

Growth of Human Tumor Xenografts on Chorioallantoic Membrane of Chick Embryo

Kikuo Okamura, Yoshikazu Tsuji, Toshio Shimoji
and Koji Miyake

From the Department of Urology, Nagoya University School of Medicine

We evaluated the success rates of transplantation and growth conditions of 33 human tumor xenografts transplanted on the chorioallantoic membrane of chick embryos. Eleven out of 14 renal cell carcinomas (78.6%), 7 out of 9 urothelial carcinomas (77.8%), 2 out of 3 testicular tumors (66.7%), an adenocarcinoma of the colon and an adenocarcinoma of the ovary but none of the 5 prostatic carcinomas were transplanted successfully. Histologically, carcinoma cells survived on the chorioallantoic membrane forming organoids the structure of which was well preserved. Several regions of the grafts frequently became necrotized. However, the viability of carcinoma nests was not influenced by the inflammatory changes surrounding necrotic tissues. Renal cell carcinomas survived diffusely with prominent angiogenesis. Immunostaining with anti-bromodeoxyuridine (BrdU) and Ki-67 monoclonal antibodies demonstrated a strong correlation between the %BrdU labeling index of the cancer cells on the chorioallantoic membrane and %Ki-67 index of the original tumors. No difference in %BrdU indexes was found between small and large cancer nests. Growth conditions remained constant for 8 days after inoculation. The growth potential of cancer nests surviving on the chorioallantoic membrane, which was identical to that of the original carcinomas, appeared to be unchanged during incubation. However, it might be difficult to exploit the chorioallantoic membrane for anticancer chemosensitivity tests except for renal cell carcinoma since few cancer cell nests were produced in spite of the high transplantation success rates.

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INTRODUCTION

An ideal anticancer chemosensitivity test should be designed for each patient, since human tumors have different growth potentials and drug sensitivities. Several *in vitro* techniques for drug sensitivity screening¹⁻³⁾ have been employed. However, *in vivo* techniques have the following advantages: 1) The procedure of making single cell suspensions, which leads to significant loss of viability, is not required. 2) The net response of solid tumors composed of heterogeneous cell populations can be evaluated. 3) A masked compound such as cyclophosphamide can be used.

In 1987, Uchida and associates⁴⁾ presented an antitumor chemosensitivity test using the chorioallantoic membrane of chick embryo (CAM). Their method by which the results are quickly obtained is

simple, inexpensive and practicable in any laboratory. They evaluated the effects of anticancer agents on experimental murine tumors based on the weight of the CAM implants. However, the growth rate of primary human tumor xenografts is anticipated to be different from that of experimental models. In fact, histological examination of such xenografts in a conventional mice subrenal capsular assay showed low viability of tumor cells and intense lymphocytic infiltration⁵⁻⁷⁾ The quality of the primary culture of human tumors on the CAM, such as take rates and viability should be studied histologically.

In the present report, the growth conditions of human tumor xenografts on the CAM were investigated to examine whether the CAM satisfied the prerequisites for individual anticancer chemosensitivity tests.

MATERIALS AND METHODS

Tumor tissues

Fresh surgical tissue specimens were obtained from 14 renal cell carcinomas, 9 urothelial carcinomas, 3 testicular tumors (2 seminomas and 1 embryonal carcinoma), 5 prostatic carcinomas, 1 ovarian adenocarcinoma metastasizing to the adrenal gland and 1 adenocarcinoma of the sigmoid colon invading the bladder. These specimens were taken from the surgical room to the laboratory in cold Eagle's modified minimum essential medium supplemented with 10% newborn calf serum and 30 $\mu\text{g}/\text{ml}$ of gentamicin. One portion of the specimen was snap-frozen and stored at -80°C for immunostaining with monoclonal antibody Ki-67 (DAKO) in order to determine the growth fraction of the original tumor. The carcinoma tissues were freed of stroma and any necrotic tissue and then minced finely to less than 0.5 mm^3 with two scalpels.

Inoculation of tumor tissues onto CAM

Embryonated chicken eggs were kept in an incubator at 37°C for ten to eleven days. The eggs were candled, and the Y-shaped junction of blood vessels in the chorio-allantoic membrane was pencil-marked on the shell. The shells were wiped with 70% ethanol and a 1.0×1.0 cm window was cut around the mark. The shell membranes were pricked and the chorioallantoic membranes were depressed by applying suction to the air sac. The shell membranes were removed with forceps, and 30~40 μl of minced tissue was pipetted onto the Y-shaped vascular junction before the window was sealed with cellophane tape. A portion of each minced sample was fixed with 10% neutral buffered formalin to assess of the quality of the transplanted tumor sample. The eggs were again incubated at 37°C . Bromodeoxyuridine (BrdU) incorporation

Embryos were killed 8 days after inoculation with tumor tissue. The embryos inoculated with 3 selected tumors, but, were killed serially between day 1 and day 8 to examine whether BrdU labeling was constant. One and a half to 2 hours be-

fore death, 1,000 $\mu\text{g}/0.1$ ml of BrdU (Sigma) was injected into the yolk sac. The tumors were excised from the chorio-allantoic membrane and freed of adhering chick tissues. The specimens were fixed with 10% neutral buffered formalin and embedded in paraffin.

Immunostain techniques and determination of BrdU labeling index and Ki-67 index

The 3.5 μm paraffin sections from three different parts of the implants, which had been cut at intervals of 100 to 200 μm , and 5 μm cryostat sections of the original lesions were prepared for hematoxylin-eosin staining and immunostaining. Immunostaining was performed by the avidin-biotin-complex method with an anti-BrdU monoclonal antibody (DAKO) for the paraffin and Ki-67 (DAKO) for the cryostat sections. Paraffin sections were immersed in 0.3% H_2O_2 methanol for 20 minutes, denatured for 20 minutes in 4-normal hydrochloric acid, and immersed in 0.1 mol. $\text{Na}_2\text{B}_4\text{O}_7$ for 3 minutes, while cryostat sections were immersed in acetone for 10 minutes and air-dried. The sections were blocked with normal horse serum, and then the cryostat sections and the paraffin sections were incubated at room temperature for 30 minutes with 1: 6 Ki-67 and 1: 40 anti-BrdU monoclonal antibodies, respectively. All sections were then treated with biotinylated horse anti-mouse immunoglobulin and finally with diaminobenzidinehydrogen peroxide substrate and counterstained with hematoxylin.

Using the grid technique in several high power fields ($\times 400$), %BrdU labeling index or %Ki-67 index was calculated by the following formula:

$$\frac{\text{labeled cancer cells/}}{(\text{labeled} + \text{unlabeled cells})}$$

Success transplantation, take rates and growth conditions of xenografts

By observing cancer nests, inflammatory changes and necrosis of the implants on CAM histologically, we evaluated the success of transplantation and take rates which were defined as the number of cancer cell nests divided by the number of chicken

Table 1. Characteristics of human tumor xenografts on the chick embryo chorioallantoic membrane.

Carcinoma No.	Pathology of original tumor	Take rates (%)	S-phase fraction (%)
1001	clear cell ca. G2	12/12 (100)	not evaluated
1002	clear cell ca. G2	8/ 9 (89)	not evaluated
1003	clear cell ca. G2	2/12 (17)	not evaluated
1004	granular cell ca. G2	2/12 (17)	not evaluated
1005	clear cell ca. G2	5/ 7 (71)	4.0± 1.4
1006	clear cell ca. G2	7/ 9 (78)	3.0± 1.0
1007	clear cell ca. G2	6/ 7 (86)	2.2± 0.4
1008	granular cell ca. G2	8/ 8 (100)	2.1± 1.4
1009	clear cell ca. G3	0/ 5 (0)	/
1010	clear cell ca. G2	6/ 6 (100)	1.3± 0.4
1011	clear cell ca. G2	6/ 8 (75)	2.7± 1.2
1012	clear cell ca. G1	6/10 (60)	2.8± 2.7
1013	clear cell ca. G1	10/10 (100)	1.2± 0.7
1014	clear cell ca. G3	10/10 (100)	3.9± 1.4
2002	TCC, G2, pTa*	0/10 (0)	/
2003	TCC, G3, pT4	12/ 7 (157)	22.9± 7.8
2004	SCC, G3, pT3b**	10/10 (100)	11.3± 4.4
2005	TCC, G3, pT3b	7/11 (64)	9.1± 3.0
2006	TCC, G2, pTa	6/ 8 (75)	2.7± 0.2
3001	TCC, G2, pT1	6/ 8 (75)	not evaluated
3002	TCC, G3, pT3a	15/11 (136)	14.0± 5.9
3003	TCC, G2, pTa	22/10 (220)	0.8± 0.7
3004	TCC, G2, pTa	0/11 (0)	/
4001	poorly diff. adenoca.	0/10 (0)	/
4002	poorly diff. adenoca.	0/ 9 (0)	/
4003	mod. diff. adenoca.	0/ 8 (0)	/
4004	poorly diff. adenoca.	0/10 (0)	/
4005	mod. diff. adenoca.	0/ 5 (0)	/
5001	seminoma	0/ 9 (0)	/
5002	seminoma	2/ 6 (33)	28.1± 8.8
5003	embryonal ca.	20/ 7 (286)	32.5±10.2
6001	mod. diff. adenoca.	6/ 5 (120)	14.3± 5.9
6002	anaplastic adenoca.	8/ 7 (113)	21.8± 9.1

* TCC : Transitional cell carcinoma

** SCC : Squamous cell carcinoma

embryos.

%BrdU labeling indexes of cancer cells in xenografts were compared with the % Ki-67 indexes of the original tumors. We compared the labeling indexes of small (30 to less than 100 cancer cells) nests and large (equal to or more than 100 cells) nests in 6 tumors to examine whether the nest size influenced the %BrdU index of the xenografts. We also determined the %BrdU indexes of 3 transplants between day 1 and 8 after inoculation.

Statistics

All values were expressed as means±SD. Differences were analyzed by Wilcoxon's

rank sum test, and the correlation between 2 sets of variables were determined by the linear regression model. Differences with P values of less than 0.05 were considered significant.

RESULTS

Success of transplantation and Take rates

Transplantation was successful in 11 of 14 renal cell carcinomas (78.6%), 7 of 9 urothelial carcinomas (77.8%), 2 of 3 testicular carcinomas (66.7%), the sole colon carcinoma and the sole ovarian carcinoma, but none of the 5 prostatic carcinomas

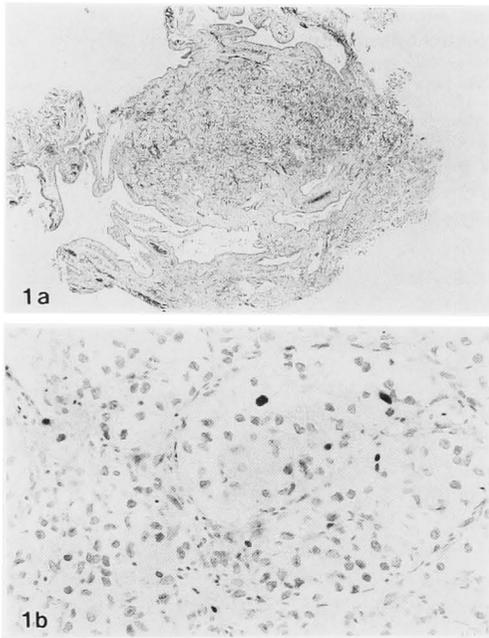


Fig. 1. Xenograft of renal cell carcinoma (No. 1013). A: Cancer cells survived diffusely, and angiogenesis was prominent. Staining with hematoxylin-eosin, $\times 40$. B: A very small number of cancer cells were labeled by BrdU. Immunostaining, $\times 400$.

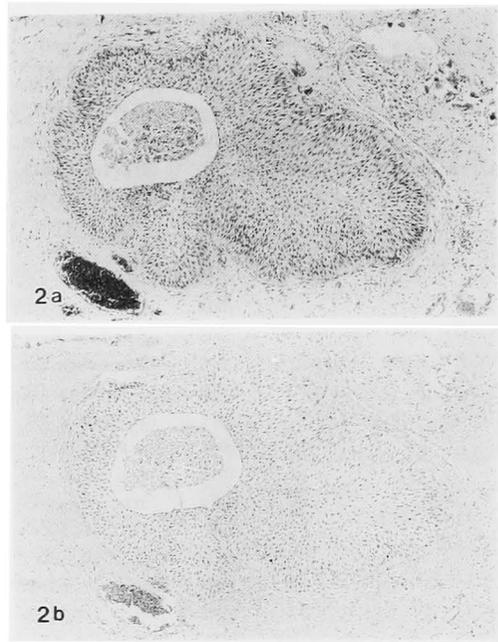


Fig. 2. Xenograft of transitional cell carcinoma G2 (No. 3003). A: Cancer cells formed a large organoid. Staining with hematoxylin-eosin, $\times 100$. B: Low S-phase fraction reflected the low malignant potential of the original tumor. Immunostaining, $\times 100$.

(Table 1). No. 1003 and No. 1004 were not considered as successful transplantations because of paucity of viable carcinoma cells. The overall success rate was 66.7%. Renal cell carcinomas survived diffusely on CAM, while only part of the minced tissue from the other carcinomas survived on CAM. The mean take rates of these urothelial carcinomas, testicular carcinomas and adenocarcinomas of the colon and ovary were $85 \pm 65\%$ ranging from 0% to 286%.

Histology of the xenografts

Figures 1a to 4a show the results of hematoxylin-eosin staining of the xenografts. Renal cell carcinomas, some with and some without central necrosis, had cancer cells spread all around the transplanted area. In the other carcinomas, cancer cells formed organoids of various sizes, which were scattered throughout the xenografts. The malignant characteristics of the cancer nests, expressed by cellular and structural atypia, corresponded to those of the origi-

nal tumors. Implanted minced tissue falling into necrosis was observed more frequently. Edema, inflammatory cell infiltration, foreign body giant cell reaction and fibrotic reaction were found in most specimens, but they did not obstruct the tumoral structure of the live cancer nests. Inflammatory change occurred surrounding the necrosis. Neovascularization was identified in most cases and was a prominent feature of renal cell carcinomas.

Growth conditions of xenografts

BrdU labeled cells were clearly identified by immunostaining (Fig. 1b to 4b). Fig. 5 shows that the Ki-67 labeling index correlated well with the BrdU labeling index according to the formula: $\% \text{Ki-67 index} = 1.69 \times \% \text{BrdU index} - 0.99$ ($r = 0.89$, $p < 0.05$). Small and large nests proved to have identical mean $\% \text{BrdU}$ labeling indexes (Table 2), although nests with less than 30 cells provided unreliable data. No change in $\% \text{BrdU}$ labeling index with

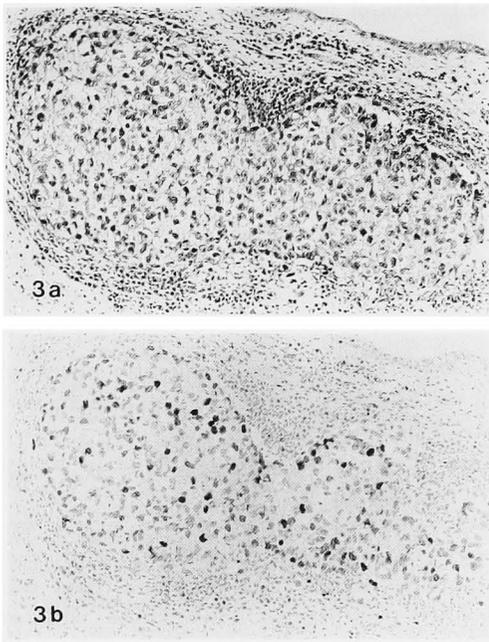


Fig. 3. Xenograft of embryonal carcinoma of testis (No. 5003). A: Cancer nest composed of cells with prominent nucleoli. Hematoxylin-eosin, $\times 200$. B: A great number of labeled cells were demonstrated with moderate angiogenesis. Immunostaining, $\times 200$.

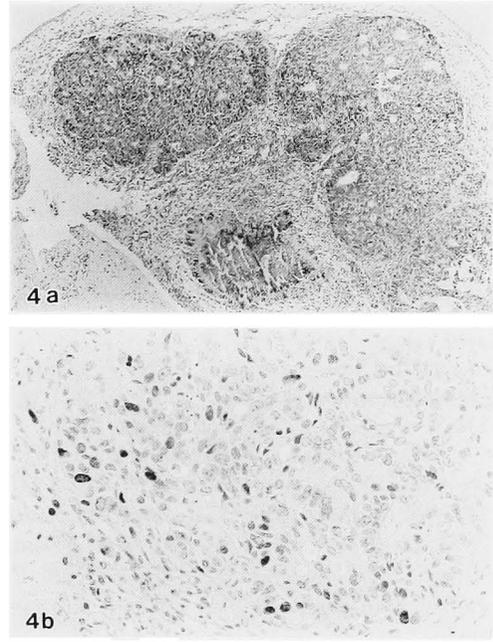


Fig. 4. Xenograft of adenocarcinoma of the colon (No. 6001). A: Adenocarcinoma cells similar to the primary tumor formed large organoids. Inflammatory reactions occurred around rejected inoculum. Hematoxylin-eosin, $\times 100$. B: A moderate number of labeled cells was found in the outer layer of the organoid. Immunostaining, $\times 200$.

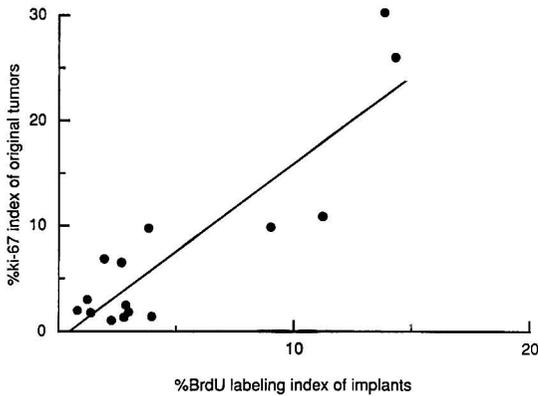


Fig. 5. The relationship between % Ki-67 index of the original tumors and the % BrdU index of the implant. A linear correlation was observed. $\%Ki-67 \text{ index} = 1.69 \times \%BrdU \text{ index} - 0.99$ ($r=0.84$, $P<0.05$). This suggested identical growth patterns of implants in CAM and original tumors.

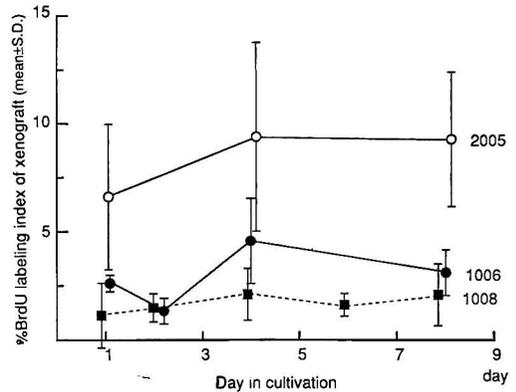


Fig. 6. Variation in %BrdU labeling index with length of incubation after inoculation.

Table 2. Comparison of S-phase fraction of small and large nests

Carcinoma No.	Small nests % BrdU labeling index (nests)	Mean size (cells)	Large nests % BrdU labeling index (nests)	Mean size (cells)
2003	26.3±10.1 (8)	57	24.0± 1.7 (4)	174
2004	9.9± 4.0 (3)	70	12.0± 4.7 (7)	310
3002	13.3± 4.0 (9)	62	15.1± 8.4 (6)	322
3003	0.8± 1.3 (12)	67	0.7± 0.7 (10)	529
5003	37.2± 7.8 (9)	65	30.4± 7.8 (11)	322
6002	22.6±13.7 (3)	58	21.4± 7.7 (3)	208

incubation time occurred in any of the 3 selected tumors (Fig. 6).

DISCUSSION

Heterologous tissue transplantation onto CAM was first reported by Murphy⁸⁾. Some animal and human cell lines can be readily transplanted⁹⁻¹¹⁾. Several authors attained lesser success rates with human tumor transplantation than with chicken or mouse tumors¹²⁻¹⁴⁾, while other studies recorded better results^{15,16)}.

A serially transplantable tumor originating from a human cancer was observed to grow well in CAM¹⁷⁾, and Uchida et al. presented an anticancer chemosensitivity test by measuring the weight of xenografts⁴⁾. In the present study, we also measured the weight of implants, but no apparent enlargement was observed. If weight had been the only criterion for chemosensitivity, assessment might become inaccurate and unreliable. In a subrenal capsular assay, the histological examination revealed discrepancy between the size of xenografts and tumor viability⁵⁻⁷⁾. Unless CAM preserves tumoral structure and growth conditions identical to those of the original tumors, CAM cannot be used for chemosensitivity testing. Hence, we examined the growth conditions of xenografts on CAM.

Several human tumors, but not prostatic carcinomas could be cultivated on CAM by transplanting minced tumor tissue. All transplanted prostatic carcinomas became necrotized, which might have been due to changes in hormonal conditions. Normal prostatic ducts and acini showing no

S-phase fraction were frequently observed in the implants surrounded by human normal connective tissues (data not shown). This phenomenon also occurred in breast cancer, which is sensitive to estrogen¹⁸⁾. Thus, hormonal manipulation might improve the transplantability of prostatic carcinomas. Renal cell carcinoma tissue was readily transplanted onto CAM with or without central necrosis. The quality and the quantity of the tissue sample should influence the take rates. Relatively large blocks of viable tissue can be easily excised from renal cell carcinoma in comparison with the other carcinomas, but renal cell carcinoma survived favorably on CAM with prominent angiogenesis. The other carcinomas also survived on CAM, and formed organoids of various sizes. Histological features of the successfully implanted cancers corresponded with those of the original tumors. Inflammatory reactions occurred mainly surrounding the necrotic area, but the tumoral structure was well-preserved. Although the successful transplantation rate was greater than 66.7%, cancer nests were unfortunately only diffuse, while necrotic implants were frequently encountered.

The growth potential in various human tumors has been identified by the BrdU labeling index or Ki-67 index^{19,20)}, and Sasaki et al. related the two indexes in the following equation: Ki-67 index = 1.59 × BrdU labeling index + 1.15²¹⁾. A similar correlation was observed in the present study, and the growth pattern in primary culture of tumor cells on CAM was believed to be identical to that of the original tumor. Growth conditions were not

changed regardless of the size of the organoids if the cancer nest consisted of more than 30 cells. Nests with less than 30 cells provided unreliable data. The structure of the cancer nests surviving on CAM was well-preserved, and growth rates were constant for the 8-day study period after inoculation. The successful grafting of tumor cells onto CAM indicates that this system gives accurate and reproducible data regarding growth potential.

In conclusion, human urological carcinomas other than prostatic carcinoma survived on CAM. Especially in renal cell carcinomas, cancer cells survived diffusely on CAM and it would be possible to test the anticancer chemosensitivity reliably based on the histological criteria including growth condition. However, in the other carcinomas, it might be difficult to exploit CAM for anticancer chemosensitivity testing because the take rates were low.

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和文抄録

鶏卵尿漿膜上に異種移植されたヒト腫瘍の増殖動態

名古屋大学医学部泌尿器科学教室 (主任: 三宅弘治 教授)

岡村 菊夫, 辻 克和, 下地 敏雄, 三宅 弘治

鶏卵尿漿膜上に異種移植された33例のヒト腫瘍の移植成功率と増殖動態を検討した。移植成功は、腎癌14例中11例(78.6%)に、尿路上皮癌9例中7例(77.8%)に、精巣腫瘍3例中2例(66.7%)に、結腸と卵巣の腺癌の各1例に認められたが、前立腺癌では5例中1例も生着しなかった。組織学的には、癌細胞はorganoidsを形成するように尿漿膜上に生着し、その構造はよく保たれていた。移植片のかなりの部分は、高頻度に壊死に陥っていた。癌巢のviabilityに関しては、壊死組織周囲の炎症性反応による影響は認められなかった。腎癌では、著明な血管新生と広範な生着

を認めた。抗bromodeoxyuridine monoclonal抗体とKi-67 monoclonal抗体を用いた免疫染色により、尿漿膜上の癌細胞のS期分画と原発巣の増殖分画には強い相関が認められた。癌巢の大きさによるS期分画には差を認めなかった。接種後8日間、S期分画の変動を認めなかった。生着した癌の増殖性は原発巣と同等であり、また、培養期間中その増殖性は安定していると考えられた。しかし、高い移植成功率を認めるものの生着する癌巢は疎らであり、腎癌以外では鶏卵尿漿膜は抗癌剤感受性試験に利用することは困難であると考えられた。

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