Primitive Neuroectodermal Tumor of the Midbrain in a Murine Model of Retinoblastoma

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The first heritable model of retinoblastoma was established by retina-specific expression of simian virus 40 T-antigen (SV40 T-ag) in transgenic mice. Bilateral, multifocal ocular tumors were observed in 100% of transgene-bearing mice. Central nervous system neoplasms occurred at a lower rate (27%) and represented the murine counterpart of human trilateral retinoblastoma. The authors characterized the transgenic brain tumors and found them to be primitive neuroectodermal tumors (PNET) of the midbrain. Murine brain tumors do not involve the pineal gland and most closely resemble undifferentiated suprasellar or parasellar tumors occasionally observed in human trilateral retinoblastoma. The murine malignancies arose from the subependymal cells of the cerebral aqueduct. Immunohistochemical and ultrastructural examination revealed that the transgenic brain tumors were undifferentiated and lacked all antigens associated with normal murine neuronal, glial, and ependymal cells. Invest Ophthalmol Vis Sci 32:293–301, 1991

Retinoblastoma, the most common ocular malignancy of childhood, occurs in both hereditary and nonhereditary forms. The gene responsible for the development of retinoblastoma has been isolated.1 The retinoblastoma gene is viewed as the prototypical tumor suppressor gene or recessive oncogene after the demonstration that the presence of one or more normal retinoblastoma alleles is sufficient to prevent the development of this ocular malignancy.2 This gene has also been designated as an antioncogene due to the binding of the retinoblastoma protein product (p105-RB) by various viral oncoproteins.3

Patients with hereditary retinoblastoma inherit one abnormal locus from a carrier parent or as a new germinal mutation. These patients with heritable retinoblastoma possess one normal allele and one mutant allele in every somatic cell. Mutation or loss of the remaining intact allele in the developing retinal cells are likely events and result in the development of bilateral, multifocal eye tumors. Nonhereditary patients are born with two normal retinoblastoma alleles. Somatic mutations of both loci in the same developing retinal cell occurs infrequently. Sporadic retinoblastoma patients, therefore, usually develop unilateral and unifocal tumors.3

Before the cloning of the retinoblastoma gene, patients with familial retinoblastoma were known to be at increased risk for second, nonocular primary cancers such as osteosarcomas, soft tissue sarcomas, and brain tumors.4,5 A defective retinoblastoma locus appears to play a role in the development of these second primary malignancies and in various tumors among patients with no evidence of or predisposition to retinoblastoma. Abnormalities of the retinoblastoma gene have been observed in some osteosarcomas,1 soft tissue tumors,6 small cell lung cancer,7 breast carcinoma,8 and bladder carcinoma.9

The rare development of primary, midline intracranial malignancies in patients with heritable retinoblastoma is known as “trilateral retinoblastoma.”10,11 These ectopic, central nervous system retinoblastomas are solitary and occur in the pineal, suprasellar, or parasellar regions.12 Brain tumors are often histologically indistinguishable from ocular retinoblastoma and sometimes display differentiation in the form of Flexner-Wintersteiner rosettes.10,13,14 The appearance of trilateral retinoblastoma in the pineal gland has been emphasized due to the close phylogenetic relationship between the pineal gland and the retina.10,11

We previously described the development of heritable retinoblastoma in transgenic mice which express
a viral oncogene (simian virus 40 T-antigen or SV40 T-ag) in the retina. To our knowledge these mice are the only animal model for heritable retinoblastoma and demonstrate retinoblastoma tumorigenesis through viral oncoprotein (SV40 T-ag) inactivation of the retinoblastoma protein product. We noted, in a preliminary report, the development of undifferentiated, midline brain tumors in occasional animals, thus representing trilateral retinoblastoma. In the current study, we studied many transgenic mice with retinoblastoma of various ages to elucidate the origin, immunohistochemical properties, and incidence of central nervous system tumors in these animals more completely.

Materials and Methods

Transgene construction, animal matings, and selection of transgene-bearing mice by tail blot analysis have previously been described for this transgenic mouse model. The investigations using animals adhered to the ARVO Resolution on the Use of Animals in Research. A total of 131 mice were studied. Ninety-one postnatal mice had transgene integration and served as the experimental group. Twenty-five postnatal mice did not have transgene integration and served as the control group. Tail blot analyses were not done on the 15 fetuses.

Multiple hematoxylin and eosin-stained, 6-μm thick sections of paraplast-embedded (Sherwood Medical Industries, St. Louis, MO) tissue were evaluated from the brains of mice of various ages. The number of brains examined in each age group was as follows: 10-day-old fetus, 5; 17-day-old fetus, 10; 4-day-old neonate, 6; 1–2-month-old, 13; 2–3-month-old, 14; 3–4-month-old, 5; 4–5-month-old, 30; 5–6-month-old, 18; 6–7-month-old, 13; 7–8-month-old, 8; 8–9-month-old, 8; and 9-month-old animals, 1.

Histopathologic processing of the brains consisted of fixation in 10% neutral buffered formalin of the cranial vault after ocular enucleation and removal of mandibles, followed by transection caudal to the orbits at the level of the olfactory bulbs. The cranium with the brain undisturbed was fixed for 48 hr and then decalcified in 5% nitric acid for 24 hr. The crania were sectioned in transverse or sagittal planes to include the pineal gland, pituitary gland, and cerebral aqueduct in the portion that would undergo step sectioning.

Immunohistochemical stainings to demonstrate antigens associated with neurons (neuron-specific enolase, synaptophysin, and neurofilament triplet proteins), glia (vimentin, glial fibrillary acidic protein [GFAP], S-100 protein, and myelin basic protein), and ependyma (vimentin, S-100 protein, and cytokeratin) were done on four of the midbrain tumors. The avidin-biotinylated peroxidase complex method was used; it has been described. Primary antineuron-specific enolase (dilution 1:500), anti-S-100 protein (1:1000), and anti-myelin basic protein (1:200) rabbit antisera were obtained from (Dakopatts, Copenhagen, Denmark). Murine monoclonal antibodies to synaptophysin (Clone SY38; Boehringer Mannheim, Germany), HNK-1 epitope (anti-Leu-7; Becton Dickinson, Mountain View, CA), vimentin (Clone V9, Sigma, St. Louis, MO), cytokeratin (Clone Lu5; Boehringer Mannheim) and 160-kD neurofilament triplet protein (Amersham, Arlington Heights, IL) were commercially obtained. Mouse monoclonal anti-GFAP, anti-200-kD and anti-160-kD neurofilament triplet protein antibodies, and polyclonal rabbit antisera against vimentin, GFAP, and 68-kD neurofilament triplet protein have been previously described.

Results

Bilateral, multifocal ocular retinoblastoma was evident in 100% of transgene-bearing mice. A detailed characterization of ocular tumors is described elsewhere. The data presented here will be limited to primary midbrain tumors occurring in these mice.

Primary midbrain tumors occurred in 19 of the 91 (21%) postnatal, transgenic mice (4 days–9 months of age). Twenty-five control mice in this age group did not develop ocular or midbrain malignancies. The 19 mice with midbrain tumors (age range, 2.5–7 months; mean age, 5 months). Eighty-five (71 transgenic and 14 controls) brains from mice older than 2 months were examined. The incidence of brain tumors in this line of mice may then be interpreted to be 27% (19 tumors per 71 transgenic mice). Eleven of the 19 affected mice were females. Clinically, mice with midbrain tumors demonstrated head tilt, gait disturbances, or tremor at 3–5 months of age, leading to death by the age of 7 months. Ten brains were examined from mice 7.5–9 months old; midbrain tumors were not observed in this age group.

Gross Findings of Midbrain Tumors

Midbrain tumors were not visible by gross examination despite the fact that tumors extended to the meninges in the area of the pituitary (two mice) or to the caudodorsolateral surface of the midbrain (one mouse). Tumors were restricted to the midbrain and were centrally and symmetrically located. The tumors ranged in size from less than 1 mm to 4.5 × 4.0 mm. The mean size of the 19 tumors was 3.0 mm in greatest dimension. Tumor size did not always correlate with age. The two smallest tumors occurred...
in mice aged 2.5 and 4.0 months. Tumors as small as 1.0 mm occurred in 5- and 7-month-old mice.

Microscopic Findings of Midbrain Tumors

Tumor cells did not form a distinct expansile collection, but their distribution suggested multicentric origin. Most of these cells were in the location of subependymal matrix cells; collections of benign matrix cells were not evident in normal mice of this age group. Slightly larger tumors apposed, and rarely replaced, aqueduct ependymal cells and did not penetrate into the aqueduct lumen. Inflammation, gliosis, or compression of the neuropil was not observed. Their absence was interpreted as a lack of expansile growth (Fig. 1).

All of the 19 mice with primary brain tumors had solitary, midline neoplasms that began below and often adjacent to the cerebral aqueduct. Twelve of the 19 tumors had cells that contacted the outer surface of some of the ventral cerebral aqueduct ependymal cells. In two other brains, tumor cells approached, but did not touch, ependymal cells. Hydrocephalus occurred in six mice due to obliteration or compression of the aqueduct by the tumor. The smallest tumor contained small clusters of cells apposed to the ventral ependymal cells of the aqueduct at its anterior margin at the level of the pineal gland.
Medium-sized tumors, when viewed on transverse sections were midline and symmetric and occurred at or near the cerebral aqueduct (Fig. 2). The base of the tumors reached the meninges in the area of the pituitary in 12 mice and invaded the meninges at that site in three. One mouse had meningeal invasion near the pituitary and at the dorsocaudal lateral edge of the midbrain. Midsagittal sections revealed that the small tumors were conical and below the cerebral aqueduct with the narrow end pointed anteroventrally toward the origin of the middle cerebral arteries.

Larger tumors had a "claw-hammer" shape (Fig. 3) with their posterior component occupying most of the caudodorsal posterior colliculus and apposed to the anterior ependymal layer of the fourth ventricle. The "striking end" of the hammer was dorsal to the anteroinferior aspect of the fourth ventricle (Figs. 3, 4). The tumor portion corresponding to the "claws" of the hammer extended anteriorly below the floor of the cerebral aqueduct or extended above the aqueduct to the superior portion of the third ventricle. The hammer "handle" pointed anteroinferiorly toward the pituitary gland at the point of entrance of the middle cerebral arteries. While seven of the larger tumors touched the posteroverentral portion of the cerebral aqueduct and the anteroinferior ependymal

Fig. 2. Primitive neuroectodermal tumor displaying neoplastic cells which are adjacent and appear to replace or alter a portion of the ventral ependymal lining of the cerebral aqueduct. The tumor does not enter or distort the aqueductal lumen (H&E). Magnification x315.
Fig. 3. “Claw hammer”-shaped primitive neuroectodermal tumor of the midbrain contacting the ventral cerebrospinal aqueduct (arrows) and extending caudally to the anterior ependymal lining of the fourth ventricle (V). The short “handle of the hammer” extends anteromedially toward the middle cerebral artery in the region of the pituitary (P). The pineal gland (PG) and the choroid plexuses (open arrows) of the third and fourth ventricles are shown (H&E). Magnification ×31.5.

cell layer of the fourth ventricle, none of the tumors invaded the ventricular system; this was a striking difference from tumor extension observed in both transgenic mice and humans.

The following cytologic features were noted in all tumors: anisokaryosis, euchromatin, hyperchromatism, giant cells, and indistinct cytoplasm. Giant nuclei had irregular outlines and multilobulation, but none had distinct multinucleation (Fig. 5). Scattered foci of mineralization were seen in 15 of 19 tumors. The mitotic index per ten high-power fields ranged from 35–200 (average, 87). Cellular patterns included scattered individual cells, compact cell groups, and irregularly clustered nuclei in poorly defined wavy and branching linear palisades (Fig. 4). Incomplete rosettes (palisading nuclei) were observed, but true Flexner-Wintersteiner and Homer Wright rosettes or fleurettes were absent.

The internuclear regions appeared as a lightly eosinophilic and faintly fibrillar matrix. The matrix was most prominent between groups of palisading nuclei and suggested that the cytoplasm of the cells had fibrillar processes (Fig. 5).

None of the animals with primary midbrain malign-
nancies had other nonocular primary or metastatic tumors. The pineal gland was sectioned in 15 of the 19 mice and was tumor free.

**Immunohistochemical and Ultrastructural Findings**

Midbrain tumors were highly undifferentiated as judged by immunohistochemistry. The neoplastic cells did not react with any antibodies to neurofilament proteins tested, nor were they reactive for neuron-specific enolase or synaptophysin, all of which were detected in normal neuronal cell bodies and processes around the tumor. Such normal cells could occasionally be entrapped in the tumor. All four midbrain tumors examined contained variable amounts of glial cells that were labeled with antibodies to GFAP and S-100 protein. These cells had many slender processes, their nuclei were smaller in size and more vesicular than those of the neoplastic cells, they lacked mitoses, and transitional cells resembling anaplastic tumor cells were absent. The morphology of the positive glial cells was thus consistent with their being reactive astrocytes. In one case, there were distinct foci of neoplastic cells which differed greatly in the number of reactive glia, suggesting the possibility of multicentric growth. All brain tumors were entirely negative for cytokeratins, which were detected in normal murine ependymal cells.
Fig. 5. Pleomorphic, midbrain tumor demonstrating anisonucleosis, hyperchromatic nuclei, numerous mitoses, poikilonucleosis, and giant multilobulated nuclei. The irregularly and often widely scattered nuclei are separated by an eosinophilic fibrillar substance. (H&E). Magnification ×315.

Detailed electron microscopic studies demonstrated that midbrain tumors consist of undifferentiated tumor cells with variably sized round nuclei, dispersed chromatin, numerous mitochondria, and lamellated membrane structures. Ultrastructural evidence of photoreceptor differentiation was not observed. Immunohistochemical and electron microscopic studies, therefore, indicate that the midbrain neoplasms were undifferentiated.

Discussion

This report describes a heritable, primitive neuroectodermal tumor (PNET) of the midbrain in apposition to or near the inferior ependymal cells of the cerebral aqueduct in a single strain of transgenic mice with heritable retinoblastoma. Midbrain tumors occur in 27% of transgene-bearing animals, and ocular tumors occur in 100% of transgenic mice. The midbrain tumors frequently begin in a linear alignment along the ventral surface of the anterior cerebral aqueduct often at the level of the pineal gland. Several of the tumors arose in scattered but specific areas suggesting tumor transformation occurred in primitive neuroectodermal cells that were destined for specific areas of migration. This theory is supported further by the midline origin, subependymal location, tumor symmetry, and the absence of expansile...
growth in most of the tumors. These progenitor cells most likely originated in the subependymal matrix layer of the mesencephalon inferior to the cerebral aqueduct.

The term PNET is used occasionally to amalgamate the sometimes histologically indistinguishable, solid central nervous system neoplasms of childhood.20,21 Included in this group are retinoblastoma, pinealoblastoma, cerebellar and cerebral medulloblastoma, ependymoblastoma, primitive polar spongioblastoma, and cerebral neuroblastoma.20,21 Rubinstein20 prefers to subdivide these tumors according to location and, most importantly, to the differentiating potential of their constituent cells. Thus PNET is usually reserved for tumors which do not display evidence of ultrastructural or immunohistochemical differentiation.21 The designation of PNET for the above transgenic midbrain tumors is, therefore, appropriate given their undifferentiated state.

Many hypotheses for the histogenesis of intracranial retinoblastoma have been proposed. Jakobiec et al10 noted the possible existence of ectopic retinal cells along the optic tract and hypothalamic regions. As an alternative explanation, they proposed that the subependymal, germinal layer of benign, primitive neuroblasts is susceptible to oncogenesis well into the neonatal period. Neuroectodermal matrix cells giving rise to the pineal and optic cup and found elsewhere in the diencephalon were also emphasized by Zimmerman.22 A histopathologic demonstration of normal pineal tissue adjacent to large, bulky pinealoblastomas in two cases of human trilateral retinoblastoma also implicates this layer as the progenitor.10,23 The development of PNET arising from the subependymal cells in transgenic mice lends further support to the hypothesis that germinal matrix cells undergo transformation and give rise to ectopic, intracranial retinoblastoma in humans.

The phylogenetic and ontogenetic relationship of the pineal to photoreception has been emphasized to explain the occurrence of pineal tumors in human, trilateral retinoblastoma. Although pineal tumors are the most common intracranial lesions observed in trilateral cases, suprasellar and parasellar neoplasms are also seen.12,23 A review of the literature concerning human trilateral retinoblastoma showed that rosette or photoreceptor differentiation is observed in approximately one half of the pineal or suprasellar–parasellar tumors. Anaplastic tumors account for the other tumors seen. Given the absence of pineal involvement in transgenic mice with retinoblastoma, murine brain tumors most closely resemble the undifferentiated suprasellar–parasellar tumors occasionally observed in human trilateral retinoblastoma.12,24–27 Indeed, all murine midbrain lesions did not show any neuronal properties and were antigenically less differentiated than human ectopic pineal tumors which have consistently reacted with antisera to neuron-specific enolase.28

Primitive matrix cells are also the likely progenitors for other central nervous system tumors of childhood.20,21 This common precursor layer may account for the similar histologic characteristics (ie, rosette formation) displayed by these tumors located in the cerebellum, pineal, retina, cerebrum, or spinal cord. Other viruses, with oncoproteins known to bind the retinoblastoma protein product, have produced ependymomas, medulloblastomas, PNETs, pineocytomas, and retinoblastomas in nonheritable animal models.29 A subependymal origin of tumors in transgenic mice raises the notion that neuroectodermal matrix cells are susceptible to abnormalities at the retinoblastoma locus or to retinoblastoma inactivation by viral oncoprotein expression in these cells.

It is probable that the subependymal expression of T-ag is a necessary event in the oncogenesis of transgenic central nervous system malignancies. Given the lower incidence of brain tumors in both heritable human and transgenic mouse retinoblastoma, additional oncogenic events (other than retinoblastoma gene loss or protein inactivation by viral oncoproteins) must occur in central nervous system tumorigenesis. The undifferentiated nature of our transgenic midbrain malignancies indicates that these tumorigenic events occur relatively early in murine development. The role of the retinoblastoma gene and other oncogenic mechanisms in childhood brain tumors (including trilateral retinoblastoma) requires further investigation.

Key words: retinoblastoma, transgenic mice, primitive neuroectodermal tumor, trilateral retinoblastoma, brain tumor

Acknowledgments

The authors thank Rene Bernards and Emmett Schmidt for reviewing this manuscript.

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