDH+ populations as high as 37.7%. Interestingly, there is a trend toward more ALDH+ cells in midgut carcinoids compared to pancreatic NETs, although this difference is not statistically significant. (*p-value = 0.087 by Mann Whitney U Test)

CONCLUSIONS

- NET cells can be cultured from human surgical specimens for up to 4 weeks in vitro and retain NET markers
- Primary culture of NET cells is enhanced in low serum medium initially, within 7 days, the NET cells require increased serum concentrations.
- NET Tumors contain an ALDH+ subpopulation, which demonstrates increased sphere formation and may represent a NET cancer stem cell population

REFERENCES

1. Van Buren G 2nd, Rashid A, Yang AD, et al. The development and characterization of a human midgut carcinoid cell line. Clin Cancer Res. 2007 Aug 15;(16): 4704-12.
2. Stojkovic P, Lako M, Stewart R, et al. An autogeneic feeder cell system that efficiently supports growth of undifferentiated human embryonic stem cells. Stem Cells. 2005 Mar;23(3):306-14.
3. Lundqvist M, Oberg K . In vitro culture of neuroendocrine tumors of the pancreas and gut. Acta Oncol. 1989;28(3):335-9.