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Chick Embryo Assay as Chemosensitivity Test for Malignant Glioma

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To predict the efficacy of anticancer drugs such as ACNU [1-(4-amino-2-methyl-5-pyrimidinyl)-methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride] and MCNU [1-(2-chloroethyl)-3-(methyl-α-D-glucopyranos-6-yl)-1-nitrosourea] in the treatment of malignant gliomas, the usefulness of the chick embryo assay as a chemosensitivity test was studied. Fifty-seven surgical specimens including benign tumors were examined by this method. All tumor specimens tested could be grafted on the chorioallantoic membrane of chick embryo; the evaluable ratio was 100%. Twenty-one patients with previously untreated malignant glioma could be evaluated to test the predictability of the clinical effects, judged by computed tomography. There were 7 (78%) instances in which the assay response corresponded to a clinical partial response (true-positive). There were 2 (22%) false-positives for the assay, 0 (0%) false-negative and 12 (100%) true-negatives. The over-all predictive accuracy was 90% (19/21). Thus, a high-degree of positive association exists between the chick embryo assay and the clinical outcome. This *in vivo* assay system for malignant glioma is advantageous for chemosensitivity tests because of its convenience, rapidity, and inexpensiveness.

Key words: Chemosensitivity test — Chick chorioallantoic membrane — Human malignant glioma — ACNU — MCNU

Despite advances in chemotherapy, the response rate of malignant gliomas to chemotherapy has been disappointingly low. This is, in part, due to the limited efficacy of currently available therapeutic agents for malignant gliomas and also the difficulty of choosing suitable drugs for individual patients. Individual human tumors of the same histological type vary widely and unpredictably in their response to chemotherapeutic agents. Improved prognosis for patients with malignant glioma requires the development of methods for rapid and accurate prediction of clinical response to specific chemotherapeutic agents. A number of chemosensitivity tests either *in vivo* or *in vitro* have been employed in attempts to predict the efficacy of anticancer agents for individual patients before their administration. However, these tests have various problems such as low graft ratio, high cost, taking a long time for evaluation, and inaccuracy of criteria for judgment of the test.

In 1912, Murphy tried to inoculate rat and mouse tumors onto the chorioallantoic membrane (CAM) of chick embryos, which are naturally immunodeficient hosts that can accept inoculation of various tumors,[1] and then Dagg *et al.* applied this method to human tumors.[2,3] This chick embryo assay system has the advantages of rapidity, convenience and low cost. In this study, we demonstrated the usefulness of the chick embryo assay as a chemosensitivity test for malignant gliomas.

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MATERIALS AND METHODS

Human malignant gliomas Fifty-seven specimens (Table I) obtained at operation were used for evaluation of takes onto the CAM in this method. Of these specimens, 21 specimens of malignant glioma were used for evaluation of the correlation between the result of this method and clinical effect.

Inoculation of malignant gliomas onto the CAM Chicken eggs (Plymouth Rock×White Leghorn) one to two days old were obtained from the Goto Chicken Farm (Gifu). They were kept in an incubator at 37°C in a humidified atmosphere (relative humidity, about 70%). Eggs 10 days after fertilization were used as recipients of tumor cells. Each egg was candled and a Y-shaped junction of blood vessels in the CAM was marked on the shell with a pencil. The egg shell was cleaned with 70% alcohol and a square hole about 1 cm^2 was made through it. The CAM was depressed by applying gentle suction at the air sac. The shell membrane was carefully stripped off, exposing the CAM (Fig. 1). Surgical specimens as free of necrotic parts as possible were minced with scissors and transplanted on the CAM in samples of 100 μl. The window of the shell was then sealed with OpSite (T.J. Smith and Nephew Ltd., Welwyn Garden City, England).

Administration of anticancer drugs After tumor transplantation, eggs were incubated at 37°C in a humidified incubator. Three days later, when the embryos were 13 days old, the growth of the transplanted tumor was

Table I. Histology of Human Brain Tumors Transplanted onto the CAM

Histology	No. of transplantations	No. of takes
Glioblastoma	21	21
Anaplastic astrocytoma	7	7
Low-grade astrocytoma	4	4
Oligodendroglioma	2	2
Medulloblastoma	3	3
Ependymoma	3	3
Malignant lymphoma	2	2
Metastatic tumor	11	11
Meningioma	4	4
Total	57	57

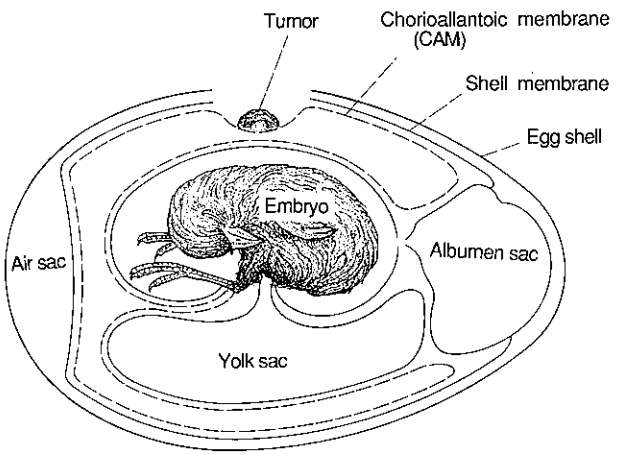

Fig. 1. Schematic drawing of the chemosensitivity test system using the chorioallantoic membrane (CAM) of fertilized chick egg.

confirmed. The eggs were candled and a line was marked on the shell over the large vessel in the CAM, where a groove was ground through the shell and a rectangular shell piece was removed. A drop of paraffin oil was put on the shell membrane, which made the vessel more visible. Anticancer drugs were injected through the vessel with a 30G needle. The drugs tested in this study were ACNU [1-(4-amino-2-methyl-5-pyrimidinyl)-methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride] and MCNU [1-(2-chloroethyl)-3-(methyl-α-D-glucopyranos-6-yl)-1-nitrosourea]. These drugs are in widespread use for patients with malignant glioma in Japan. Each was injected into the CAM vein with 0.1 ml of 0.9% NaCl solution. The dose was 100 μg/egg for each drug, which is about 10 times larger than the clinical dose per body weight.

Evaluation of anticancer effect in the chick embryo assay Four days after administration of the drug (7 days after tumor transplantation), the embryos were killed; they were 17 days old. The tumors were excised carefully from the CAM, washed with 0.9% NaCl solution, and weighed. Portions of the tumor were preserved for microscopic examination. According to Uchida *et al.*,[4] the growth inhibition ratio was calculated by means of the following formula: Inhibition ratio (%) = [(Wc − Wt)/Wc] × 100, where Wc is the mean tumor weight of the control group, and Wt is that of the treated group.

Clinical treatment and evaluation In clinical treatment, the first course of chemotherapy was given at the beginning of radiation therapy. Patients with malignant glioma were administered 2 mg/kg of either ACNU or MCNU without restriction or information as to the results of this test, depending on the choice of their own physicians. Chemotherapy was repeated every 2 months thereafter for as long as possible, 2–3 times on average. The total dose of radiation therapy was kept below 60 Gy in 25 fractions over 5 weeks. The direct effect of the treatment was evaluated 2 months after the beginning of the treatment, namely before the second course of chemotherapy, by computed tomography (CT). The cases without residual tumor after operation were excluded from this study. The criteria for evaluation on CT were as follows[5]: complete response (CR), complete disappearance of the tumor; partial response (PR), over 50% decrease in the tumor area; no change (NC), between less than 25% increase and less than 50% decrease in the tumor area; progressive disease (PD), over 25% increase in the tumor area.

Statistical analysis Student's t test was used to evaluate the statistical significance of differences. All P values below 0.05 were taken to be significant.

RESULTS

Tumor growth on the CAM Best growth seemed to occur with the benign, non-neuroectodermal tumors, such as meningiomas. Gliomas grew less vigorously than meningiomas. Within the gliomas, however, more malignant tumors grew more quickly than less malignant ones. But all of the human brain tumors tested could grow on the CAM sufficiently to be weighed and evaluated 7 days after transplantation. Microscopically, the transplants retained their morphological resemblance to the parent tumors (Fig. 2). None of the chick embryos died of toxicity at the dosage of anticancer drugs tested.

Effects of drugs on growth of transplanted tumors The effects of administered drugs on transplanted tumors on the CAM are shown in Table II. The cases, which showed a significantly higher inhibition rate than the

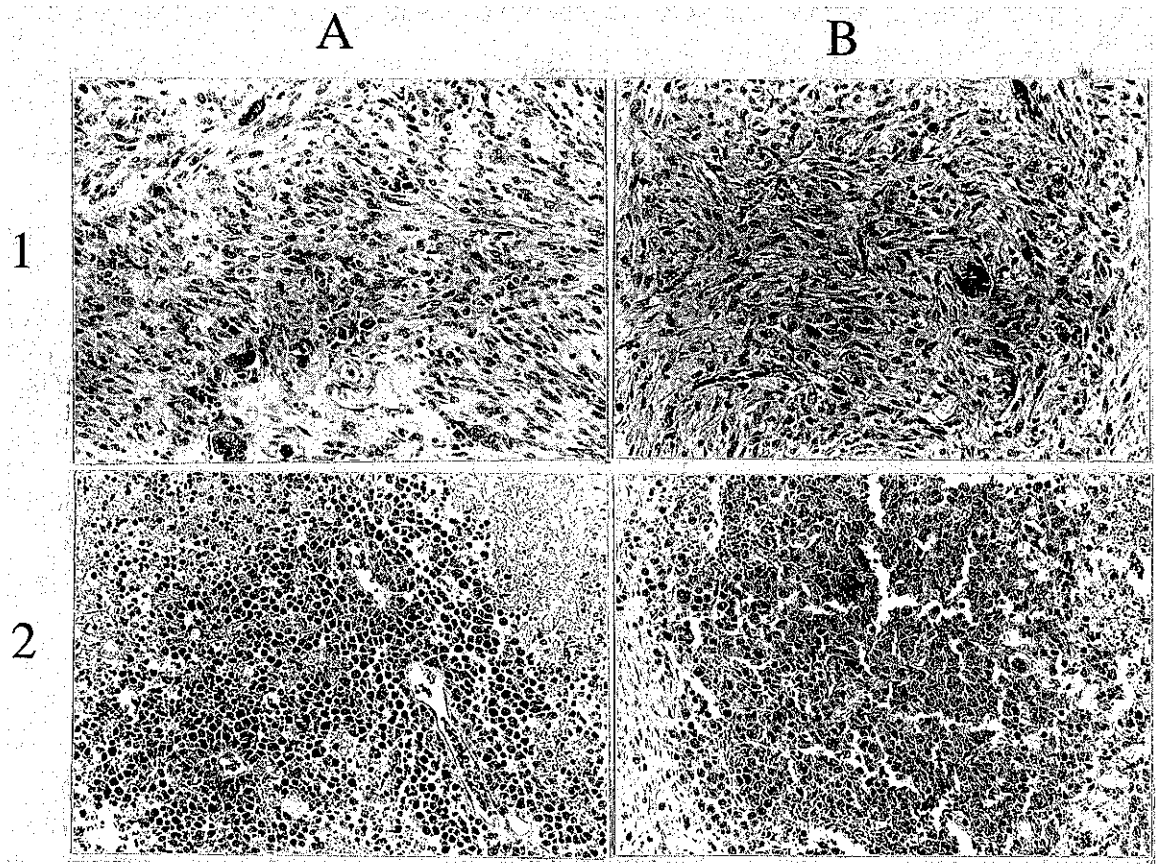

Fig. 2. Photomicrograph of the original tumor and the transplanted tumor on day 7 onto the CAM. A; the original tumor, B; the transplanted tumor. 1; glioblastoma (×200), 2; malignant lymphoma (×100). Hematoxylin and eosin stain.

control amounted to 50% (8/16) to MCNU and 29% (6/21) to ACNU.

Correlation between the chemosensitivity tests and clinical effects Twenty-one patients with malignant glioma were evaluated (Table II). Clinical effects of anticancer drugs on the brain tumor were evaluated by CT 2 months after the beginning of the treatment. Retrospectively, 9 cases had been treated with drugs to which the tumors were sensitive, judged from the sensitivity test. Among them, 7 cases (78%) showed partial response and 2 cases (22%) no change. On the other hand, 12 cases had been treated with drugs to which the tumors were not sensitive. Ten cases showed no shange and 2 cases showed progressive disease. The response ratio was defined as the ratio of the number of CR and PR to all cases. The response ratio was 78% in the group whose tumors were treated with the appropriate drug (to which the tumors were sensitive), and 0% in the group whose tumor were treated with the inappropriate drug (to which they were not sensitive).

In 21 cases, the true-positive ratio was 7/9 (78%), the true-negative 12/12 (100%), the false-positive 2/9 (22%), and the false-negative none. Accordingly, the predictive ratio of this test was 19/21 (90%).

The survival rate was evaluated by using the Kaplan-Meier method. The median survival time of the group treated with the appropriate drug was 19 months, which was slightly higher than that of the group treated with the inappropriate drug (17 months), but the difference is statistically not significant.

DISCUSSION

Despite recent advances in surgery, radiation, and chemotherapy for human malignant brain tumors, the prognosis has not been significantly improved. One of the major clinical problems is that there is no reliable method to predict clinical response to chemotherapy for individual patients. If chemotherapeutic agents to which the tumor is not sensitive are used, the patient will have only

Table II. Association between the Chick Embryo Assay Result and Clinical Response of Individual Malignant Gliomas to Chemotherapy

Case No.	Age (yr)/Sex	Inhibition ratio (%)[a]		Administered chemotherapeutic agent	CT response[d]	Correlation[e]	Survival[f] (months)
		ACNU	MCNU				
Glioblastoma							
1	60/M	18.6	–	ACNU	NC	TN	6 (D)
2	18/M	4.2	14.9	MCNU	NC	TN	15 (D)
3	63/M	19.1	–	ACNU	NC	TN	10 (D)
4	74/M	17.5[b]	2	ACNU	PR	TP	18 (D)
5	26/M	21.9[b]	36.5[c]	MCNU	NC	FP	10 (D)
6	65/M	15.9	19	ACNU	NC	TN	15
7	14/M	27	–	ACNU	NC	TN	12
8	24/M	11.3	18.3[b]	ACNU	NC	TN	8
9	45/F	15.9	–	ACNU	NC	TN	17 (D)
10	46/F	45.7[c]	14.7	ACNU	PR	TP	17 (D)
11	52/M	40.4[c]	25.4[c]	ACNU	PR	TP	19 (D)
12	68/F	4.5	21.6[b]	MCNU	PR	TP	19 (D)
13	55/M	3.9	–	ACNU	NC	TN	8 (D)
14	61/F	9.5	21.9[c]	MCNU	NC	FP	18 (D)
Anaplastic astrocytoma							
15	51/F	63.1[c]	42.3[b]	MCNU	PR	TP	29
16	41/M	18.4	12	ACNU	PD	TN	8
17	64/F	4.8	18.8[b]	MCNU	PR	TP	13 (D)
18	53/M	9	1.7	MCNU	PD	TN	18
19	41/M	19.4	4.6	MCNU	NC	TN	21
20	60/M	30.3[b]	28.1[b]	ACNU	PR	TP	17
21	39/M	10	2.5	ACNU	NC	TN	22

a) Significance of differences from the value with the factor alone in each group: *b*) $P<0.05$, *c*) $P<0.01$. *d*) PR; partial response, NC; no change, PD; progressive disease. *e*) TP; true-positive, TN; true-negative, FP; false-positive. *f*) D; dead.

side effects, such as bone marrow suppression. On the contrary, if the tumor is sensitive to the drug used, complete remission can be expected, following high-dose administration of the anticancer drug with bone marrow transplantation.[6,7] There have been several reports on assays with nude mice to predict the drug sensitivity of human tumors.[8–10] However, the growth of primary tumors implanted was poor and the graft rates were usually about 30–40%. Moreover, this assay is expensive, and it takes a long time, about 30–50 weeks or more, to evaluate the results. The sensitivity test using nude mice is more suitable as a screening test of new anticancer agents, rather than as a chemosensitivity test to choose suitable drugs for individual cases.[11] The subrenal capsule assay was reported to have a high evaluable ratio and high predictive value.[12–14] But its disadvantages are that the tumor cells do not always grow under these conditions, that it requires a large number of mice to test several drugs,[15] and that the criteria of judgment of the test have not yet been clearly defined.

Among *in vitro* tests, the human tumor clonogenic assay[16] is one of the best tests in evaluating the efficacy of anticancer drugs.[17,18] With human malignant brain tumors, Rosenblum *et al.* reported an *in vitro-in situ* correlation using this assay.[19] They reported that the *in vitro* predictability levels of clinical sensitivity and resistance were 71% and 100%, respectively. However, the plating efficacy is so low and growth of colonies is so slow that it takes a long culture time to obtain the results.[20] Moreover, pro-drugs, which require metabolic activation, can not be evaluated.[21]

The immune system of chick embryos does not mature until about day 18 of incubation.[22,23] Therefore, various tumors transplanted onto the CAM grow without the effect of host immune responses. The graft rates are high both for surgical specimens and for cultured cell lines.[4] At least in the first generation of transplantation, the tumors histologically resembled the parent tumors.[24] In our present study, the graft rate was 100% and the histopathological characteristics of the parent tumors

were maintained. According to Ossowski *et al.*, at least within the first generation of transplantation onto the CAM, the malignant character of the parent tumors was also maintained.[25] Seven days after transplantation, the tumors became large enough to be weighed and the data could be used for prediction of the efficacy of anticancer drugs. If the growth of tumor is insufficient to allow evaluation within the limits set by the standard 7-day assay, re-transplantation of the tumor onto a new CAM is possible, and the test is simple and inexpensive. Prodrugs, such as cyclophosphamide, which require metabolic activation,[26] can also be examined by this method.[4] Moreover, this system can predict the metastatic ability of the tumors.[27-29]

For statistical evaluation, it would be better to treat patients by chemotherapy without radiotherapy. However, since the effect of radiation in the treatment of malignant gliomas has been established, we could not exclude radiotherapy as the initial treatment of the patients with malignant glioma. We gave as uniform a radiation schedule as possible. If the radiation affected the clinical response within 2 months after beginning of radiation, CR or PR should be observed, regardless of the chemotherapy. The results showed that there was no CR or PR case in the group treated with radiation and the inappropriate drug but the response ratio of the group treated with radiation and the appropriate drug was 78%. This fact indicates that the effect of radiation might not appear at least within 2 months after beginning irradiation. We can not rule out the possibility that radiation may have some effect, but it does not seem to influence the clinical response within 2 months.

This study was preliminary, retrospective and non-randomized. But the potential value of the chick embryo assay as a predictive test for individual tumor sensitivity to chemotherapeutic agents seems clear. As noted above, there was a good association between results in the drug sensitivity test and the clinical course. There were 2 false-positive cases in the trial. This may be due to the high dose, about 10 times larger than the clinical dose per body weight, used in the chick embryo assay. But if the dose in the assay corresponded to the clinical dose, no clear results were obtained. Accordingly, the dose corresponding to 10 times the clinical one was used in this assay as a suitable dose. The correlation between the test results and clinical effects was 90%, which is not at all inferior to those of other available chemosensitivity tests, including the *in vitro* human tumor clonogenic assay.[24, 30] It is clear, however, that before routine use of this chemosensitivity test, prospective trials on the system are required. A more definitive evaluation must depend upon the result of future randomized trials. But since few chemotherapeutic agents are active against malignant gliomas, there is little likelihood of finding drugs to which an individual tumor is sensitive. Nevertheless, it should be emphasized that the predictive ratio was quite high. Therefore, by using this chemosensitivity test, we can at least avoid administering ineffective chemotherapeutic drugs, which produce only side effects. In particular, the test should be useful to eliminate ineffective drugs for high-dose administration with bone marrow transplantation, which is currently on-going in our department.

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