KOREAN JOURNAL OF ZOOLOGY
Vol. 17, No. 2, 51~56 (1974)

Spontaneous Morphological Transformation in Adenovirus Type 12 Induced Tumor Cells of Armenian and Chinese Hamsters

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Adenovirus Type 12로 誘發한 Armenian Hamster 및 Chinese Hamster의 腫瘍細胞에서 보이는 形質轉換

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摘 要

生後 24시간 이내의 어린 Armenian hamster와 Chinese hamster의 體內에 adenovirus type 12를 注入할 경우, 전자에서는 27일, 후자에서는 30~45일이 지난후 腫瘍모양을 한 작은 細胞塊가 생김을 보았다. 이들 細胞塊는 다른 個體에 移植이 가능하며, 上皮細胞와 같은 모양을 하고 있다. 本實驗에서 얻어진 57개의 細胞塊 중에서 6개만이 細胞培養 되었으며 14~20회정도 繼代培養을 계속하는 동안에 染色體의 변동은 없이 細胞의 모양이 纖維狀으로 변하였다. 이들 細胞가 上皮細胞 모양을 할 때는 腫瘍性이며, T-抗體도 발견되었는데, 纖維狀으로 변하게 되면 腫瘍性을 나타내지 않으며, T-抗體도 찾아 볼 수 없다. 다른 2개의 細胞塊를 上皮細胞 모양일 때 SV40 바이러스로 처리하면 바로 纖維狀細胞로 변한다. 이들 細胞에는 adenovirus에 의한 T-抗體는 없으나, SV40의 T-抗體가 존재하며, 다른 hamster에 移植할 경우 皮腫 모양의 細胞塊를 이룬다.

INTRODUCTION

Since the discovery of tumor induction in hamsters by DNA-containing adenovirus type 12 (Trentin et al., 1962), many investigators have been concentrating their efforts on the study of highly oncogenic adenovirus type 12 (Strohl et al., 1967).

There is no evidence that the adenovirus type 12 completes an infectious cycle in hamster cells (Smith and Melnick, 1964 ; Yohn and Stich, 1967) ; thus the development of tumor is attributable directly to the inoculated virus. This tumor virus/host relationship in the hamster differs from certain other DNA oncogenic viruses,

such as polyoma and SV40, which are known to replicate in hamster cells (Stanton and Otsuka, 1963 ; Girardi and Hilleman, 1964).

It was ascertained that, although the viruses themselves cannot be formed in the hamster cells, they leave antigens. Trentin, Yabe and Taylor (1963) reported that some animals with primary and transplant tumors develop neutralizing antibodies.

Recently, the Chinese hamster, *Cricetulus griseus* (2n=22) and Armenian hamster, *Cricetulus migratorius* (2n=22) have been employed principally in experimental cytogenetics and cell biology in cancer research (Yerganian et al., 1966). The physiological traits of the Armenian hamster are more like those of the Syrian hamster, *Mesocricetus auratus* (2n=44), which has a high susceptibility to oncogenic agents and a greater tolerance to both homologous and heterologous transplantable tumors, while its cytological chracteristics resemble those of the Chinese hamster.

In the present study, epithelial derivatives of tumors induced by adenovirus type 12 exhibit several morphological features which alter dramatically during the course of *in vivo* and *in vitro* continuous propagation both in Armenian and Chinese hamster cells.

MATERIALS AND METHODS

Both Chinese and Armenian newborn hamsters were inoculated with human adenovirus type 12, prototype strain Huie, within 24 hours after birth by the intraperitoneal and subcutaneous routes. The tumors were continuously propagated in vivo by implanting into cheek pouch of animals.

The tumor tissue was removed, minced into very small fragments and trypsinized for 1 hour with a magnetic stirrer. The suspension was centrifuged at 3,000 rpm for 5 minutes, and the pellet resuspended in Eagle's minimum essential medium (MEM) without calcium, supplemented with 5% dialyzed calf serum and 10 % fetal calf serum. The cell suspension was inoculated in culture bottles and incubated at 37°C in CO_2 incubator. The 4A12 and 6A12 of sublines were exposed to SV40 virus. For the chromosome study, approximately 5×10^4 cells were seeded on cover slips in cytology tubes and grown for 60 hours and added 0.2ml of 2.5×10^{-6} M of colchicine. The tubed culture was fixed with a solution of 3 parts of 95% alcohol and one part of glacial acetic acid, after 5 minutes of hypotonic treatment. Slides were stained with propiono-carmin and made by squashing technique, and then examined under a phase microscope. The morphology of the cells were obtained from cover slips in cytology tubes grown for one week. The cover slips were fixed without colchicine treatment and stained with methylene blue. Spontaneous revertant cell type and SV40 transformed cells were implanted into cheek pouch at level of

$4\sim6\times10^6$ cells. For cloning efficiencies, diluted cell suspenisions were seeded in Petri dishes and grown for 7 days (500 to 1000 cells per Petri dishes).

For the histology, the tumor was fixed in neutral formalin. The tissue were blocked, sectioned, and stained with hematoxylin and eosin by standard methods.

Immunofluorescence was conducted by indirect technique. Fixed monolayers were washed briefly with phosphate buffered saline, drained, covered with unlabeled serum and incubated, resting on a paper clip, in a Petri dish containing a wad of wet cotton, at 36°C for 40 minutes. After a 5 to 10 minutes wash, with three changes of cold phosphate buffered saline, they were drained and incubated a second time, covered with a drop or two of fluorescein labeled goat-anti-hamster globulin at 36°C for 20 minutes. After a second 5 to 10 minutes wash, they were drained, mounted in 9 : 1 glycerine-saline (pH=8.5) and rimmed with fingernail polish to prevent drying. Slides were examined under the Zeiss fluorescence microscope.

RESULTS AND DISCUSSION

In the previously "resistant" Chinese hamsters, adenovirus type 12 induced tumors appeared within 27 days and in the Armenian hamsters appeared within 30~45 days after inoculating newborns with high-titre virus suspensions. Selection of the Armenian hamster was primarily due to the resistance of newborn Chinese hamster to oncogenic viruses. Unless high titres of virus stock were inoculated within 6 hours after birth, the incidence of tumor induction was drastically reduced.

With 57 tumors (both in Armenian and Chinese hamsters) induced by adenovirus type 12, it was unable to successfully propagate these cells in vitro. The difficulty resides in the need to eliminate or reduce the level of calcium below 0.05 mM/ml of medium in order to establish culture prior to gradually increasing the calcium level by the 3rd passage of subcultivation. Subsequently, each passage must be made cyclically, initially with minimal calcium level and then with the level of calcium increasing progressively each day of passage. This result agreed with Freeman et al. (1965) that they reported the adenovirus type 12-induced tumor cultures are affected by calcium levels. As a result, 6 cell lines have been successfully maintained (5 from Armenian hamster, 1A12, 2A12, 3A12, 4A12, 6A12 and one from Chinese hamster, 8A12). However, these surviving cell lines have reverted to fibroblast-like phenotypes which contrast sharply with the epithelial-like appearance of the parent tumor cells which reflect the presence of virus genome.

Tumors 1A12, 2A12 and 3A12 proliferated wholly as epithelial-like cells both in vivo and in vitro for 14~20 passages. Samplings of tumors 1A12 and 2A12 taken around the 8th passage *in vivo* transfer generation exhibited, for the first time, a mixture of both epithelial and fibroblast-like cells (Fig. 1). Within several passages,

all cells transformed to the fibroblastic phenotype. In contrast, fibroblastic cells appeared in tumor 3A12 much later (20th passage) without replacing the epitheloids.

While reculturing tissues of the 3-4th in vivo transplantation of tumors 4A12 and 6A12, and 6A12, fibroblast-like derivatives suddenly appeared and persisted for 2 additional passages along with the parental epithelial-like cells. A Chinese hamster tumor (8A12) also reverted spontaneously to the fibroblast-like state.

The sublines of 4A12 and 6A12 were exposed to SV40 virus while still epithelial-like, also reverted to the fibroblast-like morphology, starting within several hours after exposure. The transition to fibroblastic elements was virtually complete after two subcultivation

The fluorescent studies for T-antigen were negative in spontaneous revertants (positive in epithelial-like parent cells). The positive fluorescence for SV40 T-antigen was noted in the SV40-treated cells, but negative for adenovirus T-antigen. T-antigen may be a precursor of viral components or may be an enzyme necessary for the replication of virus in cells. The infection process in hamster cells may proceed to the phase of T-antigen formation; then the process may turn from the different direction which would finally lead to the transformation of cells.

Spontaneous revertant cell types, implanted into cheek pouches, failed repeatedly to form tumors (i.e., lost neoplastic properties). In contrast, similar implants of SV40-induced revertants resulted in tumor formation (sarcoma).

Cloning efficiency is twice that of normal diploid fibroblasts, but equals to that of SV40 transformed cells (Table 1).

In the Armenian hamster, being epithelial in orgin, the distinctive heteromorphism between the two X-chromosomes in female is exaggerated (the X-chromosomes are near isomorphic in fibroblast-like derivatives). In addition, the secondary cons-

Table 1. Phenotypic comparison of normal fibroblast-like, adenovirus type 12-induced tumor derivatives, and spontaneous revertant (fibroblast-like) cells

Features	Diploid cell type		
		Adenovirus type 12-induced tumors	
	Normal fibroblast-like	Epithelial-like	Spontaneous revertant (fibroblast-like)
Cloning efficiency(%)*	10~20	0	20~40
Neoplastic tendency	−	+	−
T antigen	−	+	−
Prominence of secondary constriction (X chromosomes)	+	+	+

* Except for Armenian hamsters.

triction on the short arms of sex elements appears as a very prominent gap in about 50% of the tumors (Fig. 2). This was absent among the fibroblast-like transformation.

Spontaneously-formed, non-oncogenic fibroblast-like revertants are considered to reflect either the release of the viral genome or repression of viral incorporation sites at a critical period of in vitro cultivations.

These results suggest that there are structural parameters that may correlate well with functional aspects in particular cell types.

SUMMARY

Newborn Armenian and Chinese hamsters inoculated with adenovirus type 12 developed undifferentiated small cell tumors as early as 27 days after inoculation in the Chinese hamsters and within 30~45 days in the Armenian hamsters. These tumors were transplantable and epithelial-like cell in morphology. Cultures of 6 tumors underwent spontaneous reversion to fibroblast-like morphology during the 14~20 in vitro passages in the absence of chromosomal disturbances. While epithelial-like tumor derivatives were oncogenic and positive for the T-antigen, fibroblast-like revertants were non-oncogenic and negative for the T-antigen. Two other tumor derivatives reverted to fibroblast-like forms, immediately following exposure to SV40. These lacked the adenovirus T-antigen but were positive for the SV40 T-antigen and formed sarcomas in animals.

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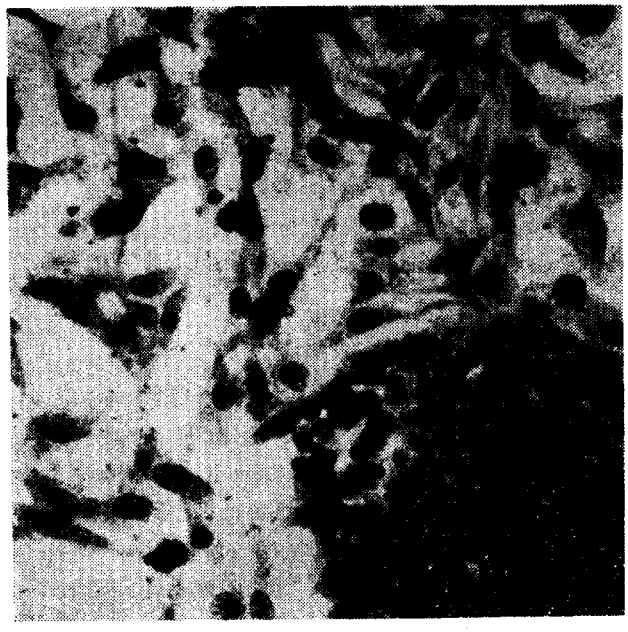

Fig. 1. Spontaneous reversion to fibroblast-like forms during 5-9 transfer generations of tumors 1A12 and 2A12.

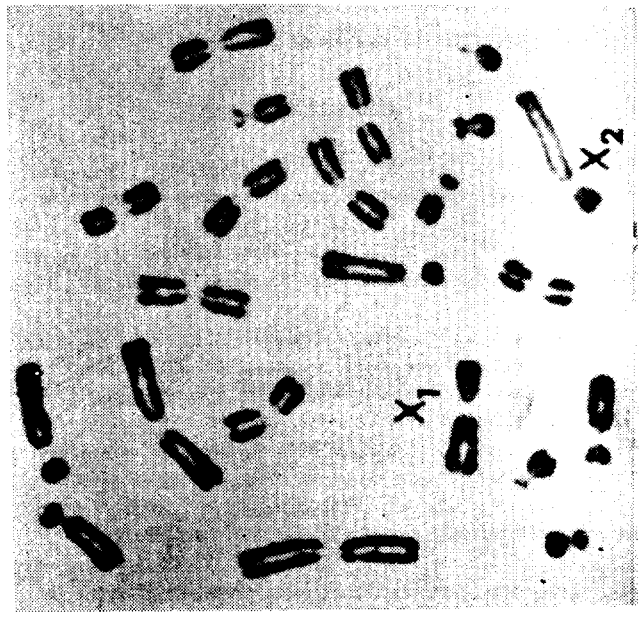

Fig. 2. Diploid (2n=22) chromosome complement of tumor 1A12 (Armenian hamster).