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Abstract

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IN VITRO STUDIES ON TARGET CELLS OF ONCOGENIC ADENOVIRUSES IN HAMSTER BRAIN

II. *IN VITRO* TRANSFORMATION OF BRAIN CELLS OF HAMSTERS AT VARIOUS AGES BY HUMAN ADENOVIRUS TYPE 12

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Abstract: In vitro transformations of brain cells of hamsters of various ages were examined after the administration of human adenovirus type 12 (Ad 12) to determine the type and origin of the target cell. Hamster brain cells at all examined ages were transformed by Ad12. Although the virus was not isolated, virus specific tumor antigen was demonstrated in the transformed cells. The histological features of tumors that developed by transplantation of transformed cells closely resembled Ad12-induced brain tumors. The transformed cell focus tended to appear near the embryonic brain cell (EB cell) or glioblastic cell (GB cell). The transformed cells were morphologically similar to the EB or GB cell. Some subcultured transformed cells showed a rosette-like pattern, and the surrounding space arrangement was similar to that of the ventricular wall. The incidence of brain cell transformations decreased with increased hamster age. This decreased incidence with age corresponded to the decreased numbers of EB or GB cells present in progressively older hamsters. From these results, it is concluded that the target cells of Ad12 in hamster brain cell cultures are probably the EB or GB cells.

Since the first description by Trentin, Yabe and Taylor (1) of tumor induction by adenovirus type 12 (Ad12) in newborn hamsters, several possible target cells have been reported (2, 3, 4, 5, 6).

Ogawa and his associates investigated the oncogenesis of Ad12 in hamsters, mice and rats and postulated that Ad12-induced tumors originated from the undifferentiated peripheral nerve supporting cells (4) and the subependymal immature cells of the central nervous system (5, 7, 8). This postulation was supported by Mukai and Kobayashi (9, 10).

There have been several reports of *in vitro* transformation of fetal or newborn brain cells by simian virus 40 (SV40) (11, 12), polyoma virus (13) and adenovirus type 12 (14, 15, 16). In these investigations, no attempts were made to determine the type and origin of the target cells *in vitro* except in

one report (11) that dealt with the transformation of human fetal neuroglia by SV40. Brain cells of older animals were not used in any of these investigations.

The author attempted to study the *in vitro* transformation of hamster brain cells at various ages by Ad12 and to determine the type and origin of the target cell of Ad12 using the standards of the previous morphological study (17) of normal hamster brain cells *in vitro*.

MATERIALS AND METHODS

Tissue culture: The whole brains of fetuses (15-16 gestational days), newborns and 3, 7, 21 and 90-day old random bred hamstars were washed in phosphate buffered saline (PBS), cut into small pieces with scissors and digested with a mixture of 0.25% trypsin and 0.5% pancreatin for 15 minutes at 37°C. The dispersed brain cells were centrifuged and resuspended in a medium consisting of Eagle's minimum essential medium (MEM) and 10% fetal calf serum. These cells were cultured on cover slips in Leighton's tubes containing 2ml of medium. The first subcultured cells were used for the experiment.

The first subcultures of transformed cells were trypsinized, and the cells were passed to other tubes after resuspension in the medium. Subsequent subcultivations of transformed cells were carried out at 7 to 10 day intervals.

Virus: Human adenovirus type-12, Huie strain, was supplied by the courtesy of Dr. Y. Yabe of the Cancer Institute, Okayama University Medical School. This virus was propagated in HeLa cells, which had been maintained in Eagle's MEM with 2% bovine serum. The culture was disrupted by freezing and thawing seven times and centrifuged at 1,000 rpm for 5 minutes. The supernatant fluid was used for the experiment. The virus titer was tentatively determined with the same cells by 50% end point 7 days after infection. The virus titer used for the experiment was $10^{2.5}$TCID$_{50}$/0.1 ml.

Procedure of transformation: Ten or 13 inoculated and non-inoculated cultures were prepared for each age group. The first subcultures in semimonolayer were washed with PBS, and 0.2ml of virus suspended in 1.8ml of Eagle's MEM was inoculated to each tube. The control cultures were sham infected with 0.2 ml of MEM. After adsorption for 2 hr, the fluid was discarded and the cells were cultured in a stationary position at 37°C with the medium being changed every 3 to 4 days.

Stains: Cover slips with transformed cell foci were stained with hematoxylin and eosin (H-E) and phosphotungstic acid hematoxylin (PTAH). Cover slips with subcultured transformed cells were stained with H-E, PTAH and Bodian's nerve fiber stain. Tumors developing in hamsters after transplantation of transformed cells were stained with H-E, PTAH, Mallory's azan method, Pap's silver impregnation for reticulin and Bodian's nerve fiber stain.

Transplantation of transformed cells into hamsters: In order to examine tumorigenicity, 10^3 cells from each transformed cell line (5 7th passage level) were injected subcutaneously to newborn hamsters.

Isolation of virus from transformed cells: For isolation of the virus, 10^6 transformed cells were inoculated into a monolayer culture of HeLa cells which had been maintained in Eagle's MEM with 2% fetal calf serum. The mixture was cultured for 21 days changing the medium once every 3 to 4 days.

Detection of T-antigen: Tumor specific antiserum conjugated with fluorescein isothiocyanate was prepared as described by Murao (7). Transformed cells cultured on cover slips were stained with the conjugate by direct immunofluorescence technique.

RESULTS

Cytopathic changes in inoculated brain cultures: As described previously (17), five cell types were identified by form, staining quality and growth pattern. The proportion of these cell types varied with the age of the animal. A-1 and A-2 cells, EB cells, GB cells and ME cells probably corresponded to astrocytes, embryonic brain cells, glioblasts or oligodendroblasts and mesenchymal cells, respectively.

After inoculation of Ad12, all cell types became granular and rounded in shape, and their nuclei became plump (Fig. 1). These phenomena disappeared within about one week. Thereafter, no difference in growth pattern and morphology was noticed between inoculated and non-inoculated cultures until the transformed cell focus appeared.

Morphological transformation in inoculated brain cultures: Morphological transformation became apparent within 18 to 56 days in some inoculated cultures at each age group. The incidences are shown in Table 1. The brain

TABLE 1. HOST AGE AND FREQUENCY OF TRANSFORMATION OF HAMSTER BRAIN CELLS BY AD 12

Age	No. tubes with foci/no. tubes cultured		Rate of transformation (%)	Latency period (days)
	Inoculated	Control		
Fetus*	7/13	0/13	54	31-56
Newborn	11/20	0/10	55	18-48
3 day old	3/10	0/10	30	30, 35, 48
7 day old	2/10	0/10	20	28, 34
3 week old	2/13	0/13	15	33, 45
3 month old	1/10	0/10	10	27

* 15-16 gestational days

cell transformation incidence tended to decrease with increasing age of hamsters. There was no relationship between hamster age and the time of appearance of the transformed cell focus after virus inoculation. The transformed cells exhibited an enhanced growth rate forming a focus of thick multilayers

(Fig. 2). The focus was sharply defined and easily identified by low-power microscopy or by the naked eye. One transformed cell focus commonly appeared in one tube; however, two foci occasionally appeared in one tube at the same time. Transformed cell focus tended to appear near a colony of immature brain cells (EB or GB cells (17)) on top of the sheet of epithelial cells (A-1 cells (17)) (Fig. 3). The transformed cells in the focus stained with PTAH were composed of small round, polygonal or spindle-shaped cells with scanty cytoplasm and dark nucleus, and they resembled immature brain cells (EB or GB cells) (Figs. 4, 5). The brain cells of non-inoculated cultures gradually degenerated and decreased in number with morphological transformation not appearing in any tube.

Subculture of transformed brain cells: The transformed focus enlarged expansively. After observing this alteration for about two weeks, subcultures were carried out. Until the third or fifth subculture, non-transformed cells were mixed; thereafter, subcultured cells were composed of only transformed cells. Subcultures of inoculated cultures without transformed cell focus or non-inoculated cultures could not be carried out more than two or three times.

Subcultured transformed cells were relatively uniform and consisted of small polyhedral cells with a small number of spindle-shaped cells and giant cells. The nuclei were round or oval in shape and stained darkly. PTAH and Bodian's stain revealed no specific findings in transformed cells. These cells exhibited an epithelial morphology and often surrounded the spaces (Figs. 6, 7). These cells were sometimes arranged in rosette-like pattern (Fig. 8).

Tumors in hamsters after transplantation of transformed cells: Twenty-six cell lines obtained from brain cultures of various age hamsters were similar in transplantability. When dispersed cells from each cell line were transplanted subcutaneously to newborn hamsters, palpable tumors appeared at the site of the transplantation within 10 to 15 days and increased in size leading to death of the host within 20 to 40 days after transplantation. Macroscopically, these tumors were solid, soft in consistency and grayish-white often mixed with yellowish patches due to marked necrosis. The histological features of these tumors resembled brain tumors induced in newborn hamsters by intracranial inoculation of Ad12. Histological variations were present resembling human medulloblastoma (Fig. 9), ependymoblastoma (Fig. 10), spongioblastoma polare (Fig. 11), and anaplastic glioblastoma with similarity to glioblastoma multiform (Fig. 12). In most tumors these four types were mixed, although one type was usually dominant in any one tumor. There was no relationship between the histological type and hamster age from which the brain cells were derived.

Isolation of virus from transformed cells: The observation for 21 days failed to reveal a cytopathic effect by the virus in HeLa cells.

Detection of T-antigen in transformed cells: Transformed cell lines from each of the six age groups were examined at the fifth to seventh passage level. T-antigen was detected mainly in the cytoplasm as fluorescent rods and granules in all cell lines (Fig. 13).

DISCUSSION

In 1967 Yamane and Kusano (14, 15) reported that brain cells of fetal and newborn hamsters were transformed by Ad12 *in vitro*. In the present experiment, cultured brain cells from hamsters of various ages were also transformed by Ad12. It is clear that the transformation was elicited by Ad12: (a) Transformation was observed only in inoculated cultures; (b) transformed cells when transplanted to newborn hamsters produced tumors with histological features resembling brain tumors induced by Ad12; and (c) virus specific tumor antigen was detected in the transformed cells but the isolation of the virus from the transformed cells was negative as in other reports (14, 18).

As described previously in cultures of normal hamster brain (17), EB and GB cells (immature brain cells) grew mainly on top of sheets of A-1 cells with epithelial morphology. The transformed cell foci appeared near a colony of EB or GB cells and on top of the A-1 cells, and the transformed cells had a morphological similarity to EB or GB cells. The transformed cells in the subculture sometimes showed a rosette-like arrangement and surrounded the spaces, which appeared similar to the ventricular wall. These findings seem to suggest that the transformed cells have the characteristics of immature brain cells, because Miyake *et al.* (19) reported that the matrix cells cultured from human fetal brain showed rosette formation and tubular structure simulating a neural tube. Furthermore, the histological features of tumors developed by transplantation of transformed cells were very similar to those of tumors induced by intracranial injection of Ad12. In the investigation of the latter, the target cells of Ad12 in the central nervous system were considered to be the susbependymal immature cells from their histological features (5), the location of early tumor nodules (7) and from immunofluorescent studies (7). Therefore, the EB or GB cells may be target cells of Ad12 in the culture of hamster brain cells.

Yabe, Trentin and Taylor (20) have shown that tumor development decreases as the age of inoculated animal increases. This tendency may be due to the acquisition of a competent immune system as the animal aged or the decline of a sensitive population of cells through cell differentiation.

Although immune competence of the host might influence the occurrence of tumors, this factor was neglected by Casto (21) who used an *in vitro* experimental system not influenced by immune mechanisms. He examined the *in vitro* transformation of lung cells of various age hamsters by simian adenovirus 7 (SA7) and showed that lung cells from progressively older hamsters revealed a corresponding decline in sensitivity to transformation by SA7. In the present experiment, the incidence of transformation also tended to decrease with increasing hamster age in the same manner as Casto's results. The virus dose, virus inoculation procedure and the culture system were the same for cultures of each age group. Therefore, the decline in the transformation by Ad12 as the hamster aged probably depends upon a corresponding decrease of target cells in the culture. In the previous study (17), it was shown that EB and GB cells decreased in number with increasing age, and the decline in sensitivity to transformation by Ad12 almost corresponded to this decrease of EB and GB cells. These data further support the conclusion that the target cells of Ad12 in cultures of hamster brain cells may be the EB or GB cells.

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Legends to Figures

Fig. 1. The cytopathic changes after inoculation of virus. Most cells were round. Live. ×400

Fig. 2. A focus in the culture from 3-day old brain tissue showing multilayered growth in the center. Live. ×40

Fig. 3. A focus in the culture from newborn brain tissue. The focus originated from the colony of EB or GB cells on top of A-1 cells (arrow) and spread expansively to the left upper region. PTAH. ×40

Fig. 4. The transformed cells at the periphery of the same focus shown in Fig. 3. Transformed cells are located in the left region of the photograph. The EB or GB cells are located in the right and upper regions. It is difficult to determine whether cells having dark nuclei in the central area are the transformed cells or EB cells. PTAH. ×200

Fig. 5. The transformed cells from the same focus in Fig. 3. The transformed cells having dark nuclei are seen in the left-lower half of the figure. The larger cells in the right upper region are the A-1 or A-2 cells. PTAH. ×200

Fig. 6. The subcultured transformed cells are arranged surrounding the spaces. Live. ×40

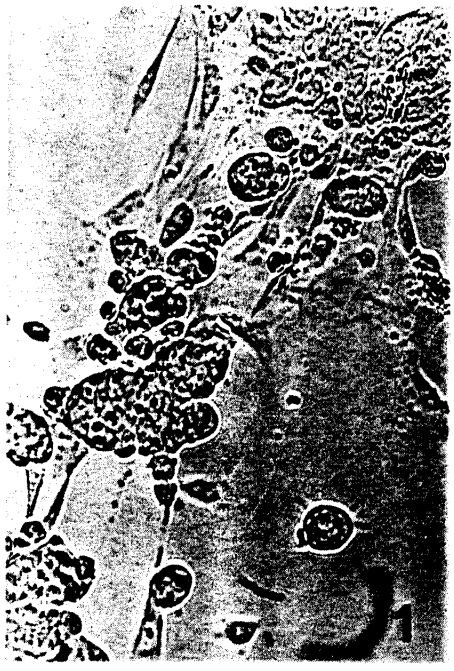
Fig. 7. The subcultured transformed cells are arranged surrounding a space and appear similar to the ventricular wall. PTAH. ×100

Fig. 8. The subcultured transformed cells show a rosette-like arrangement. PTAH. ×200

Fig. 9-12. Histological micrographs of tumors developed by the transplantation of transformed cells.

9. Medulloblastomatous picture (Type M). H-E. ×200
10. Ependymoblastomatous picture (Type E). H-E. ×200
11. Spongioblastomatous picture (Type G). H-E. ×200
12. Anaplastic glioblastomatous picture with some resemblance to glioblastoma multiform (Type A). H-E. ×200

Fig. 13. The fluorescent dots and flecks are seen mainly in the cytoplasm of the transformed cells. Stained with anti-T conjugate. ×200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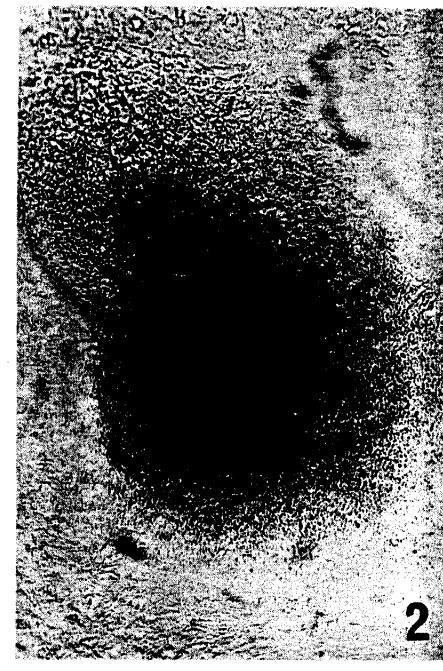
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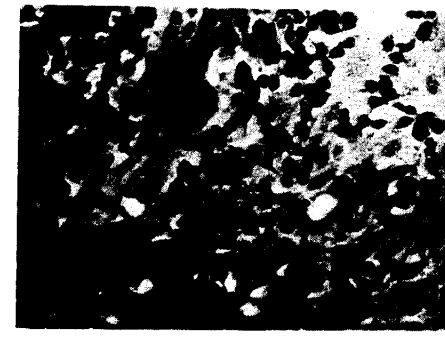

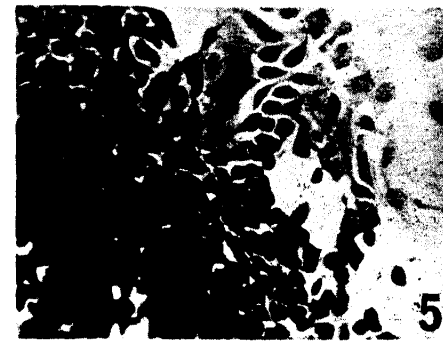
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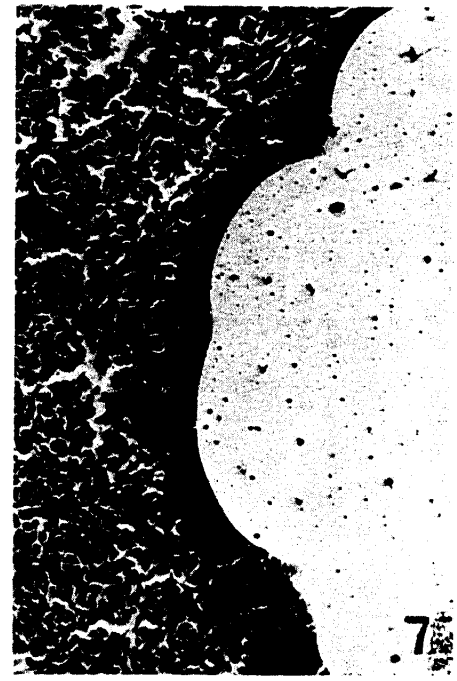
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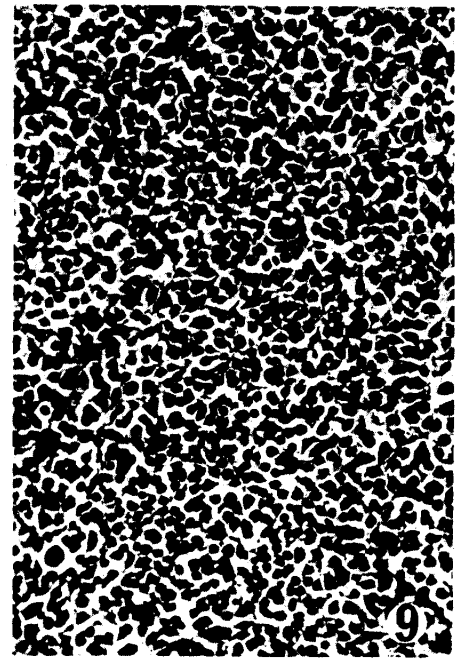
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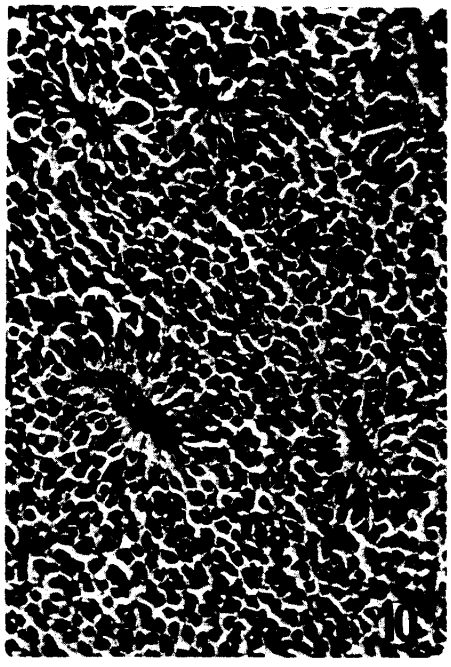

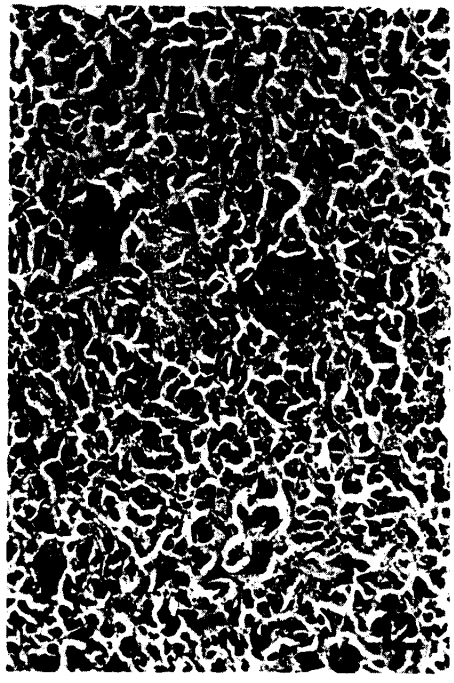


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