

# Use of a Newly Established Human Cell Line (SU-CCS-1) to Demonstrate the Relationship of Clear Cell Sarcoma to Malignant Melanoma<sup>1</sup>

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## ABSTRACT

A new tumor cell line, designated SU-CCS-1, was established from the malignant pleural effusion of a 16-year-old Caucasian girl with clear cell sarcoma. Morphological studies at the light- and electron-microscopic levels revealed similar features between the SU-CCS-1 cells and the primary tumor. Ultrastructural and cytochemical techniques showed that both the SU-CCS-1 cell line and the original tumor were amelanotic in nature. The malignant derivation of the SU-CCS-1 cell line was demonstrated by intracranial and s.c. heterotransplantation in the nude, athymic mouse and by cytogenetic analysis which showed that the cell line had a hypodiploid chromosome number and several karyotypic abnormalities. Live-cell radioimmunoassay procedures using a large panel of monoclonal antibodies directed against tumor-associated antigens revealed that, phenotypically, SU-CCS-1 closely resembled melanoma tumor cell lines. Immunological assays for the detection of neuroendocrine-associated peptides, hormones, and enzymes revealed that, like melanoma, the SU-CCS-1 cell line was actively producing  $\alpha$ -melanotropin, S-100 antigen, and nerve growth factor. A notable difference between these tumor types was the capacity of SU-CCS-1 to produce bombesin, an active neuropeptide whose synthesis has been found in cell lines from patients with small cell carcinoma of the lung. From these studies, we concluded that the SU-CCS-1 cell line is phenotypically similar to melanoma, yet displays unique characteristics which distinguishes it from other sarcomas. The availability of an established clear cell sarcoma cell line will greatly facilitate further studies aimed at uncovering the histogenesis of this rare cancer.

## INTRODUCTION

Clear cell sarcoma is a rare tumor that occurs in the deep soft tissues usually near tendons and aponeuroses as originally described by Enzinger (10) in 1965. Morphologically, this tumor consists of cords or nests of pale, fusiform cells with indistinct cytoplasmic borders, centrally positioned round nuclei, and prominent nucleoli. It occurs principally in young adults, predominantly in women, and is common to the foot and knee where it is closely associated with the tendons and aponeuroses. Clinically, clear cell sarcoma has a tendency to recur at the primary tumor site and eventually to metastasize after a protracted clinical course.

Electron microscopic studies of biopsy specimens show that

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about half of these tumors are composed of cells which contain cytoplasmic melanosomes. In 1975, Bearman *et al.* (3) first suggested that melanotic clear cell sarcoma was a soft tissue variant of malignant melanoma. Ultrastructural studies by other investigators (5, 21, 26, 34, 35, 43, 44) have since confirmed the presence of melanosomes in the majority of clear cell sarcomas. The rarity of clear cell sarcoma and the absence of permanently established cell lines have greatly hampered further biological characterization.

In this paper, we present the establishment and characterization of the first reported clear cell sarcoma cell line, designated SU-CCS-1. Heterotransplantation and cytogenetic studies were performed to confirm the malignant derivation of the cell line. In addition, evidence is presented to show that SU-CCS-1 resembles melanoma by virtue of its cell surface phenotype and ability to synthesize neuroendocrine-associated active peptides, hormones, and enzymes.

## CASE REPORT

The SU-CCS-1 cell line was established from the pleural effusion of a 16-year-old Caucasian girl with clear cell sarcoma. She had been in excellent health until July 1975, when she developed a small, painful lump in her right heel. X-rays at that time were negative. The patient continued to have increased swelling and mild, intermittent right ankle pain until she was admitted to Children's Hospital at Stanford, CA, in May, 1976, with a 4-cm right inguinal mass and multiple pulmonary coin lesions. A biopsy of the right ankle mass revealed a cream-white tumor with the morphological features of clear cell sarcoma. Negative studies included a bone scan, a liver-spleen scan, a brain computer-assisted tomography scan, an i.v. pyelogram, a bone marrow aspirate and biopsy, and a lumbar puncture with spinal fluid evaluation. The patient was treated initially with pulse administration of vincristine, actinomycin D, and Cytoxan on May 17, 1976, without an objective response. In June 1976, she received one course of vincristine, Adriamycin, Cytoxan, and 5-(3,3-dimethyl-1-triazenyl)-1H-imidazole-4-carboxamide chemotherapy followed by 4750 rads to the right groin for palliative treatment. She also was treated with high-dose methotrexate (300 mg/kg) with leucovorin rescue and high-dose Cytoxan (40 mg/kg/day) for 4 days again without response. In August 1976, she developed bilateral serosanguinous malignant pleural effusions which required Atabrine therapy. By mid-August, the patient had severe cachexia and developed congestive heart failure and pulmonary edema. Despite supportive care, she expired on September 12, 1976. Autopsy revealed extensive metastatic disease involving the lymphatics, bone marrow, lung, pleura, pericardium, liver, and a recurrent mass at the site of the primary tumor.

## MATERIALS AND METHODS

### Establishment of the SU-CCS-1 Cell Line

A pleural effusion sample was obtained for cell culture directly after thoracentesis. The effusion was filtered through sterile cotton gauze to remove clumps and fibrous material. The cells were then washed twice

and seeded into flasks in RPMI-1640 medium containing 15% fetal calf serum, penicillin G (100 units/ml), and streptomycin sulfate (100 µg/ml). Once established, the cells were routinely trypsinized for passaging once weekly. The cell line has been screened for *Mycoplasma* contamination by the Mycotrim method (Hana Media, Inc., Berkeley, CA) and found to be negative.

#### Histological and Ultrastructural Studies

A biopsy specimen of the leg mass was divided into 2 portions immediately after removal. One specimen was fixed in 10% buffered formalin for paraffin embedding. Sections were cut at 5-µm intervals and stained with hematoxylin and eosin. The other specimen was placed into 2% cold cacodylate-buffered glutaraldehyde (pH 7.4), postfixed in 1% osmium tetroxide, stained with 2% uranyl acetate, dehydrated in graded alcohols, and embedded in Epon resin. Ultrathin sections were placed on Parlodion-coated copper grids, stained with lead citrate, and examined for electron microscopy. Cytocentrifuge preparations of SU-CCS-1 cells were stained with Wright's-Giemsa for cytological examination. Pellets of SU-CCS-1 cells were fixed and processed for electron microscopic examination as described above.

#### Cytochemical Procedures

Cytocentrifuge preparations of SU-CCS-1 cells were stained with periodic acid-Schiff with and without diastase digestion, peroxidase, Oil Red O, acid phosphatase with and without NaF inhibition, β-glucuronidase, the Fontana-Masson stain for melanin granules with and without KMNO<sub>4</sub> bleaching, colloidal iron, Gordon and Sweets reticulin, alkaline phosphatase, and Sudan Black B.

#### Heterotransplantation Studies

SU-CCS-1 cells were tested for their heterotransplantability in the nude, athymic mouse. Four 8-week-old female nude mice received either s.c. or i.c. injections of SU-CCS-1 cells as described previously by Epstein et al. (11). For the s.c. injections, 5 × 10<sup>6</sup> cells in a volume of 0.5 ml were inoculated. The i.c. injections consisted of 5 × 10<sup>4</sup> cells in a volume of 0.03 ml. Mouse tissue specimens were fixed in buffered formalin and stained with hematoxylin and eosin for microscopic examination.

#### Cytogenetic Analysis

Chromosome preparations were made on cells grown from ampules frozen 3 months after the establishment of the SU-CCS-1 cell line. Cells in exponential growth were incubated at 37° either overnight or for 3 hr with 5 × 10<sup>-7</sup> M colchicine. The cells were treated with a hypotonic solution of 0.7% sodium citrate for 15 min at 37° and fixed with glacial acetic acid:methanol (1:1) at -20°. The cell suspension was kept at 4° for 30 min, centrifuged at 1500 rpm for 5 min, and fixed 4 more times. The cell suspension was then dropped onto cold, wet slides which had been cleaned in methanol. The preparations were steamed briefly and air dried. After a minimum of 3 days at 50°, the slides were stained for G-bands using trypsin-Giemsa banding techniques according to a modified procedure of Seabright (37).

#### Immunohistochemical Detection of S-100 Protein

SU-CCS-1 and Colo 38 cell pellets were fixed in formalin and embedded in paraffin. Sections of each preparation were stained with heterologous antiserum to the S-100 protein using the immunoperoxidase technique described by Cochran et al. (8). As positive and negative controls, sections of 4 human melanomas and 2 human breast carcinomas, respectively, were stained in parallel.

#### Live Cell Radioimmunoassay

**Cell Lines and Monoclonal Antibody Reagents.** In order to compare the cell surface phenotypic characteristics of SU-CCS-1 with other es-

tablished cell lines, a live-cell radioimmunoassay was performed. For this assay, the following cell lines were used: (a) Colo 38, a melanoma cell line (12) obtained from Dr. Soldano Ferrone, Columbia University; CaCL 74-36, a melanoma cell line (25), IMR-5, a subclone of the IMR-32 neuroblastoma cell line (45), and Y79, a retinoblastoma cell line (36) obtained from Dr. R. Kennett, University of Pennsylvania Medical School; (b) NCI-H69, a small cell carcinoma of the lung (15) obtained from Dr. S. Rosen, Northwestern University Medical Center; (c) A172, a glioblastoma cell line (4, 16) obtained from Dr. D. Bigner, Duke University Medical Center; and (d) HT-29, a colon carcinoma cell line (13) which was used as a negative control. All the cell lines were grown in RPMI-1640 medium containing 15% fetal calf serum, penicillin G (100 units/ml), and streptomycin sulfate (100 µg/ml). Colo 38, NCI-H69, and Y79 grew as suspension cultures. CaCL 74-36, IMR-5, A172, and HT-29 required light trypsinization for passaging.

The monoclonal antibodies used in the assay are listed in Table 2. The antigen specificity and pertinent references describing these reagents are also listed. Monoclonal antibodies L-22, SC2, and Cl45 were kindly provided by Dr. Robert Fox, Scripps Clinic and Research Foundation, La Jolla, CA; 225.28S, 376.96, 465.12S, CR1, and 763.74 were kindly provided by Dr. Soldano Ferrone, Columbia University, New York, NY; I-48.7, 3.1, 4.2, and 96.5 were generously provided by Dr. K. E. Hellström, Fred Hutchinson Cancer Research Center, Seattle, WA; and 691-19-19 NU-4B, O<sub>1</sub> 95-45, I<sub>1</sub> 82-11, G<sub>1</sub> 1543, I<sub>1</sub> 75-29, C<sub>4</sub> 20-32, 17-1A, and C<sub>14</sub>-72 were generously provided by Dr. M. Herlyn, The Wistar Institute, Philadelphia, PA.

**Radioimmunoassay Procedure.** The cell lines were dislodged from their flasks by EDTA treatment and vigorous shaking and were washed twice in cold wash buffer consisting of phosphate-buffered saline (8.0 g NaCl, 0.2 g KCl, 1.15 g Na<sub>2</sub>HPO<sub>4</sub>, 0.2 g KH<sub>2</sub>PO<sub>4</sub>, 0.1 g CaCl<sub>2</sub>·2 H<sub>2</sub>O, and 0.1 g MgCl<sub>2</sub>·6H<sub>2</sub>O/liter of distilled water), bovine serum albumin (1 mg/ml; radioimmunoassay grade; Sigma Chemical Co., St. Louis, MO), and 0.02% sodium azide. Cells (5 × 10<sup>5</sup>) resuspended in 100 µl of wash buffer were seeded into microtiter wells (Immulon Removawell Strips; Dynatech Laboratories, Inc., Alexandria, VA). The microtiter plates were pretreated the previous night with bovine serum albumin (10 mg/ml) in phosphate-buffered saline with azide in order to prevent the antibody solutions from binding to the wells. Hybridoma supernatant or ascites fluid (100 µl, diluted 1:100) was added to each well, and the plates were incubated for 30 min at room temperature in a microshaker apparatus (Dynatech Laboratories, Inc.) set at low speed. After incubation, the cells were washed 4 times with cold wash buffer by spinning the plates at 1000 rpm for 5 min, aspirating the supernatants with an 8-tip micromatic manifold (Popper and Sons, Inc., New Hyde Park, NY), and resuspending the cells in 200 µl of wash buffer using a Titertek Multichannel pipet (Flow Laboratories, Inc., McLean, VA) and the microshaker apparatus. After completion of the washes, 100,000 cpm of <sup>125</sup>I-goat anti-mouse antibody (New England Nuclear, Boston, MA), in a volume of 100 µl, were added to each well for an additional 30-min incubation period with continuous shaking. Finally, the cells were washed 6 times with wash buffer as described above, and the wells were counted in a γ-counter for 1-min intervals. All data were expressed as the mean cpm of triplicate samples minus that of the NS-1 supernatant control wells. For each test, the S.D. of the triplicate cpm was calculated and, if found to be greater than 10% of the mean cpm the assay was repeated.

#### Assays for the Determination of Neuroendocrine-associated Peptides, Hormones, and Enzymes

SU-CCS-1 cells and melanoma Colo 38 cells were tested for a number of peptides synthesized in various types of tumors. Frozen pellets of washed cells were shipped and tested for neuron-specific enolase (28) by Dr. Paul Marangos, NIH, Bethesda, MD; bombesin and Substance P (29) by Dr. Terry Moody, The George Washington University Medical Center, Washington, DC; α-MSH<sup>3</sup> (33) by Dr. Thomas O'Donohue,

<sup>3</sup> The abbreviation used is: α-MSH, α-melanotropin.

National Institute of Neurological and Communicative Disorders and Stroke, Bethesda, MD; L-dopa decarboxylase (2) by Dr. Steven Baylin, Johns Hopkins Medical Center, Baltimore, MD; and nerve growth factor (39) by Dr. Ann Sliski, Laboratory of Viral Carcinogenesis, National Cancer Institute, Bethesda, MD.

## RESULTS

### Morphological Appearance of Tumor Biopsy

The histology of the original tumor biopsy from patient M. T. is shown in Fig. 1. The tumor was composed of cells having an epithelioid appearance arranged in nests separated by slender strands of fibrous tissue. The tumor cells had pale-staining cytoplasm with indistinct borders and round to oval nuclei. The nucleoli were prominent and often were centrally located in the nucleus. Scattered multinucleate tumor cells were seen, and mitotic figures were sparse.

Ultrastructural examination of the biopsy showed the tumor to be composed of round to oval cells with irregular cytoplasmic borders (Fig. 6). The nucleus was positioned to one side of the cell and had a markedly irregular and convoluted nuclear membrane. Scattered clumps of heterochromatin were seen, and the nucleolus was central in position and prominent. The cytoplasm contained numerous mitochondria, aggregates of rough endoplasmic reticulum, and numerous polyribosomes. No structures resembling melanosomes were observed in the cells examined.

### Establishment of the SU-CCS-1 Cell Line

The SU-CCS-1 cell line was readily established from the pleural effusion of patient M. T. in August 1976 by A. Epstein while he was a student in the laboratory of Dr. Henry S. Kaplan. In culture, the cells attached to the surface of the flask but remained in a rounded configuration as shown in Fig. 2. To subpassage the SU-CCS-1 cell line, a light trypsinization was required to detach the cells from the flask. The SU-CCS-1 cell line has been maintained in culture for several years without evidence of *Mycoplasma* contamination. Its approximate doubling time is 52 hr.

Morphologically, the cells are in excess of 20  $\mu\text{m}$  in diameter and are characterized by a large bland cytoplasm, a peripheral clear zone, and frequent cytoplasmic vacuoles (Fig. 3). The nuclei are large, are most often positioned to one side of the cell, and are round to oval. Binucleate cells are common, and the nucleoli are central and prominent in most cells.

Ultrastructurally, the SU-CCS-1 cells were found to have abundant cytoplasm with numerous mitochondria and vacuoles (Fig. 7). Under the electron microscope, the nuclei were large, contained little heterochromatin, and had darkly stained and thick nuclear membranes. Rough endoplasmic reticulum, polyribosomes, and lysosomes were present in the cytoplasm. Melanosomes were not found in any of the tissue culture cells examined.

### Neoplastic Properties of the SU-CCS-1 Cell Line

**Heterotransplantation in the Nude, Athymic Mouse.** The s.c. injections of SU-CCS-1 cells produced tumor nodules both at the site of inoculation and dorsally in the s.c. space in one-half of the attempts 60 days after inoculation. The i.c. injections generated central nervous system tumors in 2 of 4 mice 30 to 40 days after implantation. An autopsy of these mice showed

tumor infiltration in the white and gray matter of the cerebral hemispheres (Figs. 4 and 5). Subarachnoid and leptomeningeal infiltrations were not noted. The cytological features of the s.c. and intracerebral heterotransplanted tumors were similar to the original biopsy specimen shown in Fig. 1.

**Cytogenetic Studies.** The modal chromosome number for the SU-CCS-1 cell line was 43, but a subset of cells was found to have 44 chromosomes. The basic chromosomal complement observed in all cells was composed of 40 chromosomes, 30 of which had banding patterns consistent with the normal human diploid complement, including 2 X-chromosomes. Ten stable markers were observed that were similar in all cells (Fig. 8). A metacentric marker, larger than a No. 1, was readily detectable in each cell, even in those of suboptimal banding quality (Fig. 8A). Portions of other markers had banding patterns that partially resembled the normal human complement, e.g.: (a) a No. 1 with unidentifiable material on pter (Fig. 8B); (b) an acrocentric chromosome resembling 9q (Fig. 8G); (c) another resembling 12q (Fig. 8H); and (d) a No. 21 with a long-arm deletion (Fig. 8J). Of the other stable markers, 4 are metacentric chromosomes (Fig. 8, C to F), 2 of which appear symmetric (isochromosomes; Fig. 8, D and F). One satellited acrocentric is intermediate in size between the D and G groups (Fig. 8I). The remaining 3 to 4 chromosomes in each cell usually consisted of chromosomes Nos. 21 and 22, and various C-group-sized markers. The presence of markers and lack of a completely stable chromosomal complement most probably resulted from recurrent breakage and reunion. Several cells with dicentric chromosomes, and acentric fragments were observed.

### Characterization of the SU-CCS-1 Cell Line

**Cytochemical Features.** The results of the cytochemical tests performed on the SU-CCS-1 cells are shown in Table 1. Of note, the cells were negative for melanin and showed positive reactivity for  $\beta$ -glucuronidase, acid phosphatase (sensitive to tartrate), naphthol AS-D acetate esterase (resistant to NaF), and periodic acid-Schiff. These results confirmed the amelanotic nature of the cell line and revealed its cytochemical profile.

**Expression of S-100 Protein.** Immunoperoxidase staining of the SU-CCS-1 cell line showed a positive reaction for the S-100 protein. All 4 human melanoma biopsies and the Colo 38 melanoma cell line were positive, and 2 breast carcinoma biopsies were negative as reported previously by other investigators (8, 14, 23, 30). Typically, immunoperoxidase staining for the S-100

Table 1  
Cytochemical characterization of SU-CCS-1

Cytochemical stain	SU-CCS-1	Description of staining reaction
Periodic acid-Schiff	+	Around periphery of cells
Periodic acid-Schiff with diastase	-	
Peroxidase	-	
Oil Red O	-	
Acid phosphatase	+	Diffuse in cytoplasm
Acid phosphatase with tartrate	-	
Naphthol AS-D acetate esterase	+	Diffuse in cytoplasm, granular pattern
Naphthol AS-D acetate esterase with NaF	+	Diffuse in cytoplasm, granular pattern
$\beta$ -Glucuronidase	+	Diffuse in cytoplasm
Melanin	-	
Colloidal iron	-	
Reticulin	-	
Alkaline phosphatase	-	
Sudan Black B	-	

protein showed a predominantly nuclear and a less intense cytoplasmic staining reaction in the melanoma tissues and the Colo 38 cell line. The SU-CCS-1 cells, however, showed a peripheral cytoplasmic staining pattern which left a distinctive clear cytoplasmic zone around the nucleus. Illustrations of the immunoperoxidase staining patterns for the S-100 protein in the SU-CCS-1 and Colo 38 cell lines are shown in Figs. 9 and 10, respectively.

**Phenotypic Analysis of SU-CCS-1 and Other Tumor Cell Lines with Monoclonal Antibodies**

In order to characterize the SU-CCS-1 cell line with respect to other established human malignant cell lines, a live-cell radioimmunoassay was performed using a panel of monoclonal antibodies. The results of these experiments are shown in Table 2. From these data, the SU-CCS-1 cell line showed remarkable similarity in phenotype with established melanoma cell lines. Compared to the SU-CCS-1 cell line, the melanoma cell lines, Colo 38 and CaCL 74-36, had concordance frequencies of 16 of 21 and 13 of 21, respectively. The concordance frequency was determined by adding together the instances when the SU-CCS-1 and the comparison cell line both had either positive or negative results with a given antiserum, divided by the total number of antisera tested. Using this method of comparison, the concordance frequencies between SU-CCS-1 and other cell lines were as follows:

16 of 21 for NCI-H69; 12 of 21 for IMR-5; 8 of 21 for Y79; 10 of 21 for A172; and 10 of 21 for HT-29. From these data, the SU-CCS-1 cell line phenotypically most resembled the melanoma cell line Colo 38 and the small cell carcinoma of the lung cell line NCI-H69. Of the solid tumor cell lines tested, only the clear cell sarcoma and melanoma cell lines were positive for the HLA-Dr antigen. Monoclonal antibodies produced against colon carcinoma were essentially nonreactive with all the cell lines except HT-29, which acted as a positive control in these experiments.

**Assays for the Detection of Neuroendocrine-associated Peptides, Hormones, and Enzymes**

Frozen pellets of the SU-CCS-1 and melanoma Colo 38 cell lines were tested for the presence of neuron-specific enolase, bombesin, Substance P,  $\alpha$ -MSH, and L-dopa decarboxylase. Most of these proteins have not been investigated in melanomas or sarcomas, and our results indicate that further studies of these tumors are warranted. As shown in Table 3, the SU-CCS-1 cell line was positive for bombesin, Substance P, and  $\alpha$ -MSH, and was negative for neuron-specific enolase and L-dopa decarboxylase. The melanoma cell line Colo 38 was positive for both neuron-specific enolase and  $\alpha$ -MSH, and was essentially negative for Substance P, bombesin, and L-dopa decarboxylase. The SU-CCS-1 cell line was also studied for the presence of nerve growth factor and, like melanoma cell lines, was found to be

Table 2  
Phenotypic characterization of SU-CCS-1 and other established human malignant cell lines using monoclonal antibodies to tumor-associated cell surface antigens

Mono-clonal antibody	Antigen specificity	Ref.	Live cell radioimmunoassay (cpm)							
			SU-CCS-1 (clear cell sarcoma)	Colo 38 (melanoma)	CaCL74-36 (melanoma)	NCI-H69 (small cell carcinoma of lung)	IMR-5 (neuroblastoma)	Y79 (retinoblastoma)	A172 (glioblastoma)	HT-29 (colon carcinoma)
L-22	Transferrin receptor	<sup>a</sup>	1,817	4,226	8,808	7,557	2,324	—	—	4,038
SC2	HLA-DR	<sup>a</sup>	5,887	8,703	2,525	—	—	—	—	—
CR1	HLA-A and HLA-B	7	16,837	19,631	25,568	3,136	—	—	9,142	4,546
225.28S	Melanoma	46	— <sup>c</sup>	19,913	1,963	—	—	2,900	2,499	—
376.96	Melanoma	22	7,832	18,789	16,283	3,499	5,313	13,427	7,784	7,093
465.12S	Melanoma	32	4,145	4,158	4,102	14,401	3,064	—	5,097	—
763.74	Melanoma	31	—	23,168	5,464	—	—	5,891	2,936	—
I-48.7	Melanoma	17	2,971	9,168	6,121	9,637	2,310	7,558	2,457	—
3.1	Melanoma	50, 51	8,469	8,273	13,316	20,501	4,719	15,697	16,182	3,249
4.2	Melanoma	49	10,140	8,666	10,215	12,749	6,349	11,645	3,355	2,409
96.5	Melanoma	6	—	—	14,736	4,430	1,808	4,661	3,995	—
691-19-19	Melanoma-astrocytoma	18	3,004	16,330	—	—	—	—	—	—
NU-4B	Melanoma-astrocytoma	18	—	—	13,287	—	—	—	3,242	—
O,95-45	Melanoma-astrocytoma	19	—	19,630	5,723	—	—	4,007	—	—
I,82-11	Melanoma-teratocarcinoma	19	3,193	3,092	—	—	—	—	2,288	—
G,1543	Melanoma-astrocytoma	19	2,771	11,006	17,858	—	—	3,183	6,802	—
I,75-29	Melanoma-astrocytoma	19	8,129	14,550	—	3,051	—	—	—	—
C <sub>4</sub> 20-32	Colorectal-pancreatic-mammary carcinoma	<sup>b</sup>	5,099	—	—	1,729	—	—	—	6,183
17-1A	Colorectal-gastric-pancreatic carcinoma	20	—	—	—	—	—	—	3,770	5,325
C <sub>4</sub> 14-72	Colorectal-gastric carcinoma	<sup>b</sup>	—	—	—	—	—	—	—	2,645
CI 45	Peripheral nerve-NK cell	<sup>a</sup>	2,137	—	8,208	5,363	—	—	—	—

<sup>a</sup> R. Fox, personal communication.  
<sup>b</sup> M. Hertyn, personal communication.  
<sup>c</sup> —, less than 1,500 cpm.

Table 3  
Protein concentration levels of specific peptides, hormones, and enzymes in SU-CCS-1 and melanoma Colo 38 cells

Protein	Range of positivity (tumor or tissue)	Ref.	SU-CCS-1	Colo 38
Neuron-specific enolase	50–5602 ng/mg (neuroendocrine tumors)	41	6 ng/mg protein	100 ng/mg protein
Bombesin	0.02–12.7 pmol/mg (small cell carcinoma)	29	0.13 pmol/mg protein	0.03 pmol/mg protein
Substance P	0.01–.6 pmol/mg (small cell carcinoma) <sup>a</sup>	29	0.5 pmol/mg protein	0.03 pmol/mg protein
α-MSH	10 pg/mg (neuroendocrine tissue) <sup>b</sup>	<sup>a</sup>	22.38 pg/mg protein	206.49 pg/mg protein
Dopa decarboxylase	0.2–300 nmol <sup>14</sup> CO <sub>2</sub> /hr/mg (small cell carcinoma)	2	0 nmol <sup>14</sup> CO <sub>2</sub> /hr/mg	0 nmol <sup>14</sup> CO <sub>2</sub> /hr/mg
Nerve growth factor	1:4–1:128 <sup>c</sup> (melanoma cell lines)	39	1:8–1:16	ND

<sup>a</sup> As described in Ref. 29, the majority of small cell carcinoma of the lung cell lines express less than 0.01 pmol/mg of Substance P, and only a small percentage express within the range of 0.01 and 0.6 pmol/mg.

<sup>b</sup> T. O'Donohue, personal communication.

<sup>c</sup> Indirect immunofluorescence titer.

positive by an indirect immunofluorescence assay. For these experiments, the melanoma cell line A375 was used as a positive control.

## DISCUSSION

Although less than 60 cases of clear cell sarcoma have been reported in the world literature [see review by Boudreaux and Waisman (5)], this rare tumor has received considerable attention because of its distinctive clinical presentation, morphological appearance, and association with melanin production. Speculation concerning the histogenesis of this tumor has been based solely upon electron microscopic and cytochemical data. To date, biological studies have not been performed because of the lack of readily available biopsy specimens and tumor-derived cell lines.

In this report, we describe the establishment and characterization of the first clear cell sarcoma cell line, SU-CCS-1. The cell line was established from the malignant pleural effusion of a 16-year-old Caucasian girl with widely metastatic disease. Light and electron microscopic studies confirmed the diagnosis of clear cell sarcoma and identified the tumor as amelanotic in nature. Morphological studies of the original tumor biopsy and derived cell line showed similarities in nuclear structure and organelle content. Electron-microscopic analysis and cytochemical studies confirmed that, like the parent tumor, the SU-CCS-1 cell line was devoid of melanin. The morphological appearance of the original tumor and derived cell line closely resembled the light- and electron-microscopic findings of other investigators (1, 3, 5, 9, 10, 21, 24, 26, 34, 35, 43, 44).

In order to confirm the malignant potential of the established cell line, SU-CCS-1 cells were injected into nude mice by s.c. and i.c. routes of inoculation. Both methods of inoculation were found to successfully produce tumors in the nude, athymic mouse. Light microscopic studies of the heterotransplanted tumors showed the characteristic morphological features of clear cell sarcoma (Fig. 5). Cytogenetic studies of the SU-CCS-1 cell line further confirmed its malignant derivation and showed that the cells were markedly aneuploid with a bimodal chromosome number of 43 and 44. All cells contained consistent marker chromosomes due to chromosomal rearrangements. Some of these findings may have been caused by the chemotherapeutic

treatments the patients received prior to the establishment of the cell line.

The establishment of the SU-CCS-1 cell line gave our laboratory a unique opportunity to study the cellular characteristics of clear cell sarcoma. Previous ultrastructural and cytochemical studies have led investigators to speculate that clear cell sarcoma may represent a soft tissue variant of malignant melanoma (3, 35). Other investigators using similar methods concluded that clear cell sarcoma is a partially dedifferentiated malignant melanotic schwannoma (34) or synovial sarcoma (24, 44). Because of the rarity of the tumor, however, rigorous proof has been lacking to verify the conclusions of these ultrastructural studies. In an attempt to characterize the histogenesis of the SU-CCS-1 cell line, we performed 2 types of studies. The first involved the use of monoclonal antibodies developed against human melanoma-associated antigens to characterize the cell surface phenotype of SU-CCS-1. Using a live-cell radioimmunoassay technique, the SU-CCS-1 cell line was studied and compared with other tumor cell lines, including melanoma, small cell carcinoma of the lung, neuroblastoma, retinoblastoma, and glioblastoma. Our results clearly demonstrated a remarkable similarity in the surface phenotype of SU-CCS-1 and the 2 malignant melanoma cell lines. Of the solid tumor cell lines tested, only SU-CCS-1 and the melanoma cell lines were positive for the HLA-Dr antigen which has been described previously as a marker for melanoma (7, 47, 48). Further studies are warranted to test the presence of this antigen on biopsy specimens of clear cell sarcoma, since it may be a useful marker in amelanotic cases. The radioimmunoassay studies also showed the SU-CCS-1 was phenotypically similar to the small cell carcinoma of the lung cell line. Small cell carcinoma of the lung has been shown to exhibit a number of neuroendocrine properties, including the synthesis of biologically active peptide hormones (40), capacity for biogenic amine synthesis (15), presence of neuron-specific enolase (27), and the presence of neurosecretory granules (29). By comparison, the other solid tumor cell lines had fewer antigenic markers in common with SU-CCS-1. Using a similar approach, Seeger *et al.* (38) demonstrated the presence of common antigenic determinants between melanoma, glioma, neuroblastoma, leiomyosarcoma, rhabdomyosarcoma, and osteogenic sarcoma cells. These and other studies demonstrate that monoclonal antibodies are invaluable reagents for assessing the presence of tumor-associated

antigenic determinants.

The monoclonal antibody studies were extended by a second experimental approach which evaluated the expression of neuroendocrine-associated peptides, hormones, and enzymes in the SU-CCS-1 cell line. Using the expertise of a number of different laboratories, we found that the SU-CCS-1 cell line synthesized bombesin, Substance P,  $\alpha$ -MSH, S-100 protein, and nerve growth factor. The malignant melanoma cell line Colo 38 was also found to produce significant quantities of some of these active molecules with notable exceptions (see Table 3). As reviewed by Tischler *et al.* (42), neuroendocrine neoplasms are characterized by their synthesis and excretion of active hormones and peptides which effect the physiological environment of their host. Some of these molecules, such as bombesin, produce multiple physiological effects including hypersecretion, anorexia, hypothermia, and hyperglycemia (29). These "bombesinergic" effects may have caused the severe cachexia noted in patient M. T. Further studies of the SU-CCS-1 cell line may reveal other active molecules which may have affected the physiological status of the patient. The identification of bombesin in the SU-CCS-1 cell line is significant, since it has not been well described in tumors other than small cell carcinoma of the lung (29). Its presence in SU-CCS-1 again reveals an unexpected similarity between clear cell sarcoma and small cell carcinoma of the lung. Therefore, our studies underscore the usefulness of applying these immunobiochemical assays in order to examine the cellular derivation of tumors.

The data obtained from both experimental approaches generally support the notion that, despite its amelanotic nature, SU-CCS-1 closely resembles established malignant melanoma cell lines and that, like small cell carcinoma of the lung, it expresses several traits of neuroendocrine differentiation. Since it does not contain dopa decarboxylase, neuron-specific enolase, and dense-core granules, it cannot be included in the group of amine precursor uptake, decarboxylase (APUD) tumors (42). Specific markers to identify the cell of origin of solid tumors are generally lacking and, hence, the histogenesis of neoplasms such as clear cell sarcoma cannot be made with certainty at this time. However, the availability of a well-characterized clear cell sarcoma cell line will greatly aid future experimentation with this and other human sarcomas.

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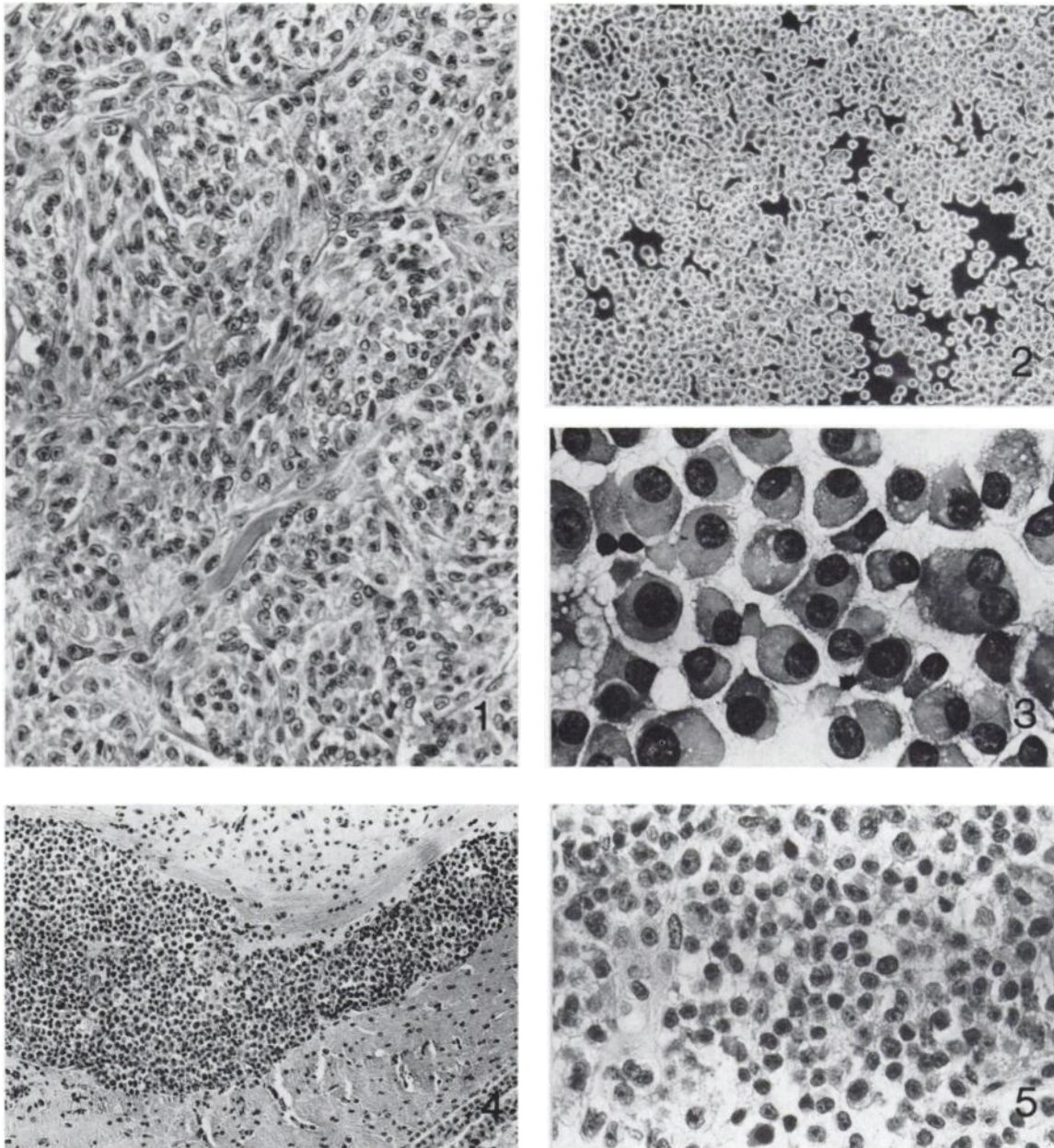


Fig. 1. Photomicrograph of excised tumor. Note the delicate fibrous septae and the prominent nucleoli. H & E,  $\times 275$ .

Fig. 2. Appearance of SU-CCS-1 cells in culture. The cells are attached to the flask but remain in a rounded configuration. Phase contrast,  $\times 100$ .

Fig. 3. Cytological appearance of the SU-CCS-1 cell line. The cells characteristically have an abundance of cytoplasm and a clear zone at the cytoplasmic periphery. Binucleate cells are common. Wright's-Giemsa,  $\times 500$ .

Fig. 4. Invasion of normal brain parenchyma of nude mouse by SU-CCS-1 at 40 days after i.c. heterotransplantation. H & E,  $\times 110$ .

Fig. 5. Higher magnification of i.c. tumor. Note similar morphological appearance of cells to the original tumor biopsy shown in Fig. 1. H & E,  $\times 400$ .



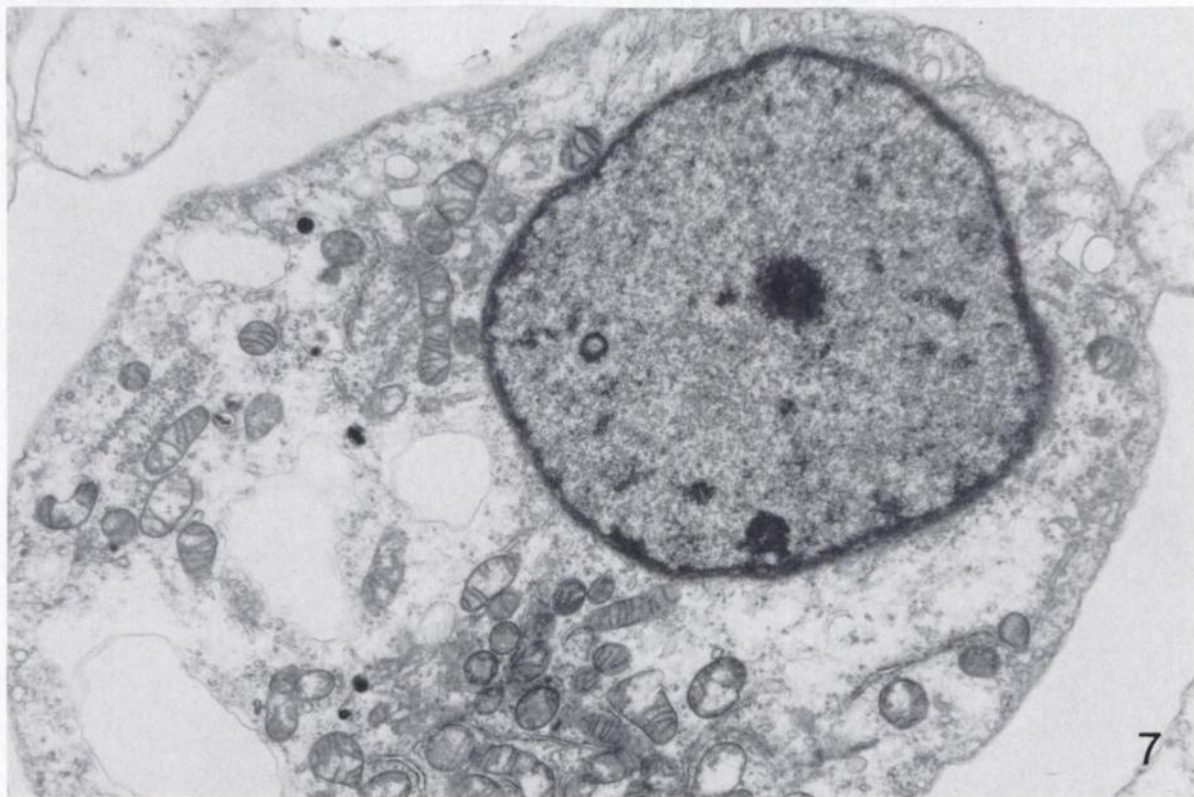
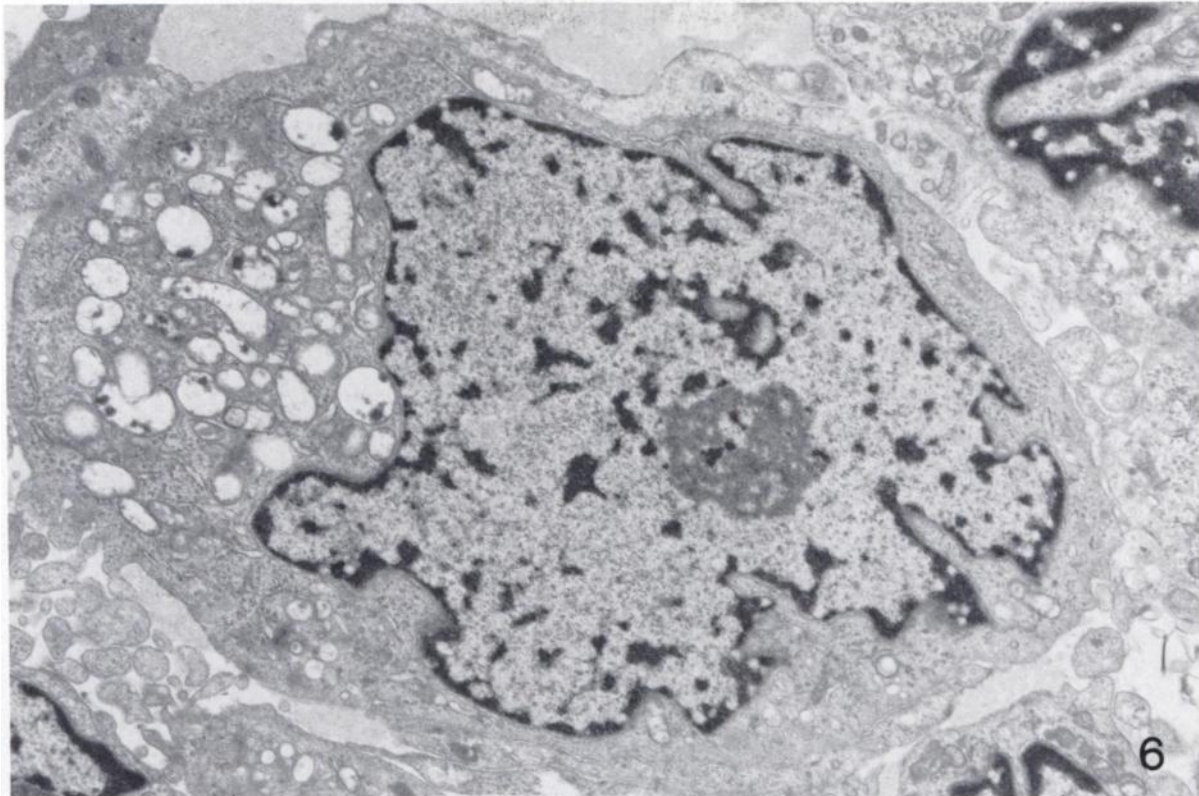


Fig. 6. Electron micrograph of tumor cell in excision specimen. No melanosomes were found.  $\times 10,000$ .

Fig. 7. Electron micrograph of SU-CCS-1 cell after 3 months in culture. Rounded dense structures found in the cytoplasm of the cells are interpreted as lysosomes.  $\times 9,900$ .

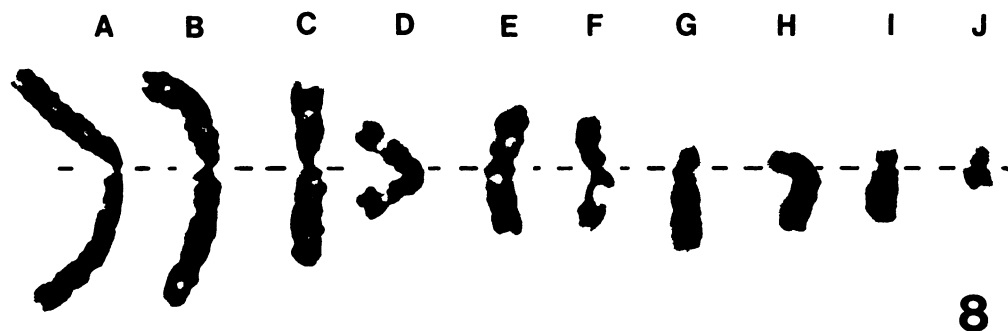


Fig. 8. Ten marker chromosomes consistently observed. Other markers appear but their composition varies among cells. trypsin-Giemsa banding.

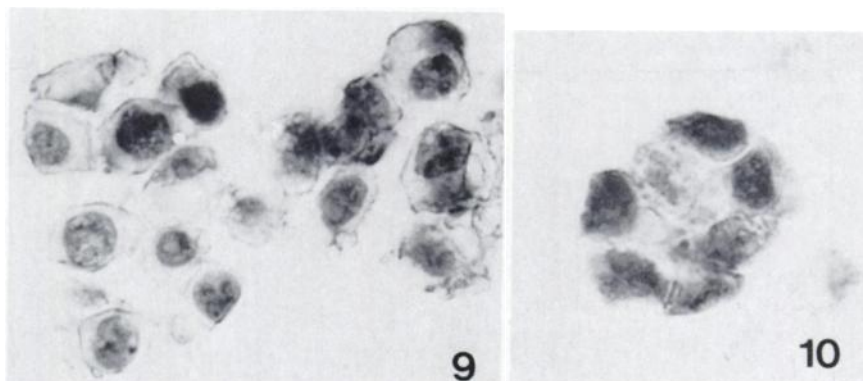


Fig. 9. Immunoperoxidase staining of SU-CCS-1 cells with heterologous antisera to the S-100 protein. Staining was restricted to the cytoplasmic periphery leaving a distinctive cytoplasmic clear zone around the nucleus. Hematoxylin,  $\times 1,250$ .

Fig. 10. Immunoperoxidase staining of Colo 38 cells with heterologous antisera to the S-100 protein. Staining was predominantly nuclear and diffusely cytoplasmic as reported previously for human malignant melanoma cell lines. Hematoxylin,  $\times 1,250$ .

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## Use of a Newly Established Human Cell Line (SU-CCS-1) to Demonstrate the Relationship of Clear Cell Sarcoma to Malignant Melanoma

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