Retinoblastoma-like Tumors Induced by Human Adenovirus Type 12 in Rats

Shoji Kobayashi and Noritsugu Mukai

Wesley C. Bowers Laboratory of Pharmacology and Experimental Pathology, Department of Retina Research, Retina Foundation, Boston, Massachusetts 02114

SUMMARY

A malignant intraocular neoplasm that resembled human retinoblastomas was produced in CD rats by injection of human adenovirus type 12. Fluid (0.01 ml), containing adenovirus type 12, 10^7 to 50% tissue culture infective doses/ml, from culture fluids of human embryo kidney cells was injected into the vitreous cavity of each newborn rat. Three of 35 virus-injected rats developed an intraocular tumor. Fifty additional rats were pretreated with methylnitrosourea on the 20th day of gestation; five of their offspring also developed tumors after virus injection. Pretreatment with methylnitrosourea remarkably reduced the latency period for tumor production. Histopathologically, tumor cells showed a marked tendency to form rosettes. A solitary cilium consisting of a typical ring of nine doublets with no axilal pair (a 9 + 0 pattern) was frequently detected in the apical region of tumor cell cytoplasm. Adenovirus 12-specific T-antigen-positive particles were detectable in cells from the primary tumor tissue cultures with the immunofluorescent procedure.

INTRODUCTION

Although several attempts at experimental production of retinoblastoma have been made, none of them has successfully produced tumors derived from the retina. Weil et al. (22) injected superheated lard and olive oil into the vitreous body of adult rats, which developed retinal cell hyperplasia without producing any malignant lesions derived from the sensory retina. Similarly, intraocular implantation of a methylcholanthrene pellet in adult mice produced melanoma and squamous cell carcinoma, but it failed to produce any solid retinal tumors (16).

Ad12 produces neurogenic tumors in the peripheral nervous system and medulloblastomas in the central nervous system of hamsters and mice (13-15). This implies that some neuronal primordia are particularly susceptible to the virus during a certain period of postnatal development. Our latest experiments strongly suggest that neuronal precursors in rats are far more susceptible to the virus than those in hamsters (11).

The production of various types of neuronal tumors in the central nervous system of the offspring of pregnant rats by i.v. injection of MNU and ethylnitrosourea is also well documented (3, 7, 8, 20, 21). The higher susceptibility of immature neuronal cells in the fetus as opposed to adults has been proven by these studies. In addition, Zülch and Mennal (24) have emphasized the importance of the high susceptibility to chemical oncogenesis of certain sensory primordia in macrosomatic rodents. These facts prompted us to test the possible affinity of the virus for undifferentiated neural cells in the rat retina and to determine the cocarcinogenic effect of MNU during viral oncogenesis.

MATERIALS AND METHODS

Preparation and Titration of the Virus Fluid. Ad12 Huie strain (Flow Laboratories, Rockville, Md.) was propagated in a HeLa cell culture as previously described (9-11). The virus containing fluid was titrated by serial 10-fold dilution in tube cultures of primary human embryo kidney cells (Microbiological Associates, Inc., Bethesda, Md.) with 4 tubes/dilution. The human embryo kidney cell culture tubes were maintained in a mixture of Medium 199 with 2% fetal bovine serum, incubated at 37°C, and observed daily for 2 weeks for cytopathic effects. The titer of this preparation was 10^7 to 50% tissue culture infective doses/ml.

Animal Experiments. Fifteen pregnant CD rats (Sprague-Dawley descent, Charles River Breeding Laboratories, North Wilmington, Mass.) were commercially obtained and gave birth in our laboratory. The pregnant rats were divided into 4 groups (Table I). Thirty-five newborns from Group 1 were inoculated within 24 hr after birth with Ad12 in the left eye and were not treated with MNU. Fifty newborns from Group 2 were also inoculated in the left eye and were pretreated with MNU (Columbia Organic Chemicals, Columbia, S. C.), which had been freshly prepared by dissolving 5 mg/ml in sterile 0.85% NaCl solution immediately before injection. As a control, 26 newborns from Group 3 (pretreated with MNU) and 15 newborns from Group 4 (not pretreated with MNU) were inoculated with the supernatant of non-virus-infected HeLa cells in the left eye.

All virus inoculations were performed as described previ-
JULY 1974 1647

Virus injection. The progeny of the groups pretreated with chemically induced malignant tumors from 74 to 118 days after the virus injection-treated eyes from Group 2 produced tumors in incidence in both groups is almost identical, as 5 of the 50 newborns of Groups 1 and 3 showed various degrees of microphthalmos without phthisis. All the newborns of Groups 1 and 4 showed normal body development, but approximately one-half of the inoculated left eyes showed various degrees of microphthalmos without phthisis. Three of the 53 virus injection-treated left eyes in Group 1 produced intraocular malignant tumors 204, 215, and 288 days after the injection (Table 1). Pretreatment with MNU during the embryonic stage (Group 2) remarkably reduced this latency period. However, the tumor incidence in both groups is almost identical, as 5 of the 50 virus injection-treated eyes from Group 2 produced intraocular malignant tumors from 74 to 118 days after the virus injection. The progeny of the groups pretreated with MNU showed a few abnormalities. No macroscopic malformation was evident. No tumors were found in the control groups. Only a few rats died following neonatal intraocular inoculation of the virus. (The number of newborn rats effectively used is listed in Table 1.)

Histological Examination. The animals that developed tumors underwent whole-body perfusion with Karnovsky's fixative (5) under anesthesia. The major portion of each tumor was processed for routine paraffin sectioning. For cytological identification, hematoxylin-eosin, phosphotungstic acid-hematoxylin, Holmes' silver impregnation, and Nissl's stains were used. Toluidine blue stain was used for Epon-embedded thin sections. A small tumor section was taken aseptically from Group 2 rats and processed for tissue culture work.

Electron Microscopy. Slabs of tumor tissue fixed with Karnovsky's fixative (5) were processed as described previously (10, 11). Ultrathin sections were cut with an LKB Ultrotome and stained with both uranyl acetate and lead citrate. Specimens were examined with Philips 200 and 300 electron microscopes.

Detection of Ad12 T-Antigen. Primary tumor cell cultures were prepared on coverslips in Waymouth's MB751/1 medium (Microbiological Associates) supplemented with 10% fetal bovine serum (Flow Laboratories) in a 5% carbon dioxide atmosphere. After suitable fixation, the tumor cells were tested by the direct method with rabbit 

## Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of litters used</th>
<th>Treated with MNU</th>
<th>No. of newborn rats used</th>
<th>Inoculation of Ad12</th>
<th>No. of cases of tumors</th>
<th>Latency period (days)</th>
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* MNU, 10 mg/kg body weight, was injected i.v. in pregnant rats on the 20th day of gestation.

RESULTS

All the newborns of Groups 1 and 4 showed normal body development, but approximately one-half of the inoculated left eyes showed various degrees of microphthalmos without phthisis. Three of the 35 virus injection-treated left eyes in Group 1 produced intraocular malignant tumors 204, 215, and 288 days after the injection (Table 1). Pretreatment with MNU during the embryonic stage (Group 2) remarkably reduced this latency period. However, the tumor incidence in both groups is almost identical, as 5 of the 50 virus injection-treated eyes from Group 2 produced intraocular malignant tumors from 74 to 118 days after the virus injection. The progeny of the groups pretreated with MNU showed a few abnormalities. No macroscopic malformation was evident. No tumors were found in the control groups. Only a few rats died following neonatal intraocular inoculation of the virus. (The number of newborn rats effectively used is listed in Table 1.)

On sectioning, a soft, grayish-pink tumor mass was observed filling the vitreous. Some parts of the tumors appeared to have undergone hemorrhagic necrosis. Histologically, the tumors of both groups were indistinguishable from each other. The major part of the tumors was composed of small hyperchromatic, undifferentiated cells that formed perivascular wreaths or rosettes. Most of the tumor cells showed irregularly shaped, round, or slightly elongated hyperchromatic nuclei and small amounts of poorly defined cytoplasm. Mitotic figures were abundant. All of the tumors showed a tendency to undergo hemorrhagic necrosis, in which degenerated tumor cells encircled nondegenerated cells located around the blood vessels. In such areas, a trabecular or papillary appearance was one of the characteristic features (Fig. 1, A and B). Tumor cells had a marked tendency to form rosettes throughout the tissue (Fig. 1, B and C). No so-called Flexner-Wintersteiner rosettes were found. In some areas, palisading arrangements of the cells were observed.

The sclera was remarkably distended by the growth of the tumor; most of the retina appeared to be invaded by tumor cells. On sectioning, a soft, grayish-pink tumor mass was observed filling the vitreous. Some parts of the tumors appeared to have undergone hemorrhagic necrosis. Histologically, the tumors of both groups were indistinguishable from each other. The major part of the tumors was composed of small hyperchromatic, undifferentiated cells that formed perivascular wreaths or rosettes. Most of the tumor cells showed irregularly shaped, round, or slightly elongated hyperchromatic nuclei and small amounts of poorly defined cytoplasm. Mitotic figures were abundant. All of the tumors showed a tendency to undergo hemorrhagic necrosis, in which degenerated tumor cells encircled nondegenerated cells located around the blood vessels. In such areas, a trabecular or papillary appearance was one of the characteristic features (Fig. 1, A and B). Tumor cells had a marked tendency to form rosettes throughout the tissue (Fig. 1, B and C). No so-called Flexner-Wintersteiner rosettes were found. In some areas, palisading arrangements of the cells were observed.

The sclera was remarkably distended by the growth of the tumor; most of the retina appeared to be invaded by tumor cells (Fig. 1D). In 5 of the 8 cases, the tumor cells infiltrated into the optic nerve (Fig. 1, A and B, arrows). Moderate infiltration of mononuclear blood cells (mainly plasma cells and large lymphocytes) was observed in the anterior chamber as well as the choroid (Fig. 1D). In some peripheral areas of necrosis, balloon-like histiocyte cells were observed. In some areas, tumor cells appeared anaplastic and at times closely resembled ganglion cells (Fig. 2A). Numerous fine protoplasmic processes were observed in some of the anaplastic tumor cells (Fig. 2A).

Electron Microscopy. Tumor cells forming pseudorosettes possessed an elongated ovoid or slightly indented nucleus with haphazardly distributed chromatin. Poorly defined cytoplasmic organelles frequently contained a single cilium, with a 9 + 0 pattern in the apical region of the tumor cell cytoplasm (Fig. 2B). A paucity of intercellular junction complexes was characteristic. A number of plasma cells appeared to intermingle with the many tumor cells encroaching upon the anterior segment of the eye.
Detection of Ad12 T-Antigen in Tumor Cells. The tumor cells under fluorescein microscopy revealed intensely positive fluorescent antigen. It appeared as slender filamentous, crescent-shaped, or sometimes dot-shaped flecks in the cytoplasm (Fig. 2C). Although most of these particles were seen at random in the cytoplasm, they were frequently located adjacent to the nuclear membrane. In addition to the cytoplasmic flecks, numerous very fine dots and flecks of fluorescent antigen were also observed in the cell nuclei of some specimens. Fluorescent particles were present in almost all cells.

A blocking test was performed to confirm the specificity of fluorescence. Unconjugated serum from tumor-bearing hamsters was overlayed on unstained fixed tumor cells for 30 min at 37°C, and then stained with fluorescein isothiocyanate-conjugated γ-globulin. Both nuclear and cytoplasmic fluorescence were markedly reduced by the serum from tumor-bearing hamsters, while normal hamster serum had no effect.

DISCUSSION

In an in vitro experiment, Albert et al. (1) transformed cultured retinal cells taken from young adult hamsters, using Ad12. When transplanted s.c. in irradiated hamsters, these transformed cells produced a solid tumor. Histologically, the tumor cells had some resemblance to retinoblastoma, but no rosettes or neural elements were identified.

Since there is no comparable experimentally induced retinoblastoma in the literature, no established criteria exist for classifying our experimental retinal tumors. However, it is reasonable to apply to some extent the diagnostic criteria and nomenclature established for human retinoblastoma. We may also refer to the analogous tumor phenotype expressed by Ad12 in the retrolubar adnexa and the central nervous system (9-11).

Ad-12-induced intraocular tumors are remarkably uniform and basically identical to those produced by the same virus in different organs. Adenovirus-induced brain tumors in hamsters and rats have been designated as medulloepitheliomatous neoplasms presumably derived from neuronal primordia of the periventricular zone. It has also been postulated that sensory neuronal precursors might be the susceptible target cells in adenovirus tumorigenesis (10).

In Ad12 tumor cells, a solitary cilium consisting of a typical ring of 9 doublets with no axial pair (a 9 + 0 pattern) is one of the characteristic features in the apical region of the tumor cell. The ultrastructural identification of the characteristic cilium and its associated pair of centrioles (highly reminiscent of the connecting sensory cilia in the retinal receptors) has been well documented in human retinoblastoma cases (2, 18, 19). In 1 study of human retinoblastoma (4), the absence of rosettes of the Flexner-Wintersteiner type was noted in 30% of the cases.

On the basis of these data, it is reasonable to assume that the malignant tumors produced by us are derived from neuronal precursor cells in the developing retina. We therefore propose that these tumors should be considered the experimental counterpart of human retinoblastomas. With regard to the tumors in rats treated transplacentally with MNU (Group 2), one could question whether the main causative factor in malignant transformation is the potent chemical carcinogen MNU or the postnatally injected virus. Although there have been many reports on the oncogenicity of N-nitrosoureas in rats, no intraocular tumors have as yet been reported in the literature (3, 7, 8, 20, 21). N-Nitrosourea-induced neurogenic tumors are classified into various types; most of them show histologically well-differentiated patterns.

All of our intraocular tumors appeared in the left eye; no tumors appeared in the non-virus injection-treated right eye. In terms of the oncogenicity of N-nitrosoureas within the fetal nervous system, MNU is known to be less carcinogenic than ethylnitrosourea. Moreover, the dose of MNU used in this experiment was deliberately small in order to avoid possible tumor production by MNU.

Histopathologically and electron microscopically, tumors from both the MNU-treated and non-MNU-treated groups showed completely identical features. The resulting detection of adenovirus-specific tumor antigen in tumor cells taken from the rats in the MNU-treated group indicates that the postnatally injected virus was the main causative factor. A marked reduction of the latency period is the only significant change when MNU is used in conjunction with Ad12. It is reasonable to suppose that MNU actively increases susceptibility of the target cells to viral oncogenicity or else actively suppresses the factors that inhibit malignant transformation. However, the precise nature of the relationship between viral oncogenesis and the N-nitrosoureas still remains to be explored.

In general, Ad12 T-antigen is not only detected in tumor cells transformed in vitro but is also produced early in the infectious cycle (17). The exact function of T-antigen during infection and tumor formation is currently unknown; however, it is believed that the production of T-antigen is a partial expression of adenovirus genes in the infected cells and tumor cells. In this study, the presence of T-antigen marks the tumor cells as being produced by the virus. The shape and localization of the T-antigen is identical to that reported by Pope and Rowe (17).

The incidence of intraocular tumors in the present study is much lower than in the central nervous system of rats (11). One of the probable reasons for this is the technical difficulty presented by the size of the vitreous body in newborn rats. According to the paper of Yabe et al. (23), the incidence of tumors induced is directly proportional to the amount of virus injected. The limited dose of virus used, together with the presumably lower population of susceptible precursors in the retina, may account for the low incidence of tumor production in the present study.

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Retinoblastoma-like Tumors

REFERENCES


Fig. 1. A, photomicrograph of a tumor that replaced the entire vitreous body. Note the large necrotic area and the papillary appearance of nonneocrotic areas surrounding the blood vessels. The tumor infiltrated the optic nerve (arrows). H & E, × 10. In B, a marked tendency for tumor cells to form rosettes throughout the tissue was observed. Arrows, tumor invasion of the optic nerve. H & E, × 40. C, higher magnification of the tumor. Note characteristic rosettes. Vascular lumen stands empty due to perfusion. H & E, × 200. In D, the tumor completely invaded the retina (arrows). Note infiltration of mononuclear cells in the choroid. Holmes' silver impregnation, × 250.

Fig. 2. In A, i some areas of the tumor, giant cells similar to ganglion cells were observed. Epon-embedded section, toluidine blue stain, × 1,000. B, poorly differentiated organelles of tumor cell cytoplasm showing a few mitochondria with evenly distributed free ribosomes. Within the apical portion of the cytoplasm of many tumor cells, a solitary ciliium was detected (arrow). Direct, × 12,500. Inset, cross-section of a typical ciliium, showing the 9 + 0 patterning of the doublets (tubules). Direct, × 36,000. In C, virus-specific tumor antigen appeared as slender filamentous crescent- or dot-shaped flocks in the cytoplasm of cultured tumor cells. Very fine flocks and dots were observed in the cell nuclei. Fluorescent antibody stain, × 600.
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