Ultrastructural studies of human adenovirus-produced retinoblastoma-like neoplasms in Sprague-Dawley rats*

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Sixteen out of 85 Sprague-Dawley rats (18.8 per cent) developed solid medullary tumors in the left ocular cavity within 74 to 288 days after a single intravitreous inoculation of 0.015 to 0.03 ml. of fluid containing human adenovirus type 12, $10^{3-5}$ to $10^{4-1}$ TCID₅₀ HeLa cells per 0.1 ml. The left eye was inoculated within 24 hours after birth. Tumor-bearing rats were perfused with Karnovsky's fixative and their enucleated eyes were subjected to epon-embedded thin sectioning. The remarkably uniform microscopic appearance of all cases resembled human retinoblastoma with incomplete rosettes. Ultrastructurally, the tumor cells forming incomplete rosettes and perivascular wreaths possessed oval or slightly indented nuclei without noticeable nuclear membrane abnormalities. A slender luminal pole of cytoplasm in each cell contained poorly differentiated organelles. One cilium per cell with a $9 + 0$ pattern of doublets associated with a pair of centrioles was detectable in all cases. A strong tendency for many tumor cells to undergo bizarre ganglioneuronic maturation was also characteristic.

Key words: human adenovirus type 12, retinoblastoma-like neoplasms, incomplete rosettes, unipolar tumor cells, ganglioneuronic differentiation.

That a DNA virus of human origin is capable of producing a retinal tumor in vitro was first demonstrated by Albert, Rabson, and Dalton. Their experiments indicate that even adult hamster retinas exposed to human adenovirus type 12 give rise to typical malignancy. In addition, transformed retinal cells transplanted subcutaneously into irradiated young hamsters result in remarkably uniform retinoblastoma-like neoplasms without rosettes. In vivo experiments with rodents also provide ample evidence that certain neuronic precursors ordained for the sensory neural complex are preferentially susceptible to malignant transformation induced by the virus. The highly selective, potent oncogenicity of human adenovirus type 12 in sensory neuronic primordia has led us to select

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Fig. 1. A, horizontal section of massive tumor showing almost total intraocular encroachment of the tumor. Note the neoplastic cell infiltration of the optic nerve (arrow). Hematoxylin and eosin, ×10. B, characteristic incomplete rosettes associated with perivascular festooning of tumor cells. Thin section, toluidine blue, ×200.

Retinal immature neurons, which are undeniably of sensory origin, as a likely target for adenovirus tumorigenesis. This paper describes electron microscopic findings in the first virus-produced autochthonous tumors of the retina. Its aim is to help understand the probable origin of human retinoblastoma.

Materials and methods

Preparation of concentrated virus fluid. Human adenovirus type 12 (Huie Strain, Flow Laboratories, Md.) was cultured in a HeLa cell monolayer prepared as described in previous papers.2-5

Inoculation of virus fluid. Each of 85 newborn, random-bred Sprague-Dawley rats (Charles River Breeding Lab., North Wilmington, Mass.) was given a single intravitreal inoculation of 0.015 to 0.03 ml. of virus fluid, 10^5 to 10^6 TCID₅₀ HeLa cells per 0.1 ml. (the 50 per cent tissue culture infectious dose per 0.1 ml., within 24 hours after birth. All inoculations were performed under an operating microscope with a fine hypodermic needle (No. 30, Metropolitan Supply, Cambridge, Mass.) connected to a microsyringe (No. 710-N, Hamilton). A fine vinyl tube of appropriate length was placed on the needle in order to prevent excessively deep penetration of the left orbit. Twenty newborn control rats of the same strain were treated with 0.015 to 0.03 ml. of supernatant fluid from nonvirus-infected HeLa cells cultured in the medium used for preparing the virus fluid.

Thin-section histology and electron microscopy. The sixteen animals bearing intraocular tumors were subjected to whole-body perfusion with Karnovsky's formaldehyde fixative.6 Perfused animals were kept at 4°C for several hours; their eyes were then enucleated. Slabs of dissected tumors, approximately 5 mm. thick, were fixed with the same solution at room temperature for two hours and then diced into small blocks under a binocular microscope. The blocks were refluxed with the same fixative for two to five hours, and then immersed overnight in 0.2 M phosphate buffer, pH 7.2, at 4°C. All blocks were postfixed with 2.0 per cent OsO₄ and embedded in Epon-812. Sections cut with an LKB-Ulrotome were stained with both uranyl acetate and lead citrate at 37°C for 45 minutes. Samples were studied with Philips 200 and 300 electron microscopes. Sections 2μ thick, stained with 0.1 per cent buffered toluidine blue, were provided for comparative cytologic studies.
Fig. 2. A, tumor cells forming incomplete rosettes frequently show mitosis; the size of the nuclei varies. Toluidine blue, ×1,000. B, clusters of bizarre, ganglionic cells are often found in a solid, medullary mass. Toluidine blue, ×1,000.

Results

Intravitreous inoculation performed in 85 newborn, random-bred Sprague-Dawley rats resulted in 16 cases of intraocular tumor (18.8 per cent) within 74 to 258 days after the virus inoculation. Four cases of extraocular neurogenic tumor and one spinal cord tumor also developed unexpectedly. A detailed description of each case will appear elsewhere. Massive intraocular growth of the adenovirus-typical tumor (Fig. 1, A) was observed in nine cases. The remaining seven cases showed microscopically detectable incipient tumors of the retina.

Light microscopy. Tumor cell infiltration into the optic nerve head was found in all cases of massive intraocular growth. (Fig. 1, A, arrow). A remarkably uniform histologic appearance, i.e., incomplete rosettes associated with perivascular wreaths formed by medullo-epitheliomatous cells, was also common to all cases (Fig. 1, B). The incomplete rosettes were composed of a unilayered cell wreath and constituted the basic structural pattern of the tumors (Fig. 2, A). Mitotic figures were frequent within tumor cells, particularly in those cells forming the incomplete rosettes. Clusters of bizarre, ganglioneuronic tumor cells were common, especially in highly vascularized or loosely meshed areas between solid medullary tumor masses where the cells retained no definite orientation and formed no rosettes (Fig. 2, B).

Electron microscopy. Tumor cells forming incomplete rosettes appeared to possess elongated, oval, or slightly indented nuclei with evenly distributed chromatin. No nuclear membrane abnormalities were found. The scanty rim of cytoplasm in each tumor cell was composed of poorly organized organelles, i.e., evenly distributed ribosomes forming a few endoplasmic reticula and a few relatively well differentiated mitochondria (Fig. 3). The apical processes of each tumor cell appeared to meet in an irregularly shaped lumen without forming a girdle-like terminal attachment (Fig. 3).
Fig. 3. Tumor cells forming an incomplete rosette possess elongated oval or slightly indented nuclei with evenly distributed chromatin, without a triple-membered membrane. Note the poorly differentiated intracytoplasmic organelles. There is a paucity of terminal bars and intercellular junction complexes throughout the cases. Note characteristic cilium (arrow) commonly seen in tumor cells. Direct, ×5,900.
There was only a trace of terminal bars. No definite tendency to form intercellular junction complexes was observed, nor was any connective tissue stroma seen. In the apical cytoplasmic processes of many tumor cells, characteristic cilia were seen (Fig. 3, arrow).

Bizarre ganglionic tumor cells usually retained a much higher degree of differentiation in their cytoplasmic organelles than did average tumor cells. The broad rim of cytoplasm of such ganglionic cells showed more endoplasmic reticula, Golgi stacks, and mitochondria (Fig. 4). Within
Fig. 5. Tumor cell which invaded the optic nerve showing transverse section of a single cilium and its associated pair of centrioles (arrow). Note poorly organized cytoplasmic organelles. Direct, ×13,500. Inset: cross-section of the cilium with a 9 + 0 pattern. Direct, ×35,000.
the apical portion of tumor cell cytoplasm, the characteristic solitary cilia were seen (Fig. 4, arrow). Plasma cells were abundant throughout the specimens examined (Fig. 4).

Many tumor cells which invaded the optic nerve also displayed one cilium per cell with a single pair of centrioles in the apical portion of its cytoplasm (Fig. 5, arrow). Without exception, all of these cilia were morphologically similar, i.e., nine pairs of peripheral doublets with no central axis (Fig. 5, inset). The average diameter of these cilia in cross-section was 270 mμ. In some places, two tightly opposed tumor cells appeared to be connected by a single cilium.

Discussion

The phenotype of adenovirus-induced retinal tumors is comprised of poorly differentiated unipolar tumor cells that closely resemble primitive neuronic precursor cells. These cells are almost indistinguishable from those produced by the same virus in the brain and in peripheral neural tissues. All adenovirus-induced neurogenic tumors have been described as possessing cells equipped with a solitary cilium consisting of a ring of nine double tubules without a central axial pair, associated with one pair of centrioles. The presence of cilia with 9 + 0 tubules and two centrioles in photoreceptive structures has been recognized as belonging to cells with an active sensory capacity or a conduction functionally modified in the direction of sensory perception. It is also well-documented that human retinoblastoma cells interpreted in association with photoreceptor differentiation contain cilia of the same morphology. The difference is that most of these cilia project into the luminal space of each well-formed rosette.

In the normal process of cell evolution, cilia of this characteristic structure are observed in human fetal retinas, in the neural epithelia of the chick embryo, and in fetal retinas of the rabbit. This type of cilium has also been found in fully developed ganglion cells and bipolar neurons of both the guinea pig and the human retina, and also in the human retinal pigment epithelium.

Mitotic cells are abundant in all adenovirus-induced retinoblastoma-like tumors, particularly in those forming rosette-like alignments. Nuclear membrane abnormalities, such as the triple-membered structure observed in some human retinoblastomas, have not been found in these malignant cells. The overall meagerness of intercellular junctions and terminal bars is considered to be a measure of the degree of malignancy, as also observed in human retinoblastoma cases.

The marked tendency for the tumor cells to invade the optic nerve is considered one of the important features of human retinoblastoma and was also found in the present study (Figs. 1, A and 5). There was no tendency to form dystrophic calcifications, although tumor cell degeneration was fairly pronounced in some areas, particularly where many plasma cells appeared among neoplastic cells.

The adenovirus-produced intraocular neoplasms, that are described closely resemble human retinoblastoma with incomplete rosette formation. This observation strongly suggests that both types of tumor share an analogous primordium cell in malignant transformation. Green and coworkers have demonstrated that the incorporation and transcription of at least part of the viral genome into target cell DNA are obligatory steps in adenovirus oncogenesis. In a previous study, we demonstrated human adenovirus-specific T-antigen in retinal tumors by the immunofluorescein microscopic procedure. Intense fluorescein flecks were found within cells throughout the retinal tumors. These facts imply that neuronic precursor populations in the rat retina are selectively vulnerable to viral transformation.

Some undifferentiated cells in retinoblastoma-like virus-induced tumors mimic
ganglioneuronic differentiation. Although no comparable ganglioneuronic maturation has been described for human retinoblastoma with incomplete rosettes, a strikingly similar maturation is well documented for human medullo-epithelioma, which is a distant oncologic counterpart of embryonic retinal neoplasms. Thus, it seems possible that human retinoblastoma with incomplete rosettes stems from neuronic primordia destined for ganglioneuronic differentiation.

Since human adenovirus type 12 is a potent oncogenic virus capable of producing, in animals, embryonal neuronic tumors similar to human embryonal neurogenic neoplasms, and since human retinoblastomas possess phenotypic similarities to the viral tumors under discussion, the viral origin of autochthonous human retinoblastomas seems a distinct possibility. Furthermore, roughly 3 per cent of pregnant women, and accordingly, their offspring, are believed to be deficient in antibodies against adenovirus type 12 infection. The potent oncogenic action of this virus on some neuronic precursors of the retina may be a factor in somatic mutation under immunologically unprotected conditions. This has been implied by genetic studies in some human retinoblastoma cases.

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