A Human Liposarcoma Cell Line Producing Hyaluronic Acid

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A human liposarcoma cell line COLO 222, derived from a primary tumor in a 62-year-old male, elaborates hyaluronic acid. COLO 222 is characterized on the basis of histochemical, ultramorphological, and cytogenetic properties, along with isozyme phenotype and cell products. A chromosome mode of 53 predominates and unique Giemsa-banded marker chromosomes are identified. An autochthonous lymphoid cell line, COLO 143v, was established after the addition of exogenous Epstein-Barr virus. Cytogenetic analysis of COLO 143v is consistent with a normal male karyotype. COLO 143v possesses B-cell characteristics. This autochthonous system has been used for immunological studies and cytotoxicity assays.

***Cancer* 45:1400–1407, 1980.**

FEW SOFT TISSUE SARCOMAS retaining *in vivo* properties have been successfully established as permanent cell lines.[1,2] The production of various mucosubstances by bone and soft tissue tumors depends both on the tissue of origin and the degree of differentiation.[20] Thus, the secretion of hyaluronic acid by a sarcomatous tumor can be indicative of cellular differentiation and/or tissue of origin. This report describes the characterization of a human tumor cell line that should be useful in studies of human liposarcomas, hyaluronic acid production, cytogenetics and tumor antigens.

Case Report

A 62-year old Caucasian male presented in early April 1975, with a four-month history of an expanding mass of his proximal right forearm. The lesion resembled a sarcoma. On April 29, 1975, the lesion was widely excised and a right axillary node biopsy was performed. The primary and metastatic tumor tissue specimens were submitted for pathology and tissue culture. A histologic diagnosis of liposarcoma was based on the presence of pleomorphic neoplastic cells with hyperchromatic nuclei, large nucleoli, and an eosinophilic cytoplasm which was vacuolated and stained positive for lipids. A peripheral blood sample was cultured on April 30, 1975. The patient's ABO blood group was O+. The patient was unsuccessfully treated with several chemotherapeutic agents and expired on July 7, 1975.

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Supported by Grant 1-RO1-CA15018, awarded by the National Cancer Institute, Department of Health, Education and Welfare and the Mary B. and L. H. Marshall Fund.

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The authors thank V. Buric for electron microscopy, D. M. Meiselbaugh, R. N., for clinical assistance, and M. Robinson for histochemistry.

Accepted for publication March 20, 1979.

Materials and Methods

Cell Culture

Cultures of the primary tumor tissue and axillary metastatic tumor tissue were initiated by mincing portions of each tissue sample in RPMI medium 1640,[31] GEM 1717 medium[56] and Nutrient medium F-12;[14] each was supplemented with 20% fetal bovine serum (FBS) (heat-inactivated at 56 C for 30 minutes; Reheis Chemical Co., Kankakee, Ill.), penicillin (100 units/ml), and streptomycin (50 μg/ml). The tissue suspensions were immediately seeded into 4-oz. flint-glass culture bottles with a thin layer of medium. The primary culture bottles were loosely capped and incubated at 37 C in humidified air with 10% CO_2 for 24 hours. Thereafter, the cultures were incubated tightly capped in a dry 37 C incubator and at four-day intervals, 2–5 ml of fresh medium was exchanged until cell growth was stabilized.

Cultures of the peripheral blood leukocytes were initiated by the method described by Moore *et al.*[31] with RPMI medium 1640 and GEM 1717 medium supplemented with 20% FBS. After 43 days, Epstein-Barr Virus (EBV) was added. The EBV was derived from a freeze/thaw lysate of B-95-8 marmoset lymphoid cell line infected with EBV.[27]

Morphological Studies

Subcultures of the tumor cells were grown on coverslips to near confluency and viewed by either phase

0008–543X/80/0315/1400 $0.95

microscopy or fixed and stained. The histochemical stains were Sudan black,[24] Baker's method for phospholipids,[3] Oil red O,[23] Alcian blue-PAS at pH 1.0 and pH 2.5,[39] and hydaluronidase digestion followed by Alcian blue-PAS. The tumor cell line was prepared for electron microscopy as described previously.[32]

Chromosome Analyses

Seven-day-old subcultures (two-days post addition of fresh medium) were incubated 5 hours at 4 C with 0.02 μg Colcemid (Ciba Pharmaceuticals, Summit, NJ) per 10 ml medium. Cells were scraped off the glass with a rubber-tipped rod and the resulting cell suspension was centrifuged, resuspended in 0.075 M KCl for 15 minutes, centrifuged, resuspended in fixative (3:1, methanol:acetic acid) for 10 minutes, centrifuged and resuspended in fresh fixative for an additional 10 minutes. Metaphase spreads were made according to the hot plate method of Lubs *et al.*[25] Giemsa-banded chromosomes were obtained as previously described.[49] Constitutive heterochromatin banding was obtained using a modification of the method of McKenzie and Lubs.[26] Marker chromosomes were described according to Paris Nomenclature, 1971.[42,43]

Cell suspensions from mitotically active lymphocyte cultures were harvested and treated for 1 hour at 37 C with 0.02 μg Colcemid/10 ml medium, treated for 10 minutes with 0.075 M KCl, and fixed as described above. These metaphase spreads were both Giemsa-banded and constitutive heterochromatin banded as described above.

Cell Products

A carcinoembryonic antigen (CEA) kit (Roche Diagnostics, Nutley, NJ) was used to assay, (1) spent medium exposed to a culture of 2.6×10^6 total live tumor cells for 14 days, (2) the cytosol from freeze/thaw lysed cells of the same culture, and (3) culture medium supplemented with 20% FBS without exposure to tumor cells.

Glucose-6-phosphate dehydrogenase (G6PD) isozyme mobility was monitored by cellulose acetate electrophoresis (Helena Laboratories, Beaumont, TX) on cytosol from 10^7 cultured tumor cells lysed by freeze/thaw in 0.5 ml of 0.9% NaCl solution. Isozyme phenotypes of the cultured tumor cells were examined with the aid of W. Wright and P. Daniels in the laboratory of J. Fogh (Sloan-Kettering Institute). The isozyme profiles analyzed were acid phosphatase (ACP), adenosine deaminase (ADA), phosphoglucomutase 1,3 ($PGM_{1,3}$), esterase D (ESD), and glutamate oxaloacetate transaminase (GOT_m).

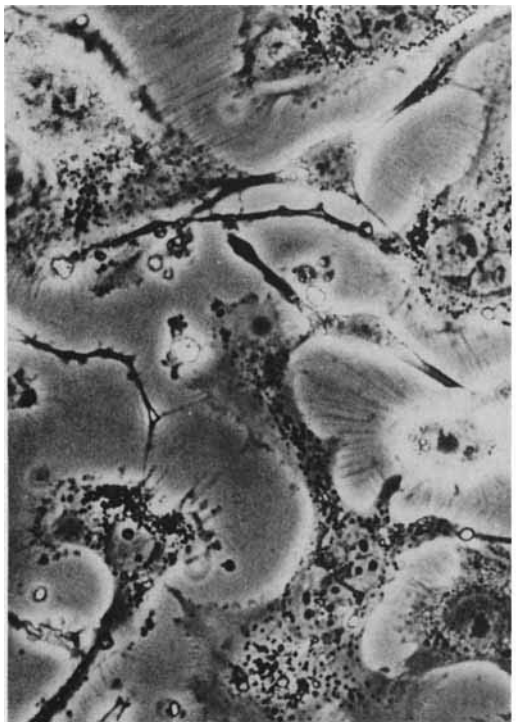
Early in the cultivation of the tumor cell line, the culture medium increased 2- to 3-fold in viscosity. The viscous-spent medium was examined for liposubstances. Both 14-day-old spent RPMI medium 1640 with 10% FBS from actively growing cultures and complete medium without exposure to cultured cells were assayed for β-lipoproteins (β-lipoprotein RID kit, Behring Diagnostics, Somerville, NJ), total triglycerides and cholesterol (Tri-Chol kit, Oxford Laboratories Inc., Foster City, CA) and total lipids (Associated Laboratories, Inc., Wichita, KA).

Viscous spent medium recovered after seven days from an actively growing tumor cell culture and a control sample of fresh medium were assayed for acid, non-sulphated mucopolysaccharides (hyaluronic acid) based on the methods of Tolksdorf *et al.*[53] and Kass and Seastone[19] in which hydaluronic acid was measured by its ability to form turbidity with acid albumin solution (250 mg/dl bovine serum albumin, fraction V, pH 3.0). Turbidity was read at 540 nm absorbance.

For incorporation of mucopolysaccharide precursors, monolayer cultures of 10^7 viable tumor cells with 15 ml of RPMI medium 1640 with 10% FBS were exposed to 5 μCi/ml of ^{3}H-glucosamine (3 Ci/mmol, New England Nuclear Corp.) for 24 hours at 37 C. The mucopolysaccharides were isolated according to the procedure of Satoh *et al.*[48] Tritiated labeled mucopolysaccharides in the 24 hour spent medium were isolated by precipitation with excess cetylpyridinium chloride after 18 hours digestion with 1 mg/ml pronase at pH 6.5 and 37 C, 18 hours exposure to 0.1 M NaOH, and dialysis against H_2O. The precipitate was washed with 30 nM NaCl and extracted with 0.4 M NaCl. Hyaluronidase susceptibility of the 0.4 M NaCl eluate was observed by measurement of dialyzable radioactivity after 24 hours of digestion at pH 5.0 and 37 C with 300 Worthington turbidity reducing units of testicular hyaluronidase. Colony formation in soft agar was determined by the method of Moore *et al.*[34]

Surface membrane immunoglobulins (SmIg) on cultured lymphoid cells were monitored by immunofluorescence using either goat or horse fluorescein isothiocyanate (FITC) conjugated antisera to human IgG, IgM, IgA, IgD, and IgE (Hyland Laboratories, Costa Mesa, CA). Epstein-Barr Virus (EBV) capsid antigen was observed on the viable cultured lymphoid cells using an indirect immunofluorescent test with the use of anti-EBV capsid antigen (Associated Biomedics Systems, Buffalo, NY).

For both SmIg and EBV-capsid antigen studies, a



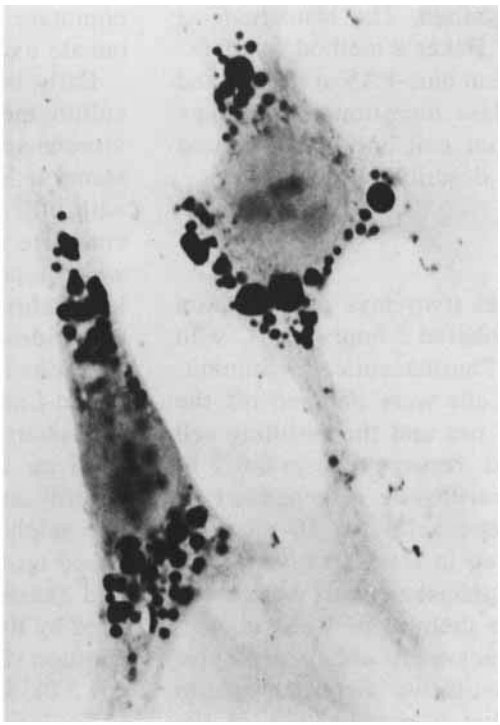


FIG. 1. (*left*) COLO 222 cells grown in RPMI medium 1640 supplemented with 10% FBS (Phase contrast ×205).

FIG. 2. (*right*) COLO 222 cells with Sudan black positive vacuoles. (×415).

series of viable cultured lymphocytes (10^6) were twice washed with phosphate-buffered saline (PBS) followed by incubation at room temperature with 0.1 ml of the appropriate antisera for 30 minutes. For SmIg, the cells were pelleted, washed with PBS, and then observed directly. In the case of EBV-antisera-treated cells, the cell pellet was stained with 0.1 ml FITC-conjugated goat anti-human IgG, washed, and then observed. Immunofluorescence was observed with the use of a Zeiss Photo III equipped with an epi-illuminator, a reflector 510 excitation 440–500 nm and 530 barrier filters, and 40× oil iris Planapochromat objective.

Negtive controls for the SmIg study were cultured human fibroblasts and the positive controls were fresh lymphoid cells isolated by Hypaque-Ficoll gradient. As controls for the EBV-capsid antigen immunofluorescence, B-95-8 cells served as positive controls, whereas MOLT-4F cells were negative controls.

Spontaneous erythrocyte rosette studies were performed according to the method of Jondal *et al.*[17] Erythrocyte-antibody-complement rosette studies using sheep erythrocytes were performed on the cultured lymphocytes.[49] The spontaneous rosette formation of the cultured lymphocytes with monkey erythrocytes (*Macaca speciosa*) were examined by the method of Pellegrino *et al.*[44]

Cultured lymphocytes were tested for cytotoxicity against autochthonous cultured tumor cells. Cytotoxicity assays were performed by seeding into each well of Test II plates (Falcon Plastics, Los Angeles, CA) a suspension of 2.5×10^4 cultured tumor cells in 250 μl of GEM 1717 medium with both 10% FBS and 1.5 × glucose. The seeded plates were incubated at 37 C for 24 hours in humidified air with 10% CO_2 to allow attachment of the cultured target cells. Following attachment of the target cells, the spent medium was aspirated and replaced with 250 μl of fresh medium which contained 5×10^5 autochthonous cultured lymphocytes. Control wells contained only target-cultured tumor cells. At predetermined intervals, control wells and wells with the cocultivated target and effector cells were time stopped, fixed, stained, and visually assessed for remaining target cells.

Spent medium and cells of both cultured tumor and lymphoid cell lines were tested for the presence of PPLO with Hoechst's fluorescent stain after the method of Chen.[6] Cryopreservation of the cultured tumor and lymphoid cell lines were as previously described.[34]

Results

Establishment

The liposarcoma tumor cell line was designated COLO 222 on August 25, 1975, (118 days post-initiation) after monolayer growth from the primary tissue minces reached semiconfluency in RPMI 1640

with 20% FBS and repeated successful subcultures were free of fibroblasts or other cell types. The monolayer was subcultured according to the procedures of Moore *et al.*[34] The lymphoid cell line was considered established after the first subculture of supernatant cells on August 1, 1975, and was designated COLO 143v. Establishment occurred 49 days after the addition of exogenous EBV or a total of 93 days post-initiation.

Morphology

Phase-contrast microscopy of COLO 222 revealed cuboidal-bipolar and cuboidal-tripolar dendritic cells with vacuolated cytoplasm (Fig. 1). Nuclei varied in size and staining properties. Figure 2 shows COLO 222 cells stained with Sudan black. Approximately 75% of the cells contained cytoplasmic vacuoles filled with lipid deposits. Table 1 summarizes the results of histochemical stains employed on COLO 222 cells.

Electron micrographs of COLO 222 revealed large cells with numerous truncate microvillar projections (Fig. 3). Membrane-bound osmiophilic and non-osmiophilic electron opaque vesicles were observed in the cytoplasm. These intracellular vesicles were intimately associated with an extensive, dilated, rough endoplasmic reticulum. These observations were consistent with the reports of Oda *et al.*,[41] Moskowitz,[36] and Moore *et al.*[33] concerning lipogenic cells. Numerous glycogen deposits, multiple Golgi apparatus, and many mitochondria were observed. Nuclei were deeply lobed and contained multiple prominent nucleoli. No tonofibrils, phagosomes, or well-formed desmosomes were observed. Tight junctions were occasionally observed. No virus or viral particles were observed during repeated ultrastructural examinations.

TABLE 1. Reaction of COLO 222 Cells for Specific Compounds Employing Histochemical Stains

Histological stain	Specificity	Reaction
Sudan black	Lipids	+
Baker's acid haematein	Phospholipids	+
Oil red O	Lipids	+
Alcian blue-PAS		
pH 1.0	Non-sulfonated mucosubstances	+
pH 2.5	Sulfonated mucosubstances	−
Hyaluronidase digestion followed by alcian blue-PAS pH 1.0	Hyaluronic acid types	−

Cytogenetics

Cytogenetic analysis of the lymphoid cell line COLO 143v was based on 62 metaphase counts of

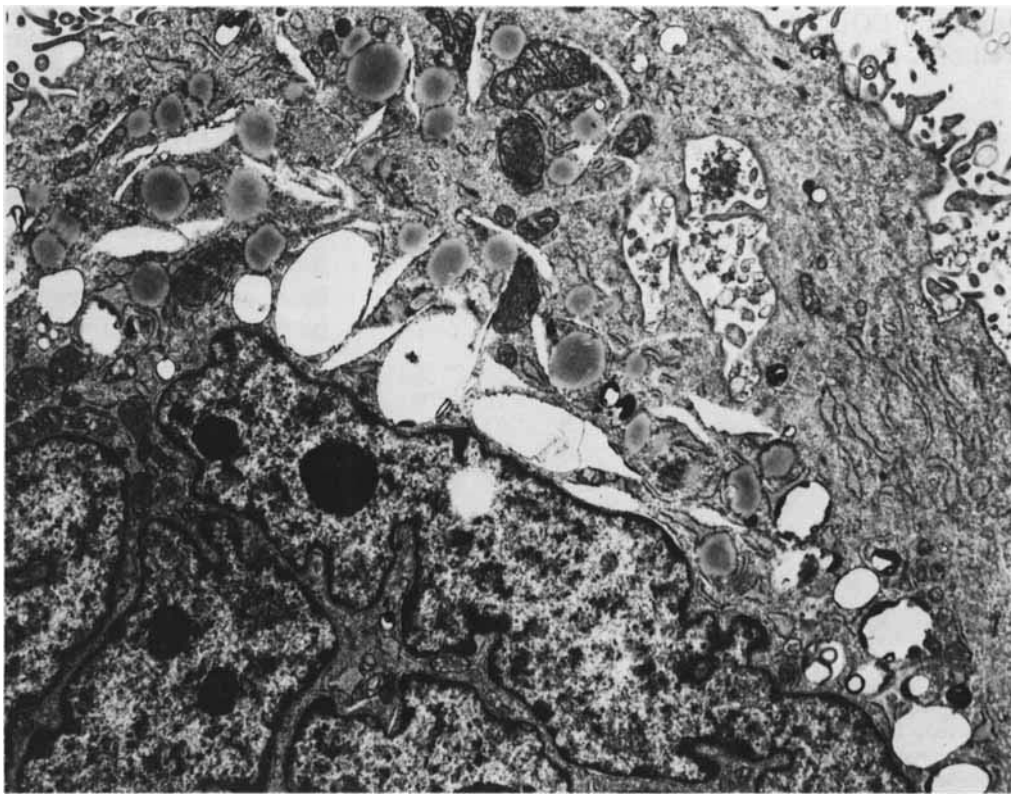


FIG. 3. Ultrastructure of COLO 222 with membrane-bound osmiophilic and non-osmiophilic vesicles (×9000).

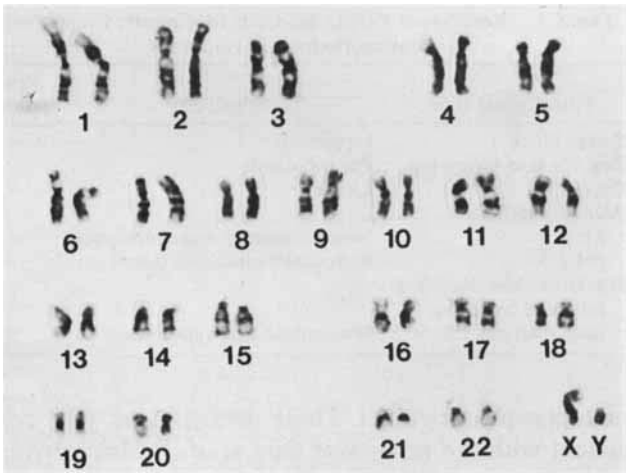


FIG. 4. COLO 143v Giemsa-banded chromosomes showing polymorphic #9 chromosome.

which 20 were photographed and eight were karyotyped. The modal number of chromosomes was 46, XY. Polyploidy was observed in 8.2% of the metaphases. The phenotypically normal karyotype of COLO 143v is depicted in Figure 4.

The cytogenetic analysis of COLO 222 cells was based on 60 metaphases of which nine were photographed and seven were karyotyped. The chromosome mode was 53 with a range of 52–55. The chromosome #9 with a variant heterochromatic region (h+) observed in COLO 143v was not evident in the tumor cell line. Frequent monosomy of chromosome #9 in the tumor cell line probably accounts for absence of this heterochromatic region in COLO 222. The Y chromosomes observed in COLO 143v and COLO 222 were equal in length. The most consistant tumor cell line markers observed in COLO 222 are depicted in a representative karyotype (Fig. 5). Our best analysis of COLO 222 markers was:

M_1 del (1) (pter → q12:)
M_2 t(1;2) (1pter → 1p22::2q2? → 2qter)
M_3 t(3;15?) (3pter → 3q?::15?q21 → 15qter)
M_4 t(1;4) (1qter → 1q25::4q3? → 4pter)
M_5 t(5;?) (5qter → cen → ?)
M_6 t(6;7) (6pter → 6q2?::7q2? → 7qter)
M_7 t(7;?) (7pter → 7q36::?)
M_8 t(12;17?) (12qter → cen → 17qter)
M_9 t(14;?) (14qter → cen → ?)
M_{10}unknown origin
M_{11}minute

The additional material on chromosome #7 of marker M_7 is heterochromatin of unknown origin and was confirmed by C-banding.

Markers M_1–M_4 and M_6–M_9 were present in 85% of the metaphases studied. Markers M_5, M_{10}, and M_{11} were less frequently observed.

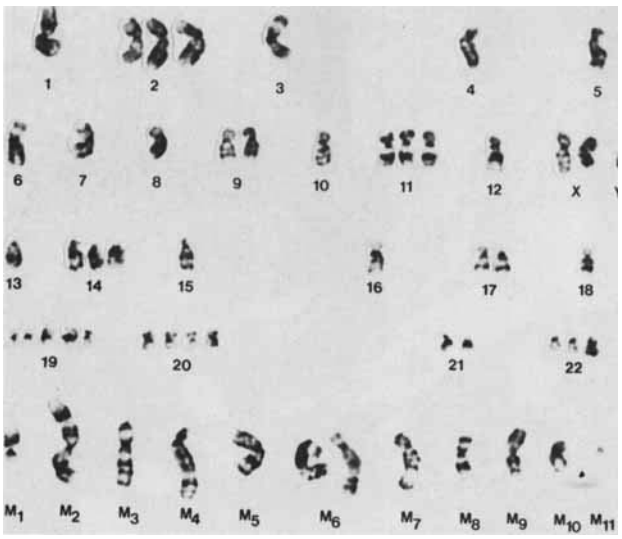


FIG. 5. Representative Giemsa-banded karyotype of COLO 222 showing normal and marker chromosomes. Two M_6 markers are displayed. The M_6 marker on the right was selected from a different metaphase to more clearly depict the distorted M_6 on the left from the original metaphase.

Cell Products

No CEA was detected in spent medium from COLO 222 or control medium. Similar negative results have been obtained after one and two years in continuous culture. The isozyme phenotypic profile of COLO 222 was G6PD type B, ACP type B, ADA type 1, PGM_1 type 1, PGM_3 type 1, ESD type 1, and GOT_m type 1. COLO 222 grew in soft agar with a 22% efficiency.

Chemical tests of the viscous spent medium for β-lipoproteins, triglycerides, cholesterol, and total lipids were either negative or no more than equal to the background values for control medium. Turbidimetric and enzymatic determinations for hyaluronic acid of spent media were positive. Hyaluronic acid level in the spent medium was 140 $\mu g/10^6$ viable tumor cells. This hyaluronic acid concentration includes subtraction of 6 μg hyaluronic acid/ml found in control fresh medium. The mucopolysaccharide fraction eluted from the cetylpyridinium chloride precipitate with 0.4 M NaCl was completely digested (>95%) by hyaluronidase.

COLO 143v lymphocytes were positive for the following classes of immunoglobulins (percent fluorescing cells); IgG (88%), IgM (58%), IgE (85%), IgA (29%), and C'3 (75%). COLO 143v cells were negative for IgD and C'4. COLO 143v was positive for EBV

capsid antigen. COLO 143v cells rosetted spontaneously with the monkey erythrocytes but did not rosette spontaneously with sheep erythrocytes.

Naive COLO 143v lymphocytes without prior exposure to COLO 222 target cells killed 100% of the COLO 222 tumor cells by 113 hours in the cytotoxicity assay. COLO 143v lymphocytes sensitized by 72 hours co-cultivation with COLO 222 cells killed fresh COLO 222 targets in 24 hours.

The liposarcoma cell line, COLO 222, and autochthonous lymphoid cell line, COLO 143v, had no evidence of PPLO contamination. Both COLO 222 and COLO 143v have been recovered from our cell bank.

Discussion

The synthesis of hyaluronic acid by cultured synovial fibroblasts has been well documented.[54,59] Short-term increase in synthesis of hyaluronic acid by cultured human and animal cells has been noted after viral transformation,[16] irradiation,[58] hybridization,[22] the addition of cyclic AMP,[55] and the addition of serum from rheumatoid patients.[4] *In vivo* synthesis of hyaluronic acid has been reported from tumors of mesenchymal origin, such as mesothelioma,[1,15] nephroblastoma,[36] reticulum cell sarcoma,[9] mastocytosis,[2] and liposarcoma.[10,20] *In vitro* secretion of hyaluronic acid by cultured human malignant cells has not previously been reported. COLO 222 secretes hyaluronic acid at levels reported by synovial fibroblasts in culture.[13]

Except for malignant melanomas, human sarcomas are difficult to establish in continuous culture. A few liposarcoma cell lines have been established and characterized. Moore *et al.*[33] described RPMI M7021. Sethi *et al.*[50] listed four liposarcoma cell lines; PLS-35, BLS-50, MLS-64, and DLS-73, but illustrated only the Papanicolaou staining reaction of PLS-35. Nelson-Rees analyzed the Giemsa-banded marker chromosomes of a liposarcoma cell line SA_4, originated by O'Conner and Shibley (Nelson-Rees *et al.*).[40]

COLO 222 is similar in ultrastructure to RPMI M7021. Both cell lines had large membrane-bound osmiophilic and non-osmiophilic electron opaque inclusions in their cytoplasm. Fewer lipid filled vacuoles were observed in RPMI M7021 than in COLO 222. One to 5% of the RPMI M7021 cells had Oil red O positive deposits; nearly 100% of the COLO 222 cells had Sudan black positive vacuoles. In both RPMI M7021 and COLO 222, no virus-like particles or foci of transformed cells, similar to those described by Morton, *et al.*,[37] were evident.

Cytogenetic analysis of COLO 222 revealed that eight marker chromosomes were present in more than 85% of the metaphases. The mode of COLO 222 was 53 whereas RPMI M7021 was bimodal with modes of 51 and 102.

The markers of COLO 222 are not similar to the banded karyotype of the liposarcoma cell line SA_4, though chromosomes #1 and #3 are involved in the formation of two markers in both COLO 222 and SA_4. Chromosome #1 and chromosome #3 have frequently been described as involved in markers of malignant cultured cells and fresh tumor preparations.[7,8,11,18,21,35,40,46,47,49,51,57] The complement of markers of COLO 222 does not appear to be the same as any other banded karyotype of a human cell line.

Isozyme patterns further identify the uniqueness of a cell line. Based on the isozymes studied, the combined chance of two individuals or cell lines being alike for all seven isozyme loci is 0.036[45] within a random Caucasian population.

COLO 222 is a unique cell line based on its composite of marker chromosomes, cell products, and isozyme phenotype. Furthermore, COLO 222 is also unique since it was derived from a primary liposarcoma. The cell products of COLO 222 aid in authentication of its sarcomatous origin. COLO 222 lacks CEA production, but does synthesize hyaluronic acid. The absence of well-formed desmosomes and tonofibrils lends evidence to its mesenchymal origin.

The autochthonous lymphoblastoid cell line, COLO 143v, phenotypically appears to be normal and exhibits numerous B-cell characteristics. COLO 143v cells produce immunoglobulins and rosette spontaneously with monkey erythrocytes but do not rosette with sheep erythrocytes.

It is uncertain whether the addition of exogenous EBV enhanced or speeded up the establishment of the lymphoid line. Without the addition of exogenous EBV, lymphocytes establish in an average of 40–60 days.[30] Days to establishment are decreased markedly if the donor is infected with infectious mononucleosis virus.[5] If the establishment of the lymphoid line was enhanced by the exogenous EBV, the time from viral addition to establishment should have been shorter than that observed.[52]

COLO 143v cells were positive for capsid EBV. The presence of capsid EBV receptors on cultured lymphocytes is not unusual since all cultured lymphocytes with B-cell characteristics possess these receptors.[28]

Mitchen *et al.*,[29] Morgan *et al.*,[35] and Semple *et al.*[49] have reported the use of cultured B-lymphocytes as effector cells against autochthonous cultured tumor target cells. The B-lymphocyte cell line COLO 143v is capable of destroying the autochthonous COLO

222 target cells in 113 hours in *in vitro* cytotoxicity assays.

COLO 143v and COLO 222 have been recovered from our cell bank after "slow freezing." They are available to other qualified investigators for their studies.

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