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SESSION III

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Studies of effects of anticancer agents in combination with/without hyperthermia on metastasized human bladder cancer cells in chick embryos using the polymerase chain reaction technique

Abstract Cultivated T24 cells derived from a human bladder cancer were inoculated into the chorioallantoic membrane vein of chick embryos. Hyperthermic treatment was performed following injection of anticancer agents 3 days after the inoculation of the T24 cells. DNA samples were obtained from the livers of the chick embryos, and the polymerase chain reaction technique was used to amplify a DNA fragment specific to the human β-globin gene. The Southern hybridization method was used to evaluate the inhibitory effects of anticancer agents in combination with/without hyperthermia on T24 cells metastasized to the liver. The hyperthermia exerted an inhibitory effect on the growth of the T24 cells in the livers of the chick embryos, and this was dependent on the thermal dose. The antitumor effects of hyperthermia performed at 42.5° C for 20 min and at 43.0° C for 10 min were evidenced by 69.2% an 82.0% inhibition of the growth of the metastasized T24 cells, respectively, as compared with the growth of untreated T24 cell. Hyperthermia performed at 42.5° C for 10 min alone produced 26.7% tumor growth inhibition, and these conditions for hyperthermia were subsequently used as a criterion for evaluating the effects of its combination with various anticancer agents. Adriamycin (20 μg/egg) alone, mitomycin C (10 μg/egg) alone, carboplatin (10 μg/egg) alone, and cisplatin (10 μg/egg) alone produced 13.5%, 58.9%, 27.3%, and 29.1% tumor growth inhibition, respectively. Adriamycin and mitomycin C applied in combination with hyperthermia showed additive inhibitory effects on the growth of the metastasized T24 cells in this chick embryo model.

Key words Polymerase chain reaction · Chick embryonic assay

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Introduction

In cancer treatment, it is generally accepted that the presence of metastases and the extent of local infiltration of cancer are exceedingly important factors influencing the prognosis of the patient. When the primary lesion is discovered at an early stage, local radical treatment may be feasible by means of surgical resection, radiotherapy, hyperthermia, and chemotherapy as well as combination therapy using two or more of these modalities. However, when the primary tumor has assumed a certain size, systemic micrometastases may be present, and these are usually treated with chemotherapy, immunotherapy, and whole-body hyperthermia. In addition, it is well recognized that there is an appreciable difference between metastatic and primary cancer cells in their sensitivity to anticancer agents. Metastatic disease is responsible for the majority of deaths caused by cancer. It is thought that better knowledge of the sensitivity of metastatic tumors to various anticancer agents as well as hyperthermia prior to institution of cancer therapy would contribute greatly to enhancing the therapeutic effects when radical treatment is performed. A highly quantitative method for detecting metastasized human cancer cells in the livers of chick embryos has been established using the polymerase chain reaction (PCR) technique [4].

In the present study, using the PCR technique we amplified a specific human tumor-cell-derived DNA sequence present in embryonic chick organ metastases, analyzed it by Southern blotting, and evaluated whether the chick-embryo assay system could serve as an in vivo

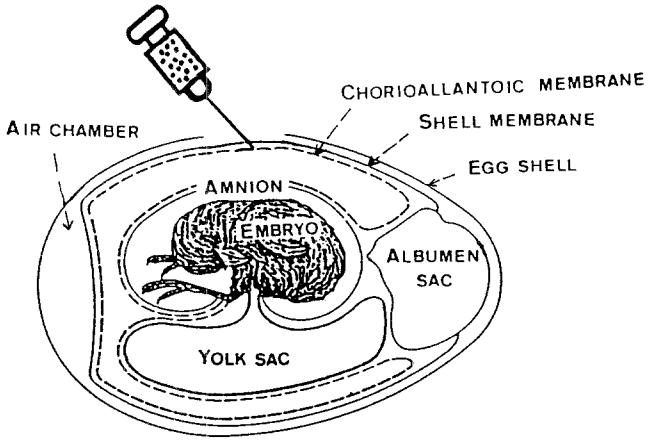

Fig. 1 Schema of a fertilized egg

primers
Huβ-1 : 5'-AGAGCCATCTATTGCTTACA-3'
Huβ-8 : 5'-TATGACATGAACTTAACCAT-3'
Probe
Huβ-2 : 5'-ACACAACTGTGTTCACTAGC-3'

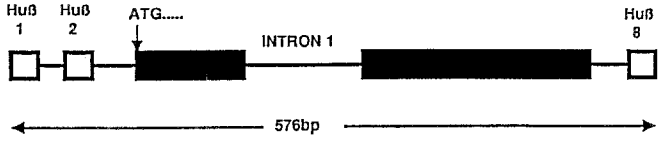

Fig. 2 DNA sequence of the human β-globin gene. Oligonucleotide primers and probe for PCR

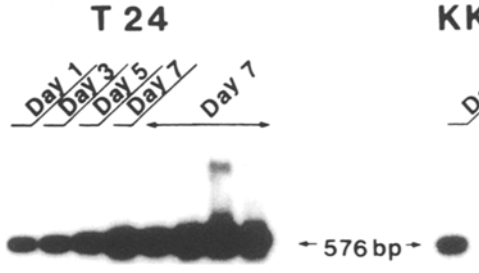

Fig. 3 Kinetics of the growth of metastatic cells in embryonic liver after the injection of T24 and KK-47 cells (10^6 cells/embryo)

model for preliminary investigation of the effects of chemohyperthermia on human tumor metastases.

Materials and methods

This method using the chick embryonic assay is explained in brief. Figure 1 shows a schema of a fertilized egg. Lying just beneath the shell is the shell membrane, below which is the chorioallantoic membrane. This chorioallantoic membrane, which is made up of partially adhering chorionic epithelium and allantoic epithelium, extends beneath the shell membrane. Blood vessels developing between these membranes play an important role in the respiration of the embryo.

In experiments concerning metastases using the chick embryonic assay, cancer cells should be injected into the veins of the chorioallantoic membrane of eggs 10 days after fertilization. On the 3rd day after cell inoculation, hyperthermia is performed following the injection of anticancer agents. After 4 additional days, namely, on the 17th day of embryonic life, the liver and lungs of the embryo are dissected and the DNA of each organ is extracted using a rapid DNA preparation method [2]. In this method, 1 mg DNA (as a template) is amplified by 25 cycles of PCR, during which the double-stranded DNA is denatured, primers are added, and complementary strands are synthesized, resulting in approximately 100,000-fold amplification of the target DNA fragment. The amplified DNA fragments are then analyzed by Southern blotting.

In the present study, a portion of the β-globin gene was selected as the human DNA-specific target for amplification. Primers and a probe were prepared from the DNA sequence of this gene for PCR. Figure 2 shows the DNA sequence of the human β-globin (*Huβ*) gene. For the metastasis experiments, *Huβ*-1 and *Huβ*-8, which flank the first and second exons, were selected as the PCR primers. The length of the DNA fragment amplified by PCR was 576 bp. *Huβ*-2, shown in Fig. 2, was labeled with ^{32}P and used as the probe. A cultured urinary bladder-cancer-derived cell line, T24 (kindly supplied by Prof. V. Peter Collins, Sahlgrenska Hospital, Gothenburg), and KK-47 cells [8] were injected into the veins of 10-day-fertilized embryos, and the metastasis-bearing organs were dissected 7 days later.

PCR was performed on the samples after DNA extraction, and amplified bands were detected by autoradiography. At day 3 after tumor cell inoculation, hyperthermia was performed by immersing eggs in a circulating water bath at various temperatures and for various periods. Then, using the same techniques, experiments on the effects of various anticancer agents in combination with/without hyperthermia were performed. In all, 1×10^6 T24 tumor cells were inoculated into the chorioallantoic membrane veins of chick embryos, and 3 days later various anticancer agents were injected i.v. in combination with/without hyperthermia. On the 7th day after inoculation the embryonic liver was dissected, the extracted DNA was amplified by PCR, and the presence of metastatic cells was examined. The inhibition rate was calculated according to the following formula:

$$\text{Inhibition rate } (\%) = [1 - A/B] \times 100,$$

where *A* represents the mean radioactivity of the treated group and *B*, that of the control group.

Results

As shown in Fig. 3, the T24 cells showed metastatic cell proliferation with time. In contrast, in the case of the KK-47 cells, no metastatic cell proliferation was found. The hyperthermic treatment showed an inhibitory effect on the growth of the T24 cells in the livers of the chick embryos, and this was dependent on the thermal dose as shown in Fig. 4. The antitumor effects of hyperthermia performed at 42.5° C for 20 min and at 43.0° C for 10 min were evidenced by 69.2% and 82.0% inhibition of the growth of the metastasized T24 cells, respectively, as compared with the growth of the untreated T24 cells. Hyperthermia performed at 42.5° C for 10 min alone produced 26.7% tumor growth inhibition, and conditions for hyperthermia were subsequently used as the criterion for evaluating the effects of combination with various anticancer agents (Table 1).

The results of experiments using Adriamycin to treat the metastatic foci formed by this method are shown in Table 2. Administration of Adriamycin (ADM) alone at 20 μg/egg resulted in a minor decrease in the amount of PCR-

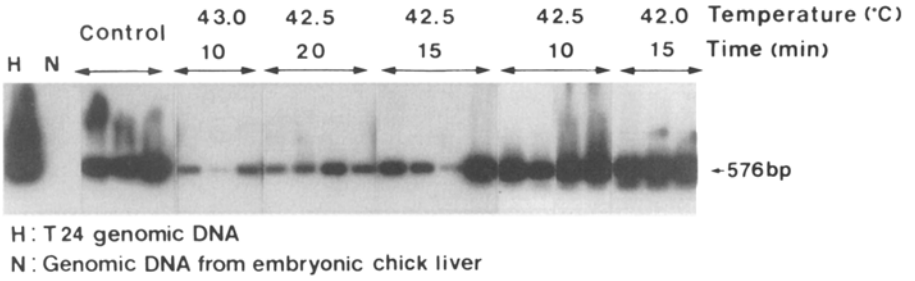

Fig. 4 Effect of hyperthermia on liver metastasis of T24 cells in embryonic chicks

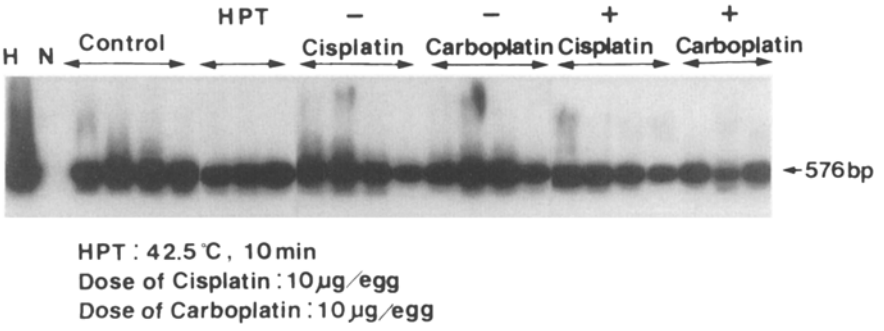

Fig. 5 Effects of cisplatin and carboplatin with/without hyperthermia on liver metastasis of T24 cells in embryonic chicks

Table 1 Effect of hyperthermia on liver metastasis of T24 cells

Temperature – time (° C, min)	Radioactivity, mean ± SD (AU/mm²)	IR (%)[a]
43.0–10	33.2 ± 30.3	82.0*
42.5–20	56.7 ± 49.2	69.2*
42.5–15	104.6 ± 118.8	43.2
42.5–10	135.0 ± 27.4	26.7
42.0–15	142.3 ± 94.0	22.7
Control	184.1 ± 76.3	

* $P < 0.01$ (Student's t-test)

[a] IR (%) = {1–(Radioactivity of treated group)/(Radioactivity of control group)} × 100

Table 2 Effect of ADM and hyperthermia on liver metastasis of T24 cells

	Hyperthermia	
Drugs (μg/egg)	None	42.5° C – 10 min
None		26.7
ADM (20)	13.5	54.2*

* $P < 0.01$ (Student's t-test)

Table 3 Effects of cisplatin and carboplatin with/without hyperthermia on liver metastasis of T24 cells

	Hyperthermia	
Drugs (μg/egg)	None	42.5° C – 10 min
None		24.5
Cisplatin (10)	29.1*	33.4*
Carboplatin (10)	27.3*	44.5*

* $P < 0.05$ (Student's t-test)

amplified DNA. The radioactivity of these bands was analyzed and compared for the groups treated with ADM alone, for those treated with ADM combined with hyperthermia, and for those left untreated. ADM in combination with hyperthermia had an additive inhibitory effect on the growth of the metastasized T24 cells in this chick embryo model. In contrast, no additive inhibitory effect was found for cisplatin or carboplatin administration in combination with hyperthermia (Fig. 5, Table 3). Then, the administration of mitomycin C alone or in combination with hyperthermia was studied. Mitomycin C used alone and with hyperthermia produced 58.9% and 88.6% inhibition rates, respectively. Thus, an even greater decrease in the amount of PCR products was seen when this agent was combined with hyperthermia.

Discussion

Most prior research on metastases has focused on elucidation of the mechanisms involved in the metastatic process. In contrast, little attention has been directed to therapeutic approaches to metastatic cancer cells, possibly because few effective experimental metastasis models have been established. The nude mouse has been the host most frequently used in xenograft experiments, although this metastasis model suffers from several drawbacks, including (1) a low rate of successful tumor implantation, (2) the necessity for special pathogen-free facilities to raise the animals, (3) a long waiting period before results are obtained, and (4) an extremely low rate of metastasis formation.

In the present study, we used the chick embryonic assay, which is simple and inexpensive to perform and yields a high rate of tumor implantation, to detect metastatic tumors

and quantify the sensitivity of metastatic tumors to various forms of therapy [1, 3–5, 7]. This chick embryonic assay has not been entirely satisfactory as a quantitative metastasis experimental model because the amount of tumor cells in the developing metastatic foci is extremely small due to the short experimental period of 7 days; thus, detection of metastatic foci has depended on the histopathological findings.

Recently, the PCR technique has been developed for highly sensitive detection by amplifying a small segment of DNA flanked by oligonucleotide primers of known DNA sequences [6]. In the present study, using the PCR we amplified a specific human tumor-cell-derived DNA sequence present in embryonic chick liver metastases and analyzed it by Southern blotting. As a result, it was shown that (1) it is possible to detact with efficiency metastatic cells in chick embryos inoculated with human tumor cells by subjecting DNA samples from the chick embryo organs to PCR to amplify a DNA fragment specific to the human β-globin gene and then detecting those amplified fragments, and (2) this method is an excellent quantitative experimental metastasis model that has considerable promise as a screening method that might provide useful information about the inhibitory effects of various anticancer agents and hyperthermia on human tumor metastases.

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