ESTABLISHMENT AND CHARACTERIZATION OF PRIMARY AND METASTATIC UVEAL MELANOMA CELL LINES

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We report on the establishment and characterization of 2 primary (EOM-3, EOM-29) and 3 metastatic uveal melanoma cell lines (OMM-1, OMM-2, OMM-3), and further cytogenetic characterization of a previously described primary uveal melanoma cell line (OCM-1). Only a few long-term growing primary uveal melanoma cell lines have as yet been established, while of metastatic uveal melanoma cell lines we have found no descriptions. The morphology of the *in vitro* cultured cells varied from spindle to epithelioid. The cell lines were characterized by immunocytochemistry, electron microscopy and cytogenetical analysis. The relative growth rate was determined by bromodeoxyuridine (BUdR) incorporation. The melanocytic origin of the cell lines was determined by positive staining with antibodies identifying melanoma-associated antigens. Melanosomes and pre-melanosomes were indeed observed by electron microscopy in all cell lines. The stem-cell karyotype was found to be normal in 3 cell lines (EOM-29, OMM-2, OMM-3) and abnormal in 3 others (EOM-3, OCM-1, OMM-1) showing a net loss of chromosome 6. The OCM-1 and the OMM-1 cell lines even demonstrated a large amount of structural chromosomal aberrations, the former being near-tetraploid and the latter triploid. The EOM-29 cell line, cultured from an ciliary body melanoma, did not show the previously described chromosome 3 and 8 abnormalities.

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Uveal melanoma is the most common primary intra-ocular tumor in adults (Egan *et al.*, 1988). These tumors spread hematogenously and preferentially to the liver. A 50% overall incidence of metastasis occurs within 15 years after initial treatment by enucleation or radiotherapy of the tumor-containing eye (Kath *et al.*, 1993). After clinical diagnosis of hepatic metastases, median survival is extremely poor: between 2 and 7 months (Kath *et al.*, 1993). The biological and molecular properties of the successive steps involved in uveal melanoma progression and metastasis could be studied in detail once established uveal melanoma cell lines were available. A limited number of cell lines obtained from primary uveal melanoma have indeed been described (Kan-Mitchell *et al.*, 1989; Aubert *et al.*, 1993; Massarelli *et al.*, 1994; De Waard-Siebinga *et al.*, 1995), but not from metastatic uveal melanoma.

In this study, we report on the successful establishment of 2 primary (EOM-3, EOM-29) and 3 metastatic uveal melanoma cell lines (OMM-1, OMM-2, OMM-3) in our institute. These cell lines were characterized by immunocytochemistry, light and electron microscopy and cytogenetic analyses. The relative growth rate was determined by bromodeoxyuridine (BUdR) incorporation. In addition, a previously described primary uveal melanoma cell line, OCM-1 (Kan-Mitchell *et al.*, 1989), was further characterized by extended karyotypic analysis.

MATERIAL AND METHODS

Tumor material

After enucleation of the uveal melanoma-containing eye, part of the ocular tumor was taken for cell culture. The eye was further histologically examined to confirm the diagnosis. When,

during follow-up of the patient, metastatic disease was suspected, a biopsy was taken for diagnosis and for cell culture. All patients had given full informed consent. Between January 1992 and May 1993, cells of the primary tumors of 16 patients and of the metastatic tumors of 4 patients were processed for culture. The patients with primary uveal melanoma ranged in age from 22 to 87 (mean: 56.8); 9 were female and 7 were male; 8 patients had a choroidal and 8 patients had a ciliary-body melanoma; 3 patients had a spindle-cell tumor, nine a mixed-cell tumor and 4 had an epithelioid-cell tumor; tumor diameter ranged from 3 to 22 mm (mean: 11.6 mm). The patients were followed until May 1995; 2 patients died of metastatic disease and one is alive, 6 months after detection of a metastatic lesion in the subcutis. From all 4 patients with metastatic disease, tumor material was obtained from sub-cutaneous lesions. From one patient the primary as well as the metastatic tumor were available.

Culturing methods

The tumor material was processed and cultured within 1 to 3 hr after dissection, as described in Luyten *et al.* (1993). In brief, the tumor-containing eye or the metastatic lesion was dissected under sterile conditions. A full-thickness biopsy was taken and transported in Dulbecco's modified Eagle's medium supplemented with 10% FCS and 1% penicillin/streptomycin (full DMEM). The tumor material was suspended with a small pair of scissors and strained through a linen cloth by continuous irrigation with full DMEM. The resulting suspension of single cells, and to a lesser extent of small clumps of tumor cells, was then washed 2 or 3 times with full DMEM. This washing was repeated, depending on the amount of pigment. The cells were seeded in a culture flask (Falcon plastic T-30) with 5 ml full DMEM at 5% CO₂ (37°C). After one week the medium was renewed, while the non-attached cells were discarded. Subsequently, the culture medium was changed twice a week. At reaching confluence, the cells were detached with 0.05% trypsin and 0.5 mM Na₂ EDTA and subsequently sub-cultured. Depending on the growth rate, the cultures were passaged at 1:2 to 1:10 dilutions.

BUdR incorporation

To measure the logarithmic growth rate of the cells in culture, the cells were incubated in culture flasks for 6 hr with 1 mM BUdR (Boehringer Mannheim, Almere, The Netherlands). After being washed and detached, the cells were centrifuged onto object glasses, fixed in 2 M HCL at 37°C for 30 min and then incubated in 0.1 M borate buffer, pH 8.5, at room temperature for 5 min. The cells were subsequently washed twice with PBS and incubated for 30 min with a

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peroxidase-conjugated immunoglobulin of a mouse MAb specific for BUdR (Boehringer Mannheim; dilution 1:33) in 1% BSA/PBS. After washing with PBS again, the incorporated BUdR was visualized with 3'3' diaminobenzidine and ureum hydroxide (Sigma, St. Louis, MO). The percentage of BUdR-incorporated cells could then be counted in one low power field (10×) using an eye-piece grid.

Immunocytochemistry

The cells were cultured on a coverslip, washed twice with PBS at 37°C and subsequently fixed in cold acetone at -20°C for 10 minutes. Endogenous peroxidase was inhibited by incubation in 0.3% hydrogen peroxide/methanol for 15 min. Subsequently the coverslip was incubated in 2% FCS/PBS for 5 min. The primary antibodies, S-100 (Dako, Glostrup, Denmark), NKI-C3 (Dako), HMB-45 (BioGenex, San Ramon, CA) and HNK-1 (Becton Dickinson, San Jose, CA) were incubated at dilutions of 1:600, 1:20, 1:50 and 1:10 respectively for 1 hr. For S-100, swine anti-rabbit peroxidase-conjugated immunoglobulin was used as a second-step antibody (Dako; dilution 1:100). For the other 3 antibodies, rabbit anti-mouse peroxidase-conjugated immunoglobulin (Dako; dilution of 1:100) was used. Both secondary antibodies were incubated for 1 hr. The activity of the peroxidase was then visualized using tablets of 3,3' diaminobenzidine HCL and hydrogen ureaperoxide (Sigma). Finally, the cells were counterstained with hematoxylin for 10 seconds. Between each of the incubation steps the coverslip was rinsed with 0.1% Tween-20/PBS.

Antibodies

The S-100 antibody recognizes an acidic intracellular Ca⁺⁺-binding protein and is a sensitive but non-specific marker for melanoma cells (Moore, 1965). MAb NKI-C3 (Vennegoor *et al.*, 1985) and HMB-45 (Gown *et al.*, 1986) binds both a cytoplasmic antigen produced by fetal melanocytes and melanoma cells of adults. The MAb HNK-1 (Abo and Balch, 1981) recognizes a family of cell-adhesion molecules (CD 57), migrating neural-crest cells and a series of neural-crest derivatives including tumors of neural-crest origin.

MHC-class-I expression

Sub-confluent cell cultures were detached with trypsin/EDTA, after which cytocentrifuge slides could be prepared. The major histocompatibility complex (MHC) class-I expression was determined using the standard NIH micro-cytoxicity test with locally obtained reagents.

Electron microscopy

After detachment by use of trypsin/EDTA, the cultured cells were centrifuged and fixed in 3% glutaraldehyde at room temperature for 30 min. Then they were post-osmified for 30 min in 1% OsO₄, and subsequently embedded in Epon. Ultra-thin sections were prepared and counterstained with uranyl acetate and lead citrate.

Cytogenetic analysis

In order to obtain metaphases, cultured cells in the logarithmic phase of proliferation were treated with colcemid for 1 to 3 hr. After trypsinization, the cells were subjected to a hypotonic (0.075 M KCL) solution. Finally the cells were gradually fixed with cold methanol/acetic acid (3:1 v/v). Air-dried slides were banded by the reverse method.

To identify the marker chromosomes, fluorescence *in situ* hybridization (FISH) was used. Slides were then hybridized with whole-chromosome plasmid-painting libraries (Pinkel *et al.*, 1988), kindly provided by Dr. J. Gray. The results presented conform to the recommendations of the International System for Human Cytogenetic Nomenclature (ISCN) (Mitelman, 1991).

RESULTS

Primary and metastatic uveal melanoma cell lines

Out of the 16 cultures of the primary uveal melanomas, 2 cell lines could be established successfully. The remaining cell cultures stopped growing after being passaged 2 to 5 times. Of the cultures of the metastatic uveal melanomas, 3 out of 4 resulted in a stably growing cell line. The cell cultures from one patient, from whom both the primary and corresponding metastatic tumor were available, were not successful. The patient data and tumor data from the established cell lines are summarized in Table I.

The primary uveal melanoma cell line OCM-1, described by Kan-Mitchell *et al.* (1989), has been growing for more than 7 years without morphological changes (Fig. 1a). The EOM-3 cell line was established from a mixed-cell primary uveal melanoma. During the first 6 passages, the cultured cells had a spindle-cell-type appearance. However, a small clone of epithelioid cells started to grow and subsequently overgrew the rest of the tumor cells. The epithelioid-cell culture underwent no further morphological changes, and has been growing now for 2 years (Fig. 1b). EOM-29 was established from a large extra-ocular extension of a ciliary-body melanoma (epithelioid-cell type) and could be passaged once a week. Gradually the passage time increased, with a decreasing proliferation rate. After passage 10, the cells had a spindle-like appearance in culture (Fig. 1c) and continued to grow without demonstrating changes in their morphology.

A sub-cutaneous metastatic lesion (mixed-cell type) was excised from a uveal-melanoma patient (OMM-1), who had undergone enucleation of his primary tumor 29 years earlier. The primary culture of this metastatic lesion and the first 10 passages consisted of adherent and non-adherent cells. Gradually more cells became adherent, showing a heterogeneous morphology of spindle and epithelioid cells. This morphological heterogeneous spectrum remained the same over 2.5 years (passage 42) (Fig. 1d). The primary culture (OMM-2) of a sub-cutaneous metastatic lesion (epithelioid-cell type) had a spindle-cell pattern in cell culture, and did not show morpho-

TABLE I - PATIENT AND TUMOR DATA OF SUCCESSFULLY ESTABLISHED UVEAL MELANOMA CELL LINES

Cell line	Patient gender/age	Survival (mo)	Tumor location	Histology	Cell-line morphology (LM)	Passage
OCM-1	F/n.a.	n.a.	Posterior	Spindle B	Mixed	> 50
EOM-3	M/62	29	Posterior	Mixed	Epithelioid	20
EOM-29	M/87	14	Ciliary body	Epithelioid	Spindle	15
OMM-1	M/74	343 ¹	Subcutis metastasis	Mixed	Mixed	42
OMM-2	M/72	40 ¹	Subcutis metastasis	Epithelioid	Spindle	23
OMM-3	M/72	41 ¹	Subcutis metastasis	Mixed	Spindle	19

¹Tumor-related death.-n.a. = not available.

FIGURE 1 – Light microscopy (scale bar, 50 μ m). (a) The OCM-1 cell line has small and large rounded cells, with one or more nuclei, growing upon a stellate and elongated cell layer with dendritic extensions; (b) the EOM-3 cell line shows a monolayer of plump polyhedral anaplastic, epithelioid cells with abundant cytoplasm, large nuclei and prominent nucleoli; (c) the EOM-29 cell line forms spindle-like patterns with large round nuclei; (d) the OMM-1 cell line: cluster of large round cells (mono- and multi-nucleated), irregularly shaped stellate and elongated cells—very large cells with abundant cytoplasm and cytoplasmic inclusions were observed; (e) the OMM-2 cell line showed a monolayer characterized by predominantly elongated cells with large nuclei, abundant cytoplasm and long dendritic extensions, forming a bundle-like, spindle-cell pattern; (f) the OMM-3 cell line has spindle-cell appearance with dendritic extensions; the nuclei are large with abundant cytoplasm and prominent nucleoli.

logical changes for 2 years. The cells have been growing slowly but continuously, with a passage time of one month (Fig. 1e). The most recently established metastatic cell line (OMM-3) was cultured from a large, partly amelanotic sub-cutaneous metastatic lesion (mixed-cell type) with a spindle-cell appearance in cell culture. This cell line has now been growing for

more than 1 year and no morphological changes have been observed (passage 19) (Fig. 1f).

Electron microscopy, immunocytochemistry

Electron microscopical morphology of the 6 cell lines is shown in Figure 2a–f. All figures are representative for the

FIGURE 2 – Legend appears on page 384.

cultured cells examined and contained melanosomes and pre-melanosomes (Table II). The immunocytochemical staining of the 5 cell lines with NKI-C3, HMB-45, S-100, HNK-1 and BUdR incorporation is summarized in Table II. The NKI-C3 stained all 5 cell lines; expression of the other 3 markers appeared to be facultative. The cell lines EOM-3 and

OMM-1 showed relatively high BUdR incorporation, thus showing high frequency of cell division. The more slowly growing cell lines EOM-29, OMM-2 and OMM-3 had indeed a lower BUdR incorporation. The primary cell lines OCM-1 and EOM-29 were found to express MHC-class-I molecules, whereas in the EOM-3 cell line no detectable MHC-class-I

FIGURE 2 – Electron microscopy. (a) OCM-1: part of an uveal-melanoma cell. The cytoplasm contains a few melanosomes (M) and pre-melanosomes type I (PM) and sporadic type II (scale bar, 0.350 μ m); (b) EOM-3: very primitive, immature cell with a large nucleus and 2 prominent nucleoli. Only a few pre-melanosomes type I (PM) are present (scale bar, 1 μ m); (c) EOM-29: heavily pigmented cells with abundant cytoplasm in which there are many melanosomes (M) and pre-melanosomes type III (PM) and lysosomal structures (L) (scale bar, 1 μ m); (d) OMM-1: 2 melanoma cells contain in the cytoplasm a large amount of osmiophilic (dense) melanosomes (M) and pre-melanosomes type III and IV, and only a few type II (PM). Note the presence of lysosomes (L) (scale bar, 0.583 μ m); (e) OMM-2: melanoma cell with an aberrant cytoplasm in which dense fusiform melanosomes (M) and pre-melanosomes (types II, III and IV) (PM) are observed (scale bar, 1 μ m); (f) OMM-3: spindle melanocytic cells. The cytoplasm contains large amounts of rough endoplasmic reticulum and pre-melanosomes types II and III (PM); a few lysosomes (L) are also present (scale bar, 1 μ m).

expression was observed. All 3 metastatic cell lines were found to express MHC-class-I antigens.

Cytogenetic analysis

The karyotypes of the 6 cell lines are described in Table III. The karyotypes were obtained from 10 to 25 metaphases from each cell line at different passages. Cells from cell line EOM-29 and OMM-3 displayed a normal karyotype. The stemline karyotype of OCM-1 was found to be tetraploid and to have many structural chromosomal aberrations (Fig. 3a). These, among other changes, have resulted in a net loss of 3q and of the distal part of chromosome 8 (8q24-qter); monosomy 6 was observed in 60% of the metaphases of OCM-1. The stemline karyotype of EOM-3 was found to be pseudodiploid with numerical changes: a loss of Y, monosomy 6, trisomy 5 and 18 (Fig. 3b). The stemline karyotype of cell line OMM-1 was near-triploid with many structural and numerical changes, a net gain of chromosome 3, 7, 12 and 20, and loss of 4, 8p, 9, 11, 15, 17, 21, among others (Fig. 3c). Cell line OMM-2 showed no clonal aberrations, but some non-clonal rearrangement, such as a monosomy 6 in 2 cells and an unbalanced translocation (14;22) in one cell.

DISCUSSION

Culturing of human uveal melanoma cells was first attempted in 1929 by Kirby (1929), but until the present date only a few cell lines from primary melanomas (Albert *et al.*,

1984; Kan-Mitchell *et al.*, 1989; Aubert *et al.*, 1993; Massarelli *et al.*, 1994; De Waard-Siebinga *et al.*, 1995), and no cell lines from metastatic uveal melanomas have been described. The relative paucity of established uveal melanoma cell lines can be explained partly by the low incidence (1:100,000) of uveal melanoma (Egan *et al.*, 1988) and by difficulties in the culturing of primary uveal melanoma tissue. Lack of therapeutic modalities for patients with hepatic metastases has led to a high mortality among them within a very short survival time, and hence provides a problem with respect to the availability of fresh metastatic tissue as well. In this study, we were able to establish 2 primary uveal melanoma cell lines and 3 metastatic uveal melanoma cell lines. Although a limited overall number of tumors could be cultured at our institute, the success rate in the establishment of long-term growing cell lines was relatively high for metastatic uveal melanomas (3/4) as compared with primary uveal melanoma (2/16). Metastatic lesions originate from selected cells that have proved to be capable of growing in secondary organs. This could be the reason for the high success rate in culturing metastatic cell lines. The present availability of primary and metastatic uveal melanoma cell lines made it possible to study the differences between primary and metastatic uveal melanoma *in vitro*.

To ascertain the melanocytic origin of the cultured cells, the cells were immunocytochemically studied by the use of melanoma-associated antibodies. S-100, NKI/C3 and HMB-45 are

TABLE II - CHARACTERISTICS OF THE ESTABLISHED CELL LINES

Cell line	Electron microscopy, melanosome type	Immunocytochemistry ¹					MHC-class-I expression on cell line
		NK1/c3	HMB-45	S-100	HNK-1	BUdR	
OCM-1	I (II)	100 ²	100 ²	100 ²	0	nd	A24, A28
EOM-3	I	100(p18)	100(p18)	0(p18)	100(p18)	24(p20)	— ³
EOM-29	III, IV	100(p5)	0(p5)	0(p5)	0(p5)	<1(p15)	A3, A30
OMM-1	III, IV (II)	100(p26)	100(p26)	100(p26)	0.5 ⁴ (p27)	49(p42)	A2, A11, B62
OMM-2	II, III, IV	100(p17)	0(p17)	0(p17)	8(p17)	<1(p23)	A1
OMM-3	II, III	100(p5)	0(p5)	0(p5)	4(p5)	2(p13)	A1, A11

¹Percentage cells binding the indicated antibody (passage number).—²Kan-Mitchell *et al.*, 1989.—³No detectable MHC-class-I expression.—⁴Initially 50% of the cells expressed HNK-1 antigens. nd, not done.

TABLE III - CYTOGENETIC DATA OF UVEAL MELANOMA CELL LINES

Cell line	Passage	Number of metaphases analyzed	Modal chromosomes	Karyotype
OCM-1	> 50	14	90-97	94,(4),XX,-X,-X,del(1)(p21)[40%],der(2)t(1;2)(p31;q37),add(3)(q12),+del(3)(q13q23),del(4)(q12)[60%],-4[40%],add(5)(p11)[40%],add(5)(q13),-6[60%],add(7)(q35),del(7)(q33),-7,add(8)(q24)×2,-9,-11,inv(11)(p15q24),del(12)(p12)×3,-12,add(13)(q31),-13 or t(13q;14q)[60%],-15,add(16)(p13),-16[40%],-18[60%],-19[60%],add(20)(q13)×2 or ×3,-20,add(21)(q21),-21,-21,del(22)(q11)×2,iso(22q)×2,+6-12 markers
EOM-3	10	22	46	46,X,-Y,+5,-6,+18
EOM-29	5	10	46	46,XY
OMM-1	8-10	25	60-68	64,(3),X,-X,-Y,der(1)t(1;3)(p31;p13),der(2)t(2;5)(q32;q14?)×2,t(2;10)(q32;q25),+3[50%],-4,del(5)(q13q24),+7,add(8)(p11),-9,-11,+12,inv(13)(q12q34),-15,add(16)(p12),-17,del(19)(p12),+20,add(21)(p13),-21,+1-3 markers
OMM-2	11-14	19	46	46,XY[11]/44-45, with random loss of 1 or 2 chromosomes, [8 of which 2× -6]
OMM-3	1-4	21	46	46,XY

known to be powerful markers for differentiating tumors of melanocytic lineage from other anaplastic tumors. In addition, electron-microscope studies revealed melanosomes and pre-melanosomes in all the cell lines described. The cell lines showed a considerable variation in passage time as measured by BUdR incorporation. The cell lines EOM-3 and OMM-1 had relatively high BUdR incorporation and were shown to have high growth rates with short passage times in culture. The cell lines EOM-29, OMM-2 and OMM-3 had low levels of BUdR incorporation and were also more difficult to maintain in culture. However, the cell lines OMM-2 and OMM-3 were stable over a long period of culture without showing any morphological changes. The cell line EOM-29 has been in culture for a relatively short period, showing no morphological changes, but immortality is not yet established. No difference in growth rate between primary and metastatic cell lines could be demonstrated by BUdR incorporation.

Although epithelioid cells have often been regarded as the most malignant cell type, 3 out of 6 cell lines in our study were of the spindle-cell type (EOM-29, OMM-2, OMM-3). This in contrast to the histological cell type of the original tumor of the patient. This can probably be explained by the selective outgrowth of certain tumor cells in a favorable culture medium such that used by us. In case of the cell line EOM-3, the spindle cells of the fresh tumor culture were gradually overgrown by undifferentiated, anaplastic cells at passage 5. After that time the uniform morphological cell type of EOM-3 remained unaltered. Whether the phenotypical change of the cell line EOM-3 is accompanied by genetical instabilities, reflecting one or more steps in tumor progression, or by simple selection is not known. Both cell lines OCM-1 and OMM-1 consist of a heterogenous population of cells with a high growth rate, showing signs of selection

of one of the morphological cell types present in the original tumor.

The normal expression of MHC-class-I molecules in 5 of the 6 cell lines indicates a possibility of a MHC-class-I-restricted T-cell response in metastatic uveal melanoma patients. In the EOM-3 cell line, MHC-class-I expression is down-regulated and, as a result of this, increased susceptibility to natural-killer-cell activity has been demonstrated (Ma *et al.*, 1995). These data indicate that enhancement of the T-cell response by the use of allogenic irradiated cell lines might be one of the most promising ways to treat patients with metastatic disease.

In cytogenetical analyses of primary uveal melanoma, the most frequently described chromosomal abnormalities found are: monosomy of chromosome 3, net loss of genetic material of chromosome 6q and 8p, and net gain of 6p and 8q (Horsman and White, 1993; Singh *et al.*, 1994). It has been suggested that monosomy 3 and the isochromosome 8q are associated with ciliary-body melanoma and poor prognosis (Sisley *et al.*, 1990). Cytogenetic analysis of our cell lines showed a normal karyotype in 3 cell lines and karyotypic abnormalities in the other 3, with partial loss of chromosome 6 among multiple other aberrations. OCM-1 and OMM-1 were near-tetraploid and triploid respectively. In these cell lines, partial loss of 3q and 8p were seen, as a consequence of structural changes. In contrast to other results on cytogenetical abnormalities in ciliary-body melanoma, our cell line EOM-29, which was derived from a massive extrascleral growth of a ciliary-body melanoma, did not demonstrate chromosome-3 and -8 abnormalities. We have found however no systematic differences in the karyotypes of the cultured cells of primary vs. metastatic tumors. The karyotyping was assessed after establishment of the cell lines and thus some chromosomal abnormalities could

FIGURE 3 – Karyotypes of the cell lines. (a) OCM-1; (b) EOM-3; (c) OMM-1.

be the consequence of *in vitro* clonal selection and progression, instead of a representation of the situation of the tumor *in vivo*. In future studies, cytogenetic analysis of primary uveal-melanoma cell cultures will hopefully provide more information on the karyotype of the tumor cells. Furthermore, complementary studies using molecular cytogenetic approaches and comparative genomic hybridization (Speicher *et al.*, 1994) could give more detailed information on chromosomal abnormalities in fresh tumor and cell lines in culture.

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