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Responses of tumour cell lines implanted onto the chorioallantoic membrane of chick embryo to anticancer agents in combination with hyperthermia

T. Uchibayashi[1], M. Egawa[1], K. Nakajima[1], H. Hisazumi[1], M. Tanaka[2], Y. Endo[2], and T. Sasaki[2]

[1]Department of Urology, School of Medicine, and [2]Department of Experimental Therapeutics, Cancer Research Institute, Kanazawa University, Kanazawa, Japan

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Summary. The chorioallantoic membrane of chick embryos was used to examine the responses of three tumour cell lines to anticancer agents, alone and in combination with hyperthermia. Fifteen minutes of hyperthermia at 42.5°C produced the most favourable anticancer effect in the B16–F10 grafts. The use of Adriamycin (ADM) alone and the combined use of hyperthermia and either cisplatin (CDDP), cyclophosphamide (CY) or ADM resulted in a significantly higher rate of tumour regression in the B16–F10 grafts from a murine melanoma. In the KK-47 grafts derived from a transitional cell carcinoma of the bladder, the use of CY alone and the combination of CY and hyperthermia produced a significant tumour regression rate. In the T24 grafts neither the use of CY or CDDP alone, nor the combination of these drugs with hyperthermia demonstrated any significant effect. This method of screening anticancer agents was found to be rapid, simple to perform and inexpensive.

Key words: Chorioallantoic membrane – Anticancer agents – Hyperthermia – Thermo-chemotherapy – Sensitivity test

In the treatment of cancer, a combination of different treatment modalities, e.g. hyperthermia and chemotherapy, has been advocated by numerous authors [1, 5]. Malignant tumours differ in their sensitivity to cytotoxic drugs or thermal injury. In vitro investigation on surgically removed specimens prior to therapy may make it possible to predict the response in vivo. Murphy [7] first reported successful cell growth and serial passage of rat and mouse tumours on the chorioallantoic membrane (CAM) of the chick embryo.

This chick embryo system might also be useful for the study of tumour growth and metastatic potential. Furthermore, this experimental system may be advantageous as a chemosensitivity test in searching for new anticancer agents. The aim of the present study was to investigate the tumoricidal effect of anticancer drugs with or without hyperthermia in implanted grafts using the modification of Murphy's method described by Sasaki [12].

Materials and methods

Sensitivity to thermo- or chemotherapy was studied according to the procedure described by Sasaki [12] (Fig. 1). Chicken eggs (Polymouse and White Leghorn) obtained from the Goto Chicken Farm (Gifu, Japan) were kept in an incubator at 37°C in a humidified atmosphere (relative humidity, 70%). Eggs 10 days after fertilization were used. Each egg was illuminated with a candle, and the Y-shaped junction of blood vessels in the CAM was marked on the shell with a pencil. The egg shell was sterilized with 70% alcohol and a square hole about 1 cm^2 was made. The CAM was depressed by suction at the air sac, and the shell membrane was removed with a pair of forceps. Single cell suspensions were made from cultured murine B16–F10 melanoma cells (kindly supplied by Dr. Tsuruo of the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research), T24 cultured cells (kindly supplied by Dr. V. Peter Collins, Ludwig Institute for Cancer Research, Stockholm) and KK-47 cultured cells established by Dr. Haruo Hisazumi in our department in 1979 [13] at a logarithmic growth rate, and then adjusted

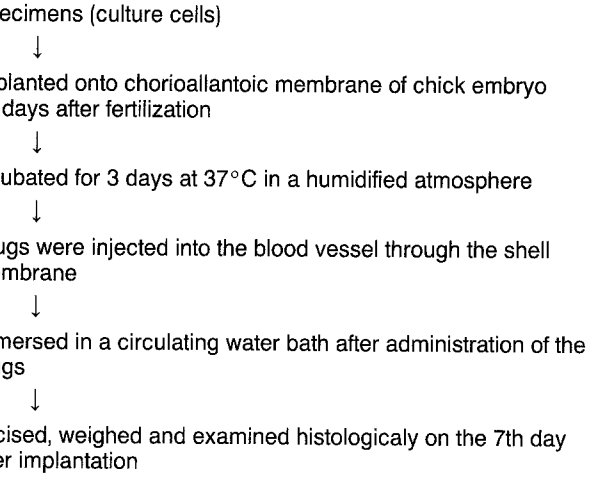

Fig. 1. Procedure for the chick embryo method

Table 1. Effect of hyperthermia on B16–F10 grafts

Temperature (°C)	Heating time (min)	IR (%)
42.0	10	18.9
	15	13.0
42.5	10	38.2*
	15	59.7**
	20	29.0*
43.0	5	4.3**
	10	51,6**
	15	all dead

* $P<0.05$, ** $P<0.01$, $n=5$

Table 2. Effect of anticancer agents with/without Hyperthermia on B16–F10 grafts

Drugs with/without hyperthermia	Dose (μg/egg)	Tumor weight (mean ± SD, mg)	IR (%)
Control	(−)	107.7 ± 77.0	(−)
CDDP	10	47.0 ± 35.5	56.4
ADM	40	21.7 ± 10.6	80.1*
CY	100	54.7 ± 24.4	49.1
HPT (42.5°C, 15 min)	(−)	59.9 ± 19.7	44.0**
CDDP + HPT	10	17.2 ± 6.9	84.0**
ADM + HPT	40	26.4 ± 14.5	75.4**
CY + HPT	100	35.9 ± 24.5	66.7**

* $P<0.05$, ** $P<0.01$, $n=5$; ADM, Adriamycin; CDDP, cisplatin; CY, cyclophosphamide; HPT, hyperthermia

Table 3. Effect of anticancer agents with/without hyperthermia on KK-47 grafts

Drugs with/without hyperthermia	Dose (μg/egg)	Tumor weight (mean ± SD, mg)	IR (%)
Control	(−)	147.6 ± 72.4	(−)
CDDP	10	123.0 ± 97.5	16.7
CY	100	80.3 ± 53.7	45.6*
HPT (42.5°C, 15 min)	(−)	140.5 ± 75.6	4.8
CDDP + HPT	10	182.1 ± 77.6	-23.4
CY + HPT	100	89.8 ± 78.9	39.2*

* $P<0.05$, $n=5$

with Ham's F12 culture medium (Nissui Pharmaceutical Co., Tokyo) supplemented with 20% fetal bovine serum (Gibco, Grand Island, N.Y.) to a concentration of 1×10^6 viable cells/ml. A 20-μl amount of this tumour cell suspension was implanted inside a sterilized Teflon ring (8 mm in diameter), which was placed on the junction of the blood vessels in the CAM of the eggs. The opening windows were sealed with Opsite (Smith and Nephew, Welwyn Garden City, UK), and the chicken eggs were kept in an incubator at 37°C for 7 days. Three days after incubation the drugs were injected into the blood vessel of the embryo. Mitomycin C (MMC) and Adriamycin (ADM) were purchased from Kyowa Hakko (Tokyo, Japan). Cyclophosphamide (CY), vincristine (VCR) and vinblastine (VLB) were products of Shionogi Pharmaceutical Co. (Osaka, Japan). Cisplatin (CDDP) was a product of Nihon Kayaku (Tokyo, Japan). Each of the drugs was dissolved in 0.9% NaCl solution to the concentrations corresponding to clinically achievable blood levels. The doses injected into the vessel were calculated on the basis of the mean body weight of the chick embryo, namely, 100 μg/egg for CY, 10 μg/egg for CDDP, 40 μg/egg for ADM, 5.0 μg/egg for MMC, 0.5 μg/egg for VCR, and 0.2 μg/egg for VLB. A portion of the shell lying directly over a blood vessel was carefully removed, and the vessels under the shell membrane were made more easily visible with a drop of paraffin oil. Injection of drugs was performed with a 30 gauge needle through the shell membrane. Some implanted eggs were injected with 0.1 ml of 0.9% NaCl solution as controls. Hyperthermia was induced after drug injection according to the following protocol; each of the eggs was immersed in a circulating water bath DX-80 (Taiyo, Tokyo, Japan), for different lengths of time and at different temperatures, while monitoring the intra-yolk temperature using a Takara thermometer (model D 925) and thermistor (model SKX-67; Takara, Kanazawa, Japan). The embryos were sacrificed 7 days after tumour cell implantation. The resulting grafts were excised from the CAM, and washed with 0.9% NaCl solution. The rinsed grafts were weighed using an AE 240 analytical balance (Mettler, Switzerland). The inhibition rate was calculated according to the following formula:

$$\text{inhibition rate (\%)} = [\,(A - B)\,/A\,] \times 100$$

where A represents the mean tumour weight of the control group and B that of the treated group. Using Student's t-test, the data were evaluated statistically.

Table 4. Effect of anticancer agents with/without hyperthermia on T24 grafts

Drugs with/without hyperthermia	Dose (μg/egg)	Tumor weight (mean ± SD, mg)	IR (%)
Control	(−)	22.1 ± 6.8	8.1
CDDP	20	16.6 ± 5.4	24.9
CY	100	17.5 ± 4.5	20.8
HPT (42.5°C, 15 min)	(−)	20.3 ± 7.5	8.1
CDDP + HPT	20	28.7 ± 9.9	-29.9
CY + HPT	100	18.3 ± 7.8	17.2

$n=5$

Results

The effects of hyperthermia on the B16–F10 melanoma grafts are shown in Table 1. In the B16–F10 melanoma grafts, 15 min hyperthermia at 42.5°C had a favourable effect. As shown in Table 2, when using ADM alone or a combination of 15 min hyperthermia and CDDP, CY or ADM, significant tumour regression was recorded ($P<0.01$), with an additive effect of hyperthermia with CDDP and CY but not with ADM. In the KK-47 grafts, as shown in Table 3, CY alone and in combination with 15 min hyperthermia produced significant tumour regression ($P<0.05$). CDDP alone or in combination with hyperthermia had no effect. No tumour regression was produced in the T24 grafts (Table 4).

Discussion

In this study, we employed the chick embryo method using the CAM as reported by Sasaki [12] to test the sensitivity of cultured tumour cells to thermo-chemotherapy. In recent years, there have been reports on tests of sensitivity to anticancer drugs and hyperthermia using cultured cells or surgical specimens, applying the human tumour clonogenic assay [6, 11, 14], nude mouse transplantation assay [9, 10] or the subrenal capsule assay [2–4]. The human tumour clonogenic assay provides a good index for tumour cell proliferation and it has been considered to be one of the best in vitro sensitivity tests for anticancer drugs and also for hyperthermia. However, plating efficiency is low and the growth of colonies is so slow that it takes about 2–4 weeks to obtain the results, making it impractical for clinical applications. The nude mouse transplantation assay has been found to be a sensitive method for anticancer agents or hyperthermia, particularly in terms of the correlation between test results and clinical effect. However, this method also has drawbacks: namely, the successful implantation rate is rather slow and an experimental period of more than 3 weeks is necessary to obtain conclusive results. Among these methods, the subrenal capsule assay is clinically the most useful as a test of chemosensitivity; however, to heat the implanted grafts whole body hyperthermia must be used. In this case, the body temperature tolerated by the mouse is approximately 42.5 °C; also local hyperthermia of grafts is extremely difficult [8].

In the present study, we examined the responses of three cancer cell lines to anticancer agents with or without hyperthermia using the chick embryo. We propose that this assay system using the CAM of the chick embryo is a useful model system for the evaluation of anticancer agents, alone or in combination with hyperthermia. The advantages of this system include the following: (1) embryos have no pathogens and are immunologically incompetent, producing favourable growth conditions for grafts on the CAM without interaction of the host's immune system; (2) the assay period is short, which makes it practical for clinical application; and (3) it is inexpensive and simple to perform.

ADM alone was effective in suppressing tumour growth of B16–F10 grafts but no additional anticancer effects were observed when ADM was combined with hyperthermia. Hyperthermia combined with CDDP or CY showed an enhanced tumour-suppressing effect on B16–F10 melanoma; however, CDDP or CY alone demonstrated no significant suppressing effect on the tumour growth of the grafts. In the KK-47 grafts, no further effect of hyperthermia was found compared with CY alone. In the T24 grafts, no significant suppressing effect was produced by the anticancer agents, even in combination with hyperthermia. From these observations on three types of grafts, it is obvious that the response to anticancer agents with or without hyperthermia varies among different cell lines.

In future, these combination therapies may become more important in clinical practice. The present results suggest that this assay system may prove to be useful as a thermo-chemotherapy sensitivity test.

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Tadao Uchibayashi, MD
Department of Urology
School of Medicine
Kanazawa University
13-1 Takara-machi
Kanazawa
920 Japan