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In vitro studies on target cells of oncogenic adenoviruses in hamster brain. III. In vitro transformation of brain cells of hamsters at various ages by bovine adenovirus type 3*

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Abstract

In vitro transformation of brain cells of hamsters at various ages was examined after the addition of bovine adenovirus type 3 to determine the type and origin of the target cells. Cellular transformations occurred only in cultures of fetus and newborn animals and at low incidences. Nine cell lines were obtained. Virus specific tumor antigens were demonstrated in the transformed cells. The present investigation suggested that bovine adenovirus type 3 might transform mesenchymal cells (ME cell) and that these cells are probably of meningeal or vascular origin. The histological picture of tumors following transplantation of the transformed cells resembled human primary sarcoma of the meninges and brain.

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**IN VITRO STUDIES ON TARGET CELLS OF ONCOGENIC
ADENOVIRUSES IN HAMSTER BRAIN**
**III. IN VITRO TRANSFORMATION OF BRAIN CELLS OF
HAMSTERS AT VARIOUS AGES BY BOVINE
ADENOVIRUS TYPE 3**

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Abstract: *In vitro* transformation of brain cells of hamsters at various ages was examined after the addition of bovine adenovirus type 3 to determine the type and origin of the target cells. Cellular transformations occurred only in cultures of fetus and newborn animals and at low incidences. Nine cell lines were obtained. Virus specific tumor antigens were demonstrated in the transformed cells. The present investigation suggested that bovine adenovirus type 3 might transform mesenchymal cells (ME cell) and that these cells are probably of meningeal or vascular origin. The histological picture of tumors following transplantation of the transformed cells resembled human primary sarcoma of the meninges and brain.

Since Darbyshire (1) reported that bovine adenovirus type-3 (BAd3), WBR-1 strain, had high oncogenicity in newborn hamsters, the oncogenic properties of this virus have been investigated (2, 3, 4). When BAd3 was subcutaneously inoculated into hamsters of various ages, subcutaneous tumors developed at high incidences in every age group. However, differing opinions are present on the origin of this tumor. Berman (2) reported that the fast-growing bovine adenovirus tumor resembled characteristic primate adenovirus tumor but that the slow-growing tumor did not resemble primate adenovirus tumor. Levenbook and Strizhachenko (5) emphasized that the tumor bore evidence mainly of vascular origin and Motoi and Ogawa (4) reported that most subcutaneous tumors of the non-progressive type were of neurogenic origin and that few progressive tumors showed an angiomatous structure. Motoi and Ogawa (6) reported the low incidence of BAd3-induced brain tumors when this agent was intracranially inoculated into newborn hamsters. These tumors histologically resembled choroid plexus papilloma or papillary ependymoma and sarcomatous tumors. The *in vitro* transformation of skin-muscle cells of hamster embryo by BAd3 has been reported (7), however there has been no report concerning the *in vitro* transformation of hamster

brain cells by BAd3.

The author attempted to study the *in vitro* transformation of brain cells of hamsters at various ages by BAd3 and to determine the type and origin of the target cells.

MATERIALS AND METHODS

Tissue culture: The whole brains were prepared from 2/3 and near-term gestational fetuses, from newborns and from 3, 7 and 21-day old hamsters of randomly bred strains. Procedures for the preparation and maintenance of cultures were described in the previous report (8). The brain cells were cultured on cover slips in Leighton's tubes containing 2ml of nutrient medium which consisted of Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum. The medium was changed once every 3 to 4 days. The first subcultured cells were used for the experiment.

Virus: Bovine adenovirus type-3, WBR-1 strain, was supplied by the courtesy of Dr. Y. K. Inoue of the Institute for Virus Research, Kyoto University. This virus was propagated in the primary calf kidney cells which had been maintained in Eagle's MEM with 2% fetal calf serum. The culture was disrupted by freezing and thawing seven times and centrifuged at 1,000 rpm for 5 minutes. The supernatant was used for the experiment. The titer of the virus was tentatively determined with the same cells by 50% end point 7 days after infection. The virus titer used for experiment was $10^{4.5}$ and $10^{5.5}$ TCID₅₀/0.1 ml.

Procedure of transformation: In Experiment I, 0.2ml of the virus fluid was inoculated into the first subcultured brain cells suspended in 1.8ml serum-free MEM. The suspension of control cells was sham infected with MEM. After adsorption for 2 hours, the cells were centrifuged and resuspended in the medium, and 2ml of nutrient medium containing 10^5 cells/ml was placed into each tube. They were cultured in a stationary position at 37°C.

In Experiment II, 0.2ml of the virus fluid diluted in 1.8ml of MEM was inoculated into each tube with the first subculture in semimonolayer. The control cultures were sham infected with 0.2ml of MEM. After adsorption for 2 hours, the virus fluid was discarded and the cells were cultured in a stationary position at 37°C.

Stains: Cover slips with subcultured transformed cells were stained with hematoxylin and eosin (H-E), phosphotungstic acid hematoxylin (PTAH) and Bodian's nerve fiber stain. Tumors developed by the 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: To examine tumorigenicity, 10^6 cells from each transformed cell line (5-7th passage level) were injected into newborn hamsters subcutaneously or intracranially.

Isolation of virus from transformed cells: For this purpose, 10^6 transformed cells were inoculated into a monolayer culture of calf kidney cells which had been maintained in Eagle's MEM with 2% fetal calf serum. The observation

period was 21 days. The medium was changed once every 3 to 4 days.

Detection of T-antigen: Tumor specific antiserum conjugated with fluorescein isothiocyanate was prepared by the method of Nishibe, Nakamura and Inoue (9). Transformed cells cultured on cover slips were stained with the conjugate and observed by the direct immunofluorescence technique.

RESULTS

Cytopathic changes and morphological transformation in inoculated brain cultures: In inoculated cultures of Experiment I, all five cell types described previously (10) became oval-shaped, and a delay in adherence to the glass surface was observed compared to the results of the non-inoculated culture. In the inoculated cultures of Experiment II, all five cell types also became slightly oval-shaped and the growth of cells in the inoculated cultures was slightly inhibited. However, these phenomena in Experiments I and II disappeared within four to five days. Thereafter, no difference was noticed between inoculated and non-inoculated cultures until the transformed cell focus appeared in inoculated cultures. In some cultures of fetal and newborn hamsters, morphological transformation was observed within 37 to 76 days, as shown in Table 1.

TABLE 1. *In vitro* TRANSFORMATION OF HAMSTER BRAIN CELLS BY BAD-3

Experiment no.	Age	Virus titer (TCID ₅₀ /0.1 ml)	No. tubes with foci/ no. tubes cultured		Latency period (days)	Observation period (days)	Cell line designation
			Inoculated	Control			
I	Fetus*	10 ^{5.5}	1/4	0/4	50	100	FMHB-1
	Newborn	10 ^{5.5}	3/20	0/15	61, 66, 76	100	NHB-1, 2, 3
	3-day old	10 ^{5.5}	0/5	0/5		180	
	7-day old	10 ^{5.5}	0/10	0/10		180	
	7-day old	10 ^{4.5}	0/7	0/7		100	
II	Fetus*	10 ^{5.5}	2/14	0/14	45, 45	100	FMHB-2, 3
	Fetus**	10 ^{4.5}	2/13	0/10	37, 52	100	FLHB-1, 2
	Newborn	10 ^{5.5}	1/12	0/12	56	100	NHB-4
	3-day old	10 ^{4.5}	0/13	0/10		100	
	7-day old	10 ^{4.5}	0/7	0/7		180	
	21-day old	10 ^{4.5}	0/10	0/10		180	

Experiment I: Virus was inoculated in suspension.

Experiment II: Virus was inoculated in semimonolayer.

* 2/3 gestation

** Near-term gestation

No morphological transformation was observed in any inoculated cultures from 3, 7 and 21-day old hamsters and from the non-inoculated control cul-

tures. The transformed cells were composed of small, round and relatively large spindle-shaped cells, and these cells formed a focus of thick multilayers (Fig. 1). The small round cells were mainly found in the central multilayers of the focus and the spindle-shaped cells were found in the peripheral areas (Fig. 2). The transformed cell focus tended to appear in degenerated areas. One transformed cell focus appeared in one tube.

Subculture of transformed brain cells: The transformed cells in the focus spread diffusely on the non-transformed cells. After observing this alteration for about two weeks, subcultures of these cells were carried out. Nine transformed cell lines were obtained. The subcultured transformed cells of eight lines were variable in shape and size and consisted of large polygonal cells with multipolar processes, spindle-shaped cells, relatively small round cells and some giant cells (Figs. 3, 4). The subcultured transformed cells of one cell line (FLHB-1) obtained from the culture of near-term fetus were rather uniform and were composed mainly of large spindle-shaped cells which formed bundles (Fig. 5). Most transformed cells had pale nuclei with two or three distinct nucleoli. None of the transformed cell lines showed an epithelial morphology. PTAH and Bodian's stain revealed no specific findings in transformed cell.

Tumors in hamsters after transplantation of transformed cells: The nine transformed cell lines were similar in transplantability. When 10^6 transformed cells from each cell line were injected into newborn hamsters intracranially or subcutaneously, tumors developed in all cases at the transplantation site. Within 8 to 14 days after transplantation, the animals manifested neurological symptoms and developed subcutaneous tumors. Macroscopically these tumors were solid and soft in consistency and revealed manifold colors from hemorrhage and necrosis. The histological features of these tumors resembled those of brain tumors induced by intracranial inoculation of BA₂3 into newborn hamsters. The tumor cells were spindle, ovoid or polygonal in shape and had a light nucleus with two to three distinct nucleoli. Some multinucleated giant cells and huge round cells were observed. The spindle-shaped cells often formed interlacing bundles (Fig. 6) and sometimes showed a whorl-like arrangement (Fig. 7). The ovoid or polygonal cells were arranged in a sheet-like pattern (Fig. 8) and showed a syncytial character (Fig. 9). These findings were commonly found concurrently in one tumor. There was proliferation of capillaries without hyperplasia of endothelial cells. PTAH and Bodian's stain revealed no specific findings in tumor cells. Pap's silver impregnation revealed a large number of reticulin fibers in one area of the tumor (Fig. 10), but there were some regions with scanty reticulin fibers (Fig. 11). Mallory's azan method revealed no collagen fibers.

Isolation of virus from transformed cells: Isolation of the virus from the transformed cells was negative during the 21 day period.

Detection of T-antigen in transformed cells: Four transformed cell lines were examined at the 15th passage level. T-antigens were detected in all cell lines as fluorescent granular spots or flecks in both the nucleus and cytoplasm or in the nucleus alone (Fig. 12).

DISCUSSION

Transformation by BAd3 occurred only in cultures from fetus and newborn, and not from older animals. This result differed from the *in vitro* transformation of Ad12 (8).

In vitro transformation by oncogenic viruses is influenced by the following factors: the virus dose, the virus inoculation procedure, the condition of culture and the existence of target cells in culture. The two virus titers ($10^{1.5}$ or $10^{2.5}$ TCID₅₀/0.1 ml) and the two virus inoculation procedures (in suspension or in semimonolayer) showed approximately the same effect upon transformation. The culture conditions throughout the present study were constant. The results indicate that the presence of target cells appears to be a critical factor in transformations of hamster brain cells by BAd3.

The author attempted to determine the type and origin of target cells of BAd3 in the culture system. The transformed cells of the focus consisted of relatively large spindle-shaped and small round cells. As the small round cells were mainly found in the central part of the focus, their shapes might be due to the multilayered growth. Therefore, spindle-shaped cells may be the essential form of the transformed cells. The transformed cells have a similarity to ME cells (10) which are considered to be mesenchymal cells of vascular or meningeal origin. Of nine subcultured cell lines, one cell line (FLHB-1) consisted of uniform spindle-shaped cells that resembled ME cells and transformed cells of the transformed focus, and the other eight cell lines were variable in shape and size. In spite of these differences, the transformed cells from all cell lines produced tumors having almost the same histological features. This indicates the possibility that the transformed cells of all cell lines were of the same origin. Furthermore, the transformed cells from all cell lines showed fibroblastic morphology, and PTAH and Bodian's stain revealed no specific structures. These points suggest that BAd3 may transform ME cells. However, ME cells exist in a higher frequency in cultures of older age animals than in fetus and newborn cultures, although transformation occurred in cultures of only the fetus and newborn. This contradiction may be explained by ME cells of older age animals being too mature to be transformed by this virus.

There has been a report (6) concerning BAd3-induced brain tumors. According to this report, one tumor developed in the lateral ventricle and had histological features of choroid plexus papilloma or papillary ependymoma, and the other tumor had sarcomatous features. However, the incidence of intracranial tumor induction by BAd3 is so low that the histogenesis has not been confirmed.

The histological features of the transplanted tumors showing sarcomatous features resembled BAd3-induced brain tumors. The tumors were very anaplastic and consisted of spindle-shaped cells forming interlacing bundles and sometimes whorl-like arrangements, and ovoid or polygonal cells were arranged in a sheet-like pattern with a syncytial character. From these findings these tumors may be of meningeal origin, and they resembled human primary sarcoma of the meninges and brain which occurs mostly in infants and children (11, 12). These histological features of the transplanted tumors support the suggestion of the *in vitro* study that BAd3 may transform ME cells.

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Legends to Figures

- Fig. 1. The transformed cell focus in culture from a newborn. Live. $\times 100$.
- Fig. 2. Higher magnification of Fig. 1. The small round cells are found mainly in the central part of the focus and the spindle-shaped cells are found in the peripheral part of the focus. Live. $\times 300$.
- Fig. 3. The subcultured transformed cells from NHB-1. The cells are variable in shape and size. PTAH. $\times 100$.
- Fig. 4. The subcultured transformed cells from FMHB-1. PTAH. $\times 200$.
- Fig. 5. The subcultured transformed cells from FLHB-1. The cells consist of uniform spindle-shaped cells. PTAH. $\times 200$.
- Fig. 6. A photomicrograph of the tumor following transplantation of FLHB-2. Spindle-shaped cells are interlaced. A huge round cell is seen (in lower part). H-E. $\times 200$.
- Fig. 7. A photomicrograph of the tumor following transplantation of NHB-4. The tumor cells are arranged in whorl-like pattern. H-E. $\times 200$.
- Fig. 8. A photomicrograph of the tumor following transplantation of NHB-1. The tumor cells consist of ovoid or polygonal cells having pale nuclei with distinct nucleoli and are arranged in a sheet-like pattern. PTAH. $\times 200$.
- Fig. 9. A photomicrograph of the same tumor in Fig. 7. The tumor cells show syncytial character. H-E. $\times 200$.
- Fig. 10. Numerous reticulin fibers are seen. Pap's silver impregnation for reticulin. $\times 100$.
- Fig. 11. A section of scanty reticulin fibers is seen of the same tumor as Fig. 10. Pap's silver impregnation for reticulin. $\times 100$.
- Fig. 12. T-antigen in the transformed cells in both the nucleus and cytoplasm. $\times 200$.





