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BIOLOGICAL AND BIOCHEMICAL PROPERTIES OF A HUMAN UVEAL MELANOCYTE-DERIVED CELL LINE

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SUMMARY

A human uveal melanocyte-derived cell line (UII) is described. The cell line has a doubling time of 27.2 hr, a plating efficiency on plastic surfaces of 10%, and a cloning efficiency in soft agar of <0.1%. UII displays marked chromosomal aneuploidy and sensitivity to ultraviolet light. Biochemical studies indicate the presence of tyrosinase, which is stimulated by several compounds, including theophylline, progesterone, and nerve growth factor.

Key words: melanocyte; melanoma; uveal; ultraviolet light; tyrosinase; chromosome.

INTRODUCTION

Melanocyte cultures derived from normal fetal uveas (1), melanoma tissue (1–4), nevi (5), split-thickness skin grafts (6), and choroidal tissue (7) have been used by previous investigators to study human melanocyte differentiation and proliferation. One study of pigmented retinal epithelium melanocytes noted the occasional spontaneous evolution of a new cell type in these primary cultures that appeared to be able to grow indefinitely in vitro (7). We have noted the same spontaneous appearance of a permanent cell line in one of our primary uveal cultures. We report here selected biological, biochemical, and chromsomal properties of this cell line that should be useful for studying diverse aspects of melanocyte biology.

MATERIALS AND METHODS

Tissue culture techniques. An eye was obtained post mortem from a 16-year-old normal male accident victim (protocol approved by the University of Arizona Human Subjects Committee). The cornea was removed, and the eyeball was incised behind the limbus and the anterior segment was removed. The gelled vitreous and retina were removed from the vitreous cavity after inversion of the posterior globe. Pigmented tissue was freed by needle dissection of the retina. Cells were obtained after incubation of the retinal remnants in 0.50% collagenase and 0.25% trypsin for 2.0 hr at 37° C and harvested by repeated aspiration of the minced tissue through an 18-gauge needle. Cell fragments were incubated in growth medium [F-10 media containing 10% heat-inactivated fetal bovine serum and 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 0.25 μg/ml of Fungizone (Grand Island Biological Company, Grand Island, NY)]. Cultures formed a confluent monolayer after 2 weeks. The cells were subsequently diluted and transferred to fresh petri dishes after removal with 0.25% trypsin. Neither the patient nor members of his family had a history of uveal melanomas, retinoblastomas, or other ocular neoplasms.

Doubling time was determined by inoculating 2×10^4 cells into each of several 25-cm² flasks. At 24-hr intervals the cell number in duplicate flasks was determined and the doubling time calculated from the plot of the log of the cell number versus the incubation time.

Plating efficiency on plastic surfaces was determined by inoculating plastic 35-cm² petri dishes with 0, 100, 500, or 1000 cells in growth medium. The cells were incubated for 14 days, washed

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twice with serum-free medium, fixed with Wright-Giemsa and stained, and clones containing 50 or more cells were counted.

Cloning efficiency in semisolid agar was determined by inoculating various cell numbers into 0.3% agar (Noble) over an underlayer of growth medium supplemented with 0.5% agar. Clones developed over 6 to 7 days and were then counted directly by means of an inverted microscope.

Analysis of viral antigens. Cells were grown to confluence, removed from the petri dish with a rubber policeman and, along with the supernatant medium, inoculated onto monolayer tube cultures of either primary rhesus monkey kidney cells, a heteroploid human epithelial cell line, or a human embryonic tonsil fibroblast diploid cell strain. These cultures were observed for cytopathic effect due to viral infection or evidence of hemadsorption for 2 weeks (8).

Acetone-fixed smears of scraped cells were also examined by immunofluorescence methods (9) for the presence of cytomegalovirus antigen, utilizing fluorescein-labeled rabbit hyperimmune sera (Flow Laboratories, McLean, VA) and for Epstein-Barr virus capsular antigen and nuclear antigen (EBNA). Detection of virus capsular antigen employed human antibody-containing sera and labeled goat antihuman immunoglobulin (Meloy Laboratories, Springfield, VA). EBNA was detected by anticomplement immunofluorescence, utilizing antibody-positive human sera, guinea pig complement, and labeled goat anti-guinea pig complement (Cappel Laboratories, Cochranville, PA) (10).

Chromsomal analysis. Metaphase spreads were prepared by standard karyotypic techniques. One hundred spreads were counted for modal number. G banding was carried out by the trypsin-Giemsa technique of Sun et al. (11). Nucleolar organizer regions were also scored on 22 metaphase spreads (12).

Growth factors. These compounds were used at concentrations that are known to have effects on melanoma cell proliferation and differentiation (13,14). Nerve growth factor (NGF, 10 ng/ml) and fibroblast growth factor (FGF, 50 ng/ml) were obtained from Collaborative Research Inc. (Waltham, MA). Insulin (5 μg/ml), N^6,O^2-dibutyryl cyclic AMP (10^{-4} *M*), theophylline (1 m*M*), prostaglandin E_1 (10^{-6} *M*), and progesterone (4×10^{-6} *M*) were obtained from Sigma Chemicals (St. Louis, MO). Porcine follicle stimulating hormone (FSH, 0.4 ng/ml) and luteinizing hormone releasing factor (LHRF, 10 ng/ml) were obtained from Beckman Chemicals (Palo Alto, CA). α-Melanocyte stimulating hormone (MSH, 2×10^{-9} *M*) was synthesized in the laboratory of V. Hruby (Chemistry Department, University of Arizona).

Tyrosinase assay. The presence of tyrosine hydroxylase (tyrosinase) activity was determined as described previously (15). Briefly, 2×10^5 cells were inoculated into 25-cm^2 flasks and incubated at 37° C overnight. Medium was then replaced with fresh medium containing 1 μCi/ml of [^{3}H]tyrosine (sp act 48 Ci/mmole, New England Nuclear, Boston, MA) and the appropriate concentration of compounds under investigation. After 24 hr of incubation, the medium was collected and fresh medium was added for an additional 24 hr. The collected media were absorbed with activated charcoal and Dowex 50W (BioRad Laboratories, Richmond, CA) to remove unreacted tyrosine and the remaining unbound ^{3}HOH counted in a liquid scintillation counter.

Melanin assay. The presence of melanin was detected by solubilizing one million cells with 1.0 ml of 1*N* NaOH and 10% DMSO for 30 min. The optical density was read at 470 nm and the melanin content was expressed as micrograms melanin per 10^6 cells.

Melanoma cell line M1RW. This cell line was established from a subcutaneous metastasis obtained from a 17-year-old male with melanoma. The cell line has been maintained for 24 month for over 90 passages. Cells on their 10th to 25th passage were used to analyze their growth properties: doubling time, 23.6 hr; plating efficiency on plastic surfaces, 15%; cloning in soft agar, 5%, tyrosinase activity (30,903 cpm/10^6 cells), and melanin content (16 μg/10^6 cells).

Response to ultraviolet radiation. For survival studies, rapidly growing cells were inoculated onto 100 cm^2 petri dishes at various cell densities (10^2 to 10^5) in 5 ml of growth medium and incubated 5 hr to allow attachment of cells.

The medium was removed and the cells were covered with 1 ml of phosphate buffered saline and irradiated with ultraviolet light (254 nm) at a dose rate of 2.8 J/m^2 per s (determined using a Black-Ray ultraviolet meter, Ultra-Violet Products, Inc., San Gabriel, CA). Following irradiation the buffer was removed and 10 ml of fresh medium was added to each plate. The plates were incubated at 37° C in 5% CO_2 and refed every 3 days with fresh medium. Clones were fixed in 3% glutaraldehyde after 15 days (human melanoma line, MIRW) or 8 days (UlI) and

stained with 10% Giemsa stain. Colonies containing 50 cells or greater were counted and the surviving fractions of colony-forming cells were determined from these counts.

RESULTS

Morphology. During the first 5 days of incubation of six human retinal fragments in primary culture, heavily pigmented cells appeared to migrate from larger fragments. Within 3 weeks these cells formed a pigmented confluent monolayer (Fig. 1*A*). In 5 of 6 cases the cells ceased to grow after dilution and transfer of the primary cells to a secondary culture. In one case the cells lost their pigmentation (Fig. 1*B*, *C*) and began to divide at a rapid rate. These cells, subsequently designated UII, exhibited no evidence of increased melanogenesis or dendritogenesis during either the exponential or stationary phase of growth. Electron microscopy revealed no detectable melanosomes, premelanosomes, onion-ringlike structures, or viral structures (16). UII cultures were next subjected to the following analyses in order (a) to investigate the changes in growth properties of these cells, which occurred during incubation of secondary cultures, and (b) to determine whether these cells retained characteristics of their uveal origin and therefore might be useful as models for studying melanocyte development.

Growth properties. UII cells grew rapidly in culture (27.2 hr doubling time) and had a saturation density of 1.2×10^6 cells/25 cm^2 on plastic. The cell line has been maintained for over 18 months and is now on its 75th passage and remains *Mycoplasma* free as determined by the [^{3}H]uridine-to-[^{14}C]uracil ratios (17). The characterization of the cell line reported below was performed on cells between their 10th and 25th passages. Since these growth characteristics are frequently exhibited by tumor cells, studies were undertaken to determine whether UII cells might have become transformed into cells with neoplastic characteristics. Tumor cells frequently exhibit comparable and high cloning efficiencies in semisolid medium (soft agar) and on plastic surfaces. In contrast, UII cells showed poor ($< 0.1\%$) colony formation in soft agar. The plating efficiency of UII cells on plastic surfaces was 10% and was linear following inoculation of 200 to 1000 cells.

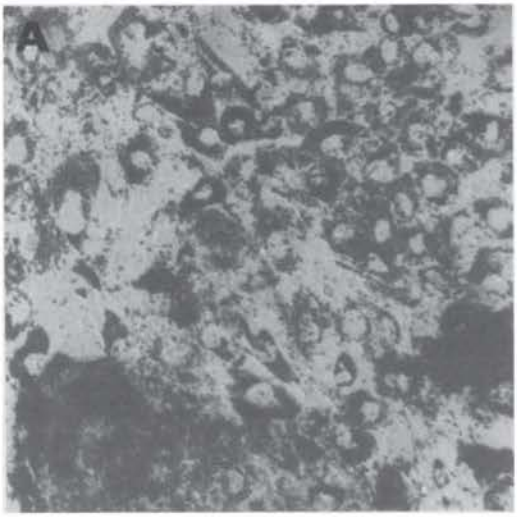
Test for viral infection. Since spontaneous transformation of normal human cells in culture is rare, but viral transformation has been found to produce malignant cell types, UII cells were tested for evidence of viral infection. UII cells were inoculated onto monolayers of monkey kidney, human epithelial, and human fibroblast cells and followed for evidence of cytopathic effects (due to herpesvirus infection) or of hemadsorption. No evidence of viral infection was found. Tests for cytomegalovirus antigen and for Epstein-Barr virus antigens (VCA and EBNA) were also negative.

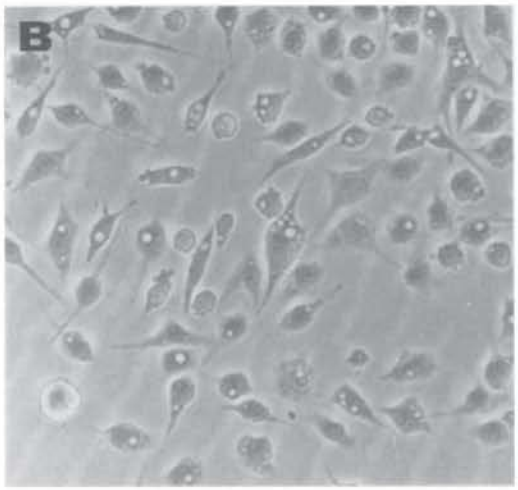
Chromosomal analysis. Out of 100 metaphase spreads counted (Fig. 2), 91% were found within the chromosomal range of 26 to 44 with no modal number. Five G-banded metaphase spreads were analyzed. Although each karyotype was both abnormal and different, several consistent abnormalities were found: centric fashion in Groups A and C; deletions in Groups A to C; whole chromosome loss in Groups, C, E, F, and G; no six chromosomes; and complete lack of abnormalities in Group D. The average number of nucleolar organizing regions per metaphase was 4.0, which is below the normal range of 6 to 10. The effect was not totally due to the loss of G group chromosomes as the number of active organizing regions on the karyotypically normal D group chromosomes was also low.

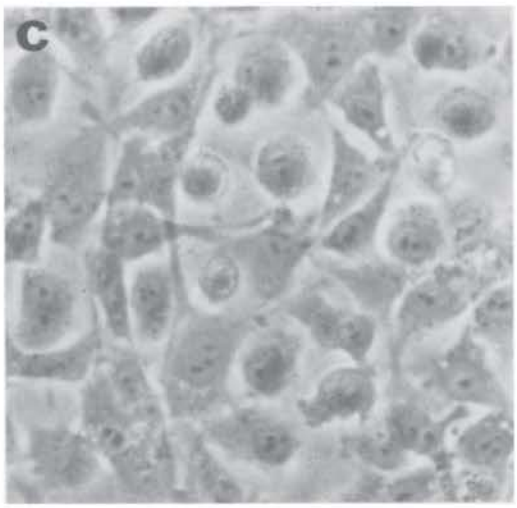
Tyrosinase activity and melanin content. Unstimulated enzyme activity was 27,522 cpm/10^6 cells. Melanin was *not* detectable even when 10^7 cells were used in the assay.

Response to chemical messengers. Various growth factors and other chemical compounds were added to cultures of UII in order to test the effects of these substances on production of tyrosinase activity and cellular proliferation. Various chemical messengers had different effects on the cellular proliferation and differentiation of UII as measured by cell counts and tyrosinase activity (Table 1). NGF and progesterone caused the greatest inhibition of cellular proliferation and stimulation of tyrosinase activity. FSH, LHRF, and insulin inhibited cellular proliferation 50, 40, and 49% but did not stimulate tyrosinase activity. MSH and dibutyryl cyclic AMP had no effect on cellular proliferation or tyrosinase activity whereas theophylline markedly inhibited cell growth and stimulated tyrosinase activity. Melanin was not detected after treatment with any of the chemical messengers.

Response to ultraviolet radiation. UII cells and human melanoma MIRW cells were irradiated with ultraviolet light and the surviving fraction of colony-forming ability was determined (Fig. 3).







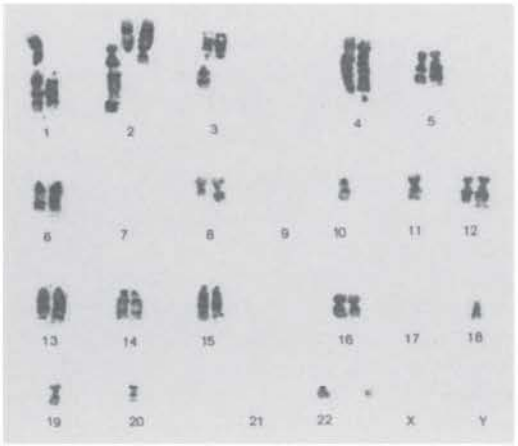


FIG. 2. Karyology of human uveal melanocyte cell line. ×2500.

UlI cells showed 15- to 250-fold greater sensitivity to irradiation than the human melanoma cells at the doses used, indicating a marked UV sensitivity of the UlI cell line compared to the melanoma line.

DISCUSSION

The most frequently studied model for melanocyte development has been the murine malignant melanoma. Although melanoma cells have yielded extensive information concerning pigment production and induction of tyrosinase activity, these cells are neoplastic and may behave quite differently from their normal melanocyte counterpart. Studies of normal human melanocytes in culture have been limited to the use of split-thickness skin grafts (6,18,19) and fetal (1) and adult (7) uveal tissues. Mannagh et al. (7) reported that two of seven "spontaneously transformed" cell lines derived from adult uveas proliferated for greater than 40 passages and were aneuploid, but no details were given regarding the expression of tyrosinase or melanin. In a more detailed study Giovanella et al. (1) described four cell lines derived from fetal uveas as having long doubling times (60 to 108 hr) and expressed melanin only at confluence. One of two lines was said to be diploid whereas the other cell line was

FIG. 1. Human uveal melanocyte cell line. *A*, Three-week primary cell culture derived from uveal tissue. *B*, *C*, Secondary culture derived from the primary culture but exhibiting altered growth and pigmentation properties. ×1000.

TABLE 1

EFFECT OF GROWTH FACTORS ON CELLULAR PROLIFERATION AND TYROSINASE ACTIVITY OF CELL LINE UII[a]

Chemical Messenger	Cell Count	Tyrosinase Activity
	% of control	*% of control*[c]
MSH	90	98
Dibutyrylcyclic AMP	100	112
Theophylline	48	208
NGF	44	278
FGF	68	131
FSH	50	0[b]
LHRF	60	0[b]
Insulin	51	73
Progesterone	26	767
Prostaglandin E_1	105	120

[a] Cell counts and tyrosinase activity were measured after 48 hr of exposure to a given compound at the cell concentrations indicated in Materials and Methods. The responses to MSH, dibutyrylcyclic AMP, and theophylline are discussed in detail elsewhere (15).

[b] Tyrosinase activity was not detectable after treatment with these compounds.

[c] In no case was melanin detectable, even when 2×10^6 cells were used for the assay.

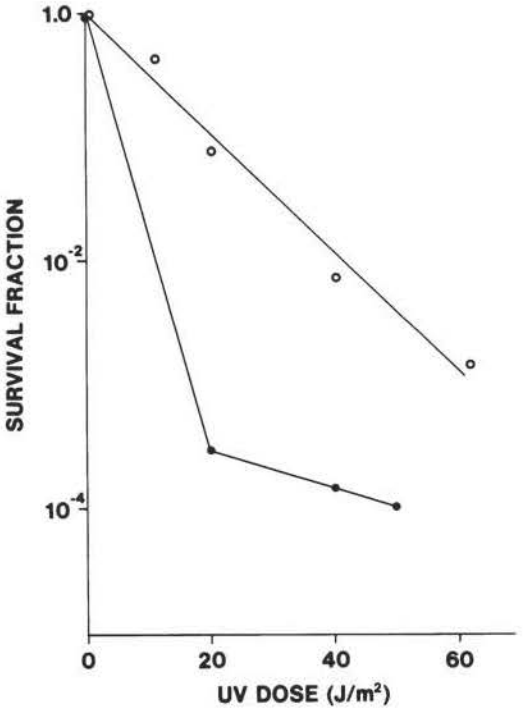


FIG. 3. Surviving fraction of human cells after ultraviolet irradiation. Survival of colony-forming ability was determined as described in Materials and Methods. Plating efficiencies for unirradiated cells were 10% for UII and 15% for the melanoma MIRW cell line. The results are the average of two experiments. Symbols: ●—●, UII; ○—○, melanoma cell line MIRW.

aneuploid. Consequently, the development of an aneuploid human melanocyte uveal-derived cell line such as UII, which expresses tyrosinase activity without melanin formation, may prove useful for studying both aneuploidy and human melanocyte regulation.

Analysis of the UII line indicates that these cells have some characteristics of melanocytes. First, these cells were derived from uveal tissue and contain the enzyme tyrosinase. Although these cells fail to demonstrate melanin or early premelanosome by electron microscopy, the presence of tyrosinase in these cells, which is increased following treatment with various chemical messengers, indicates that the cells are of primitive neural crest origin. The inducibility of tyrosinase in these cells by progesterone, theophylline, or nerve growth factor indicates that UII cells have retained some differentiated characteristics of melanocytes (6,13,18,19). However, MSH and dibutyryl cyclic AMP did not induce tyrosinase activity. Previous reports have indicated that normal human melanocytes in split-thickness skin grafts and murine (15,20), and human malignant melanoma cells (21) in some cases do show induction of tyrosinase activity in the presence of these compounds. The inhibition of UII cellular proliferation by FSH, LHRF, and insulin without a concomitant change in tyrosinase activity requires further investigation since these three compounds are known to be necessary components of serum-free medium that support murine melanoma cell growth (14).

Although UII cells have characteristics of their in vivo counterparts, they clearly differ from melanocytes in vivo and from primary melanocyte cultures by exhibiting rapid growth rates, marked chromosomal aneuploidy, apparent indefinite growth in culture, and also form tumors in nude mice (22). These properties are comparable to those seen in cultured melanoma lines and other tumor cells and suggest that the UII cells have become transformed. Also, normal cells in culture generally exhibit a diploid karyotype. By this criterion, UII cells exhibited karyotypes more characteristic of tumor cells than normal cells in culture. However, previous investigators have shown that the growth property of cells transformed in culture that most closely correlates with the ability of these cells to form tumors in animals is the ability to form clones in a semisolid medium (23). Since UII cells, unlike human melanoma cells [5 to 50% cloning efficiency, (1)] clone poorly in soft agar, these cells appear to exhibit a

more normal phenotype than melanoma cells. At present, it is not known whether the UlI cells are more closely related to normal melanocytes or melanoma cells in vivo. Therefore, further study of the UlI line may yield useful information providing insights into melanocyte development.

U1I cells also differ from melanoma cells by exhibiting marked sensitivity to ultraviolet light. In contrast, melanoma cells appear to be relatively resistant to ultraviolet irradiation as shown by data in this paper and by others (24). Normal human fibroblasts, although slightly less resistant to UV irradiation than melanoma cells (24) are considerably more UV resistant than UlI cells, indicating a possible loss of repair ability during melanocyte development. If this difference in radiation sensitivity proves to be associated with other melanocyte derived and melanoma cell lines, then further investigation of the repair capacities of these cell types may provide insight into the changes that occur during transformation of a melanocyte into a melanoma cell.

We have described a unique cell line derived from normal uveal melanocytes, which has several properties useful for exploring human melanocyte biology. Further investigations of the effects of growth factors on tyrosinase levels and cellular proliferation of UlI cells and of the sensitivity of these cells to ultraviolet light may provide a better understanding of melanocyte development and regulation.

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