Pigmented uveal tumours in a transgenic mouse model

Theresa R Kramer, Marianne B Powell, Matthew M Wilson, Jennifer Salvatore, Hans E Grossniklaus

Abstract

Aims/background—The authors have developed transgenic mouse strains at the Arizona Cancer Center using a tyrosinase promoter to target expression of the mutated T24 Ha-ras gene in melanin producing cells. Histopathology and electron microscopy (EM) were performed to characterise the intraocular tumours observed phenotypically.

Methods—Transgenic TPras mice (n=8) and normal, age matched control mice (n=6) were sacrificed at 3 weeks, 6 weeks, 7 weeks, 4 months, 5 months, 9 months, and 11 months. Six were processed in formalin for light microscopic examination and eight in a glutaraldehyde/formalin solution for electron microscopic examination.

Results—Six of the TPras mice were found to have bilateral pigmented melanocytic/RPE proliferations of the uveal tract. The cytological characteristics of the tumours included low nuclear to cytoplasmic ratios (N:C ratios), bland nuclei, and abundant intracytoplasmic melanin. By EM two populations of cells were identified, including spindle-shaped cells with round to oval melanin granules and cuboidal cells with apically located, cigar-shaped, melanin granules, and basement membrane formation. A 3 week and an 11 month old TPras mouse had a higher grade, bilateral, melanocytic proliferation of the uveal tract which, although not metastatic, was morphologically melanoma. Cytological features included increased N:C ratios, nuclear pleomorphism, and prominent nucleoli. The uveal tract was normal in both eyes in all of the control animals.

Conclusion—Pigmented intraocular tumours developed in transgenic strains of mice that express a mutated Ha-ras gene in melanin producing cells. The morphology was most consistent with a melanoma in two of the mice and a benign melanocytic/RPE proliferation in the remaining mice.

Table 1 Summary of the pathological findings in the TPras mice and the control mice

<table>
<thead>
<tr>
<th>Number</th>
<th>Age</th>
<th>Type</th>
<th>Ocular pathology</th>
<th>Neuropathology</th>
<th>EM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3 weeks</td>
<td>ras</td>
<td>iris/ciliary body moderately pigmented melanoma</td>
<td>normal</td>
<td>no</td>
</tr>
<tr>
<td>2</td>
<td>6 weeks</td>
<td>ras</td>
<td>melanocytic/RPE hamartoma</td>
<td>benign melanocytic proliferation</td>
<td>yes</td>
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<tr>
<td>3</td>
<td>7 weeks</td>
<td>ras</td>
<td>melanocytic/RPE hamartoma</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>4</td>
<td>5 months</td>
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<td>melanocytic/RPE hamartoma</td>
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<td>yes</td>
</tr>
<tr>
<td>5</td>
<td>4 months</td>
<td>ras</td>
<td>melanocytic/RPE hamartoma</td>
<td>no</td>
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<tr>
<td>6</td>
<td>9 months</td>
<td>ras</td>
<td>melanocytic/RPE hamartoma</td>
<td>benign melanocytic proliferation</td>
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<tr>
<td>7</td>
<td>11 months</td>
<td>ras</td>
<td>melanocytic/RPE hamartoma</td>
<td>normal</td>
<td>no</td>
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<tr>
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<td>11 months</td>
<td>ras</td>
<td>iris/ciliary body moderately pigmented melanoma</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>9</td>
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<td>normal</td>
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<tr>
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<td>RPE proliferation</td>
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<tr>
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<td>3 months</td>
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<tr>
<td>14</td>
<td>11 months</td>
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Choroidal melanoma is a primary intraocular cancer and is the most common ocular cancer among adults. Ocular cancers account for approximately 1% of all new cancers cases in the United States each year. Other less common intraocular cancers in adults include melanoma of the iris and ciliary body. Although rare, this condition is of special concern within the field of ophthalmology because of its life threatening potential.

Primary ocular melanoma can arise from the choroid, ciliary body, iris, conjunctiva, or less commonly from the orbital structures, lacrimal system, cornea, and retina. It is the most common primary malignancy of the eye in adults. There are several animal models of intraocular melanoma which have proved useful in studying tumour development and therapeutic modalities. In addition, several research groups have been successful in developing experimental mouse or transgenic mouse lines for the study of intraocular pigmented tumours and melanoma. The latter authors used a tyrosinase promoter to regulate expression of the SV40 early region transforming sequences in melanocytes. The mice they generate develop pigmented ocular tumours at a young age. We have developed transgenic mouse strains at the Arizona Cancer Center using a tyrosinase promoter to target...
expression of the mutated T24 Ha-ras gene in melanin producing cells. Two independent founder mice were found to have a distinct phenotype with muted agouti coat colour, deeply pigmented skin with multiple naevi and “twirling behaviour”. Histopathology of the tissue revealed hyperpigmentation and/or melanocytic hyperplasia in the skin, eyes, inner ear, and meningeal membranes in the brain. Histopathology and electron microscopy (EM) were performed to characterise the intraocular tumours observed phenotypically. In this paper, we report the histopathological characterisation of the ocular tumours.

Methods

TPRAS TRANSGENIC MICE

An expression vector using mouse tyrosinase promoter sequences to drive expression of the mutated human Ha-ras (T24) gene was used to generate the TPras transgenic mice. The pTPras construct contains a 2.5 kilobase (kb) tyrosinase promoter fragment isolated from the plasmid GL3. The promoter fragment was ligated into pR8-T24, a promoterless construct which contains the genomic sequences of the mutant T24 c-Ha-ras gene in the pIC-20R vector. A 6.5 kb fragment isolated from the pTPras plasmid was used for microinjection to generate the transgenic mice. The exact procedure used to generate the transgenic mice has been completely described by Powell and colleagues. The mice used for this study are on C57BL/6XSJL X C3He/N background. Expression of the T24 Ha-ras mRNA is detected in tissues containing melanocytes such as skin, eyes, and meningeal membrane. The mice exhibit a distinct phenotype consisting of a muted agouti coat colour, pigmented skin, and twirling behaviour. Mice were randomly chosen to represent a range of ages and sacrificed for pathological evaluation. All animals were treated in accordance with the ARVO resolution on animals used in research.

LIGHT AND ELECTRON MICROSCOPIC STUDIES

Whole mice heads were fixed in 10% buffered formalin. Specimens were then dehydrated in a series of alcohols and embedded in paraffin. Serial sections 4–6 µm thick were subsequently stained with haematoxylin and eosin for routine light microscopic evaluation. Selected samples were also fixed in 2.5% glutaraldehyde/0.1 molar cacodylate buffer solution, postfixed in osmium tetroxide, and subsequently embedded in LX 112 resin (Ladd Res Ind Inc, USA) for transmission electron microscopy. For transmission electron microscopy, semithin sections (1–2 µm) were stained with toluidine blue and sodium borate (1%) and examined by light microscopy. Ultrathin sections (60–70 nm) were stained with aqueous uranyl acetate followed by lead citrate and examined using a Jeol 100CX electron microscope.

Results

Bilateral intraocular tumours arose in the TPras mice. The tumours were identified histopathologically and ultrastructurally as melanoma and mixed melanocytic/retinal pigment epithelial (RPE) hamartomas. In addition, a benign melanocytic proliferation of the choroid plexus was identified in two of the TPras mice. In this report, we examine the ocular tissues from TPras transgenic mice at several different ages and compare them with age matched negative littermates. Table 1 summarises the histopathological and ultrastructural findings in the TPras mice (n=8) and the control mice (n=6). The ocular tissue examined from one of the normal control littermates contained an RPE proliferation.

Six of the TPras mice were found to have pigmented hamartomatous proliferations of the uveal tract. These were composed primarily of proliferations of heavily pigmented, spindle-shaped melanocytes with low nuclear to cytoplasmic ratios, bland nuclei, and abundant intracytoplasmic melanin pigment (Fig 1). Occasionally, more atypical cytological characteristics were observed in these pigmented hamartomatous proliferations. These
tumours massively filled the entire uveal tract. Four of the six tumours were examined by electron microscopy in addition to histopathological examination. The majority of the cells identified had ultrastructural characteristics which included spindle-shaped cells with oval to round nuclei and numerous intracytoplasmic oval melanosomes and premelanosomes (Fig 2). This cell type did not display intercellular junctions or basement membrane formation. Less frequently, cells which had cuboidal shape with rounded nuclei, apical to basal polarity, and intracytoplasmic cigar-shaped melanin granules were identified. These cells displayed intercellular junctions and basement membrane formation.

The remaining two TPras mice, a 3 week and an 11 month mouse had a higher grade melanocytic proliferation which was morphologically melanoma. The tumours were present in the iris, ciliary body, and the anterior chamber and were characterised histopathologically as a proliferation of moderately pigmented uveal melanocytes (Fig 3). Cytological features included increased nuclear to cytoplasmic ratios, nuclear pleomorphism, and prominent nucleoli. Ultrastructurally, the cells were spindle shaped with oval to round nuclei, increased nuclear to cytoplasmic ratios, marginned chromatin, irregular nuclear envelope, and prominent nucleoli. Intracytoplasmic melanin granules were demonstrated. The round to oval melanosomes measured 0.53 µm by 0.87 µm (range 0.41–0.75 µm and 0.75–1.08 µm) (Fig 3). The uveal melanocytic tumour in the 11 month TPras mouse also contained melanophages and areas of retinal pigment epithelial proliferation. None of the mice were shown to have metastatic tumours.

Two of the six TPras mice were also noted to have benign melanocytic proliferations in the brain. These were characterised histopathologically as small proliferations of moderately to heavily pigmented melanocytes in the choroid plexus. These melanocytes were entirely bland and morphologically characterised as plump polyhedral cells similar to naevus cells. Ultrastructural examination was not done because of the benign morphology on histopathological examination.

The uveal tract was normal in all six of the control animals (negative littermates).
Microscopically, the uveal tract was composed of arteries, veins, and melanocytes amid loose stromal connective tissue (Fig 4). Ultrastructural examination was completed on the retinal/choroidal junction. Choroidal melanocytes were spindle-shaped cells with oval nuclei containing a bland chromatin pattern. Intracytoplasmic round and oval melanin granules are identified. The melanosomes measure 0.50 µm by 0.75 µm. The RPE is composed of cuboidal cells with oval nuclei containing marginated heterochromatin. There is apical to basal polarity and basement membrane formation. Intracytoplasmic rough endoplasmic phagosomes and membranous vesicles are identified. Intracytoplasmic cigar-shaped and oval melanin granules are identified and measure 0.44 µm by 1.58 µm (original magnification ×9180).

Intracytoplasmic rough endoplasmic phagosomes and membranous vesicles were identified. An RPE proliferation, confirmed by electron microscopy, was discovered in one control mouse (5 weeks). This was characterised, histopathologically, as a melanotic proliferation of RPE cells between the retina and choroid. The cells were noted to form tubuloacinar configurations. Ultrastructural characteristics included apical to basal polarity, abundant basement membrane proliferation, and intercellular junctional complexes (Fig 5).

Discussion
In order to better understand the pathobiology of uveal malignant melanoma, numerous investigators have developed models of ocular melanoma. These models have been reviewed extensively and the potential limitations and advantages of each model have been discussed by Albert et al. These have included hetero-transplantation models, models induced by a DNA oncogenic virus (papova, C-type, retrovirus), and chemically induced models. In addition, models with transplantation of melanoma cells into the anterior chamber and the posterior compartment of the eye have been established. In the model of Grossniklaus, mice were inoculated in the anterior chamber or posterior compartment with B16F10 melanoma cells. Melanoma was demonstrated in both compartments and these melanomas metastasised to the lungs. Newer models of ocular melanoma have included transgenic mouse models. These models are based on using the promoter region of the tyrosinase gene to target expression of oncogenes to pigment producing cells of different origins. Most of the models have been successful in producing intraocular pigmented tumours including RPE tumours, carcinomas, and ocular melanosis. These models are summarised in Table 2.

Powell et al, in an attempt to develop an animal with cutaneous melanoma, reported hyperpigmentation and melanocytic hyperplasia in transgenic mice expressing the human T24 Ha-ras gene regulated by a mouse tyrosinase promoter. The mutated Ha-ras was used to generate the transgenic mice based on observations of the occurrence of ras mutations in human cutaneous melanoma. Phenotypically, these transgenic mice displayed a muted agouti coat, highly pigmented skin, and a twirling movement. Although no cutaneous melanoma developed, primarily benign melanocytic lesions were observed in the skin and tissues of the inner ear and uveal tract. In our study, we determined that the cellular composition of the spontaneously arising pigmented ocular tumours that developed in the TPras transgenic mice expressing a mutated Ha-ras gene represented a proliferation of melanocytes intermixed with RPE cells. The cytological characteristics of the tumour cells included a spindle/dendritic shape with abundant intracytoplasmic melanosomes and premelanosomes. These cells had round to oval melanin granules. A second population of cells were cuboidal with cigar-shaped...
melanin granules characteristic of RPE cells. Some of the cells had intercellular junctions and basement membrane formation characteristic of pigment epithelial cells such as iris pigment epithelium, RPE, and ciliary pigment epithelium. These cells also displayed apical to basal polarity and abundant basement membrane formation typical of epithelial cells. Thus, we have concluded that the majority of the cellular proliferations in the uveal tract of the transgenic mice represented a combined melanocytic/RPE proliferation.

The identification of cell type in the uveal tumours of the TPras mice was based on morphological and ultrastructural characteristics of the intraocular pigmented cells in the human eye. In this respect, several types of pigment containing cells are generally recognised within the eye, including iris pigment epithelium, pigmented ciliary epithelium, retinal pigment epithelium, and uveal melanocytes. Pigmented epithelial cells and melanocytes of the eye have different morphological characteristics, are known to arise embryologically from different tissue origins, and behave in biologically distinct ways.

Uveal melanocytes are generally recognised as dendritic cells with a spindle shape and an elongated, oval nucleus containing peripherally margined chromatin. Numerous oval melanosomes are present in the cytoplasm and many of the melanosomes contain a striated substructure. Intracytoplasmic melanin granules in uveal melanocytes measure 0.6–0.8 µm in diameter. Melanocytes residing in skin, iris, and choroid are neural crest derivatives. Uveal melanocytes do not begin to produce pigment until late in embryological development and continue to produce pigment after birth. Intraocular pigmented epithelial cells are morphologically epithelial rather than dendritic. The RPE cells contain larger ellipsoidal to needle-shaped pigment granules. Intracytoplasmic melanin granules in the RPE are cigar shaped and measure 1 µm in diameter and 2–3 µm in length. RPE cells arise from the outer layer of the optic cup. Melanin granules are found in the RPE at about the 10th week of gestation. Between the eighth and 14th week of gestation, granules of all stages can be found including premelanosomes and melanosomes. The RPE density also increases from birth to age 2 years. In the mouse, the first pigment in the retinal region appears around day 11–11.5 of gestation.

Uveal malignant melanoma is a cellular proliferation of cytologically malignant melanocytes. The cytological characteristics of uveal malignant melanoma were originally described by Callendar, who recognised two main cell types, spindle and epithelioid. Spindle-type cells of uveal malignant melanoma are fusiform and usually arranged in tightly cohesive bundles. These spindle-type cells have been divided into subtypes A and B. Spindle A cells have a small, slender nucleus with a fine, delicate, chromatin structure, and ill defined or absent nucleoli. Often a nuclear fold passing along the length of the nucleus giving the appearance of a nuclear streak. Mitotic figures are rarely seen. Spindle B cells have a larger, plumper nucleus containing a coarse chromatin network and a small round, sharply defined, eosinophilic nucleolus. Mitotic figures are often observed. Callendar’s
epithelioid cell is a larger, polygonal cell with considerable variation in both size and shape. These cells have abundant glassy cytoplasm. The nuclei of these cells are typically larger and rounder and the nuclear envelope is more angular with irregular indentations and outpouchings. The chromatin is very coarse and margined. A very large eosinophilic nucleolus is present in the centre of the nucleus. Mitotic figures are abundant. Gamel et al. further defined the nuclear characteristics of the various malignant melanoma cells. The modified Callender classification now recognizes spindle cell naevus, spindle cell melanoma, mixed cell melanoma, and epithelioid cell melanoma. In our study, we found that the majority of the uveal tumours were composed of a benign mixed melanocytic/RPE cell population in all but two of the TPras mice. However, focal areas of cytologically atypical cells were identified in many tumours. The remaining two TPras mice (the 3 week and 11 month old) had a higher grade melanocytic proliferation which was morphologically identical to melanoma. The uveal tumour was characterized histopathologically as a proliferation of moderately pigmented uveal melanocytes with malignant cytological features including increased nuclear to cytoplasmic ratios, nuclear pleomorphism, coarse chromatin network with margination, and prominent nucleoli. Pigment production in these cells was variable. Although the pigmented uveal tumour in two of the TPras transgenic mice most closely resembled human uveal malignant melanoma, true malignancy cannot be confirmed without known evidence of metastasis. We did not demonstrate metastases of the ocular tumours; however, we have found metastases to the lung and lymph node, from DMBA induced cutaneous melanoma in these same transgenic mice (Powell, personal communication). Additionally, injection of cell lines from these cutaneous tumours into SCID mice results in melanocytic tumours that invade adjacent muscle. In ongoing studies, we will inject these melanocytes into the anterior chamber and posterior compartment of semisyngeneic mice and assess for metastases according to the model of Grossniklaus et al. In our studies, we found that the majority of the uveal tumours were composed of a benign mixed melanocytic/RPE cell population in all but two of the TPras mice. However, focal areas of cytologically atypical cells were identified in many tumours. The remaining two TPras mice (the 3 week and 11 month old) had a higher grade melanocytic proliferation which was morphologically identical to melanoma. 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In future studies, we intend to use this model to study the process of malignant transformation of uveal melanocytes to melanoma. We have already established cell culture lines of both the benign melanocytes and/or RPE cells from the melanocytic/RPE hamartomas and we intend to establish cell cultures of the melanoma cells from the malignant melanomas. As the biology of ocular melanoma and molecular mechanisms involved in malignant transformation are poorly understood, we hope to use this model along with microarray technology to study the molecular alterations occurring during the process of malignant transformation. Should metastases develop after injection of tumour cells into the anterior and posterior compartment, we hope to analyse the molecular alterations in metastatic tumours and compare them with primary tumours. We believe that this unique transgenic model of spontaneously arising pigmented intraocular tumours may be valuable in elucidating the pathobiological mechanisms involved in the process of malignant transformation.

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