Subrenal Capsule Tumor Implant Assay with Frozen Tumor Tissue: A Preliminary Study Using Mouse Sarcoma and Human Uterine Cervix Cancer

Soon Beom Kang and Jong-Hyeok Kim

Department of Obstetrics and Gynecology, Seoul National University College of Medicine, Seoul 110-744, Korea

= Abstract = **The purpose of this study is to evaluate the possibility of application of SRCA to previously frozen tumor tissue. Mouse sarcoma-180 and human squamous cell carcinoma of the uterine cervix were used as implanting tumor specimens. The fresh tumor specimens were frozen by rapid freezing method and stored in a liquid nitrogen tank. On the 7th day of freezing, the specimens were thawed and diced into 1 cubic millimeter pieces, and implanted beneath the subrenal capsule of ICR mice. The average viabilities before freezing and after thawing were 86.7% and 10.5% in mouse sarcoma-180 respectively. And those of human uterine cervix cancer were 73.3% and 9.5% respectively. The evaluability of implanted tumor was 83.3% on the 6th day and 73.7% on the 12th day in mouse sarcoma, and in human cervix cancer, 75.0% and 66.7% respectively. The growth of previously frozen implanted mouse sarcoma was significant, 2.1 times on the 6th day and 73.9 times on the 12th day in volume. In the case of cervix cancer, the previously frozen tumor implants also grew significantly, 1.7 times on the 6th day and 2.1 times on the 12th day in volume. In conclusion, this preliminary laboratory study showed that SRCA could be applied to frozen tumor tissue as well as to fresh tissue for chemosensitivity test.**

Key Words: *Subrenal capsule tumor implant assay (SRCA), Chemosensitivity test, Frozen tumor tissue*

INTRODUCTION

Patients with advanced stage cancers are frequently treated with chemotherapeutic agents. But the chemotherapeutic agents are chosen on the basis of the statistical evaluation of clinical studies involving many patients and physician preferences, not on the responsiveness of individual tumors to chemotherapeutic agents because of the lack of a suitable tumor sensitivity test. Thus several chemosensitivity tests have been tried to predict the chemosensitivity of malignant tumors. For example, the double-layer- soft-agar method was developed. This technique is now commonly referred to as the "clonogenic assay". But this assay has several current problems, including low plating efficiencies, nonstandardized drug concentrations,

methods of exposure, existence of nonstandardized criteria for in vitro sensitivity, and methods of colony counting. Furthermore, the in vitro tissue culture systems bypass the normal metabolic processing capacity of the animal and therefore might be inaccurate in reflecting the responsiveness of the whole animal to chemotherapeutic agents. Many tumors cannot grow easily in tissue culture and up to 75% of tumor specimens may be unevaluable due to insufficient growth of control cultures.

So the subrenal capsule assay(SRCA) was adapted for "in vivo" chemosensitivity testing. Originally the subrenal capsule assay was performed with athymic mice to aid in new drug screening, but it has been modified to handle fresh human tumor specimens. This assay is a precise technique which makes it possible to quantify minute changes in the size of human tumor xenografts implanted into the sub-capsular region of the kidney in normal immunocompetent mice. The subrenal capsule assay is reliable technique for predicting clinical response to chemotherapy. This assay is relatively simple to perform and results are available within a week, so the test is quick enough to benefit the patient. Also, the relation between the efficacy of cytostatic treatment and its toxicity may be evaluated in this test. In Korea, we have already performed many SRC assays for various gynecologic malignancies to evaluate the chemosensitivities, the toxicities and the histopathologic changes of the tumor implants.

Unfortunately because fresh tumor tissue is required in SRCA, it has to be performed immediately after surgery or biopsy. Moreover when the tumor is recurrent and there is difficulty in resampling fresh tumor specimens or when the decision on the chemotherapy is delayed, it can not be performed due to the absence of original fresh tumor tissue. The purpose of this study is to evaluate the possibility of application of SRCA to previously frozen tumor tissue.

MATERIAL AND METHODS

Mice with sarcomatous ascites from the Biologic Science Department of Seoul National University were used as the sources of sarcoma-180 tissue. The ascites were tapped and centrifuged and the precipitates were injected into the subcutaneous space of ICR mice to form solid tumors. In the case of human squamous cell carcinoma of the uterine cervix, tumor specimens were obtained at the time of operation from patients, most of whom had advanced malignancies and were being treated in the Gynecologic Department of Seoul National University Hospital from January 1990 to February 1990. Specimens from six mice and six patients were used respectively.

The obtained fresh tumor specimens were cleaned of blood, necrotic tissue and fat and sectioned into about $3 \times 3 \times 3$ cubic millimeter pieces and immediately placed into sterile cryotubes containing 7.5% of DMSO(dimethyl sulfoxide; Merck, Darmstadt, West Germany), 40% FBS(fetal bovine serum; GIBCO, Grand Island, USA) and MEM(minimum essential medium; Irvine Scientific, USA). The specimens in the cryotubes(NUNC, Roskilde, Denmark) were frozen at $-20°C$ for 1 hour and at $-70°C$ for 6 hours and stored in a liquid nitrogen tank($-196°C$; MVE, USA). On the 7th day of freezing, the specimens were thawed in a $37°C$-water bath for 5 minutes.

Just before freezing and after thawing, a viability test of the tumor specimen was done with trypan blue. The thawed tumor tissue was diced into nearly 1 cubic millimeter pieces with No. 10 blades. While preparing the fragments of the tumor, young female adult ICR mice weighing 25-35 gm were anesthetized with intraperitoneal injections of 0.22 M chloral hydrate solution (Yakuri Pure Chemical Co., Osaka, Japan), and then shaved on the left dorsal area and numbered. An 5-8 mm skin incision was made on the left dorsum and the left kidney was exteriorized. Then a small nick was made in the kidney cap-
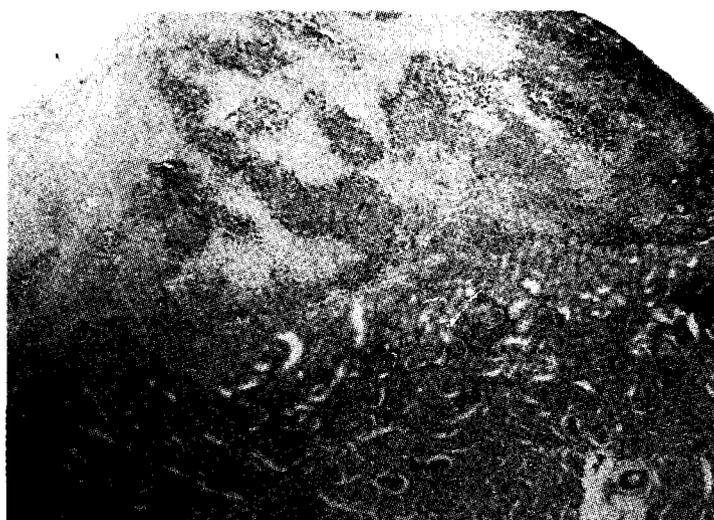
Fig. 1. Microscopic picture of mouse sarcoma-180 on the 6th day implanted in the subrenal capsule of a mouse after freezing for 7 days (× 100).
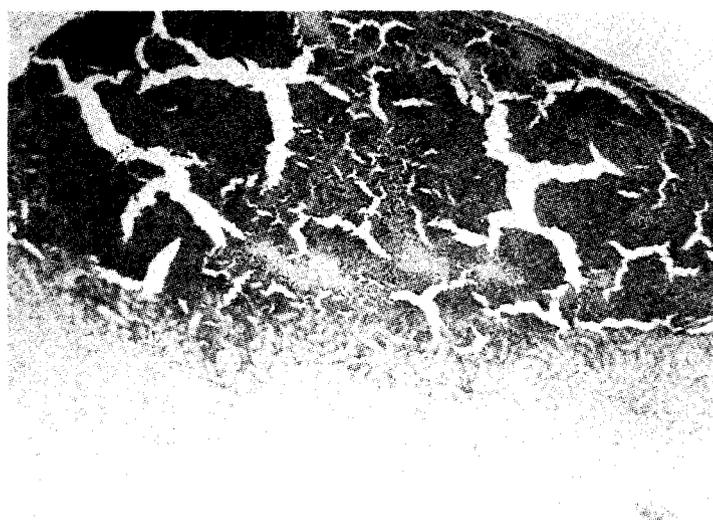

Fig. 2. Microscopic picture of mouse sarcoma-180 on the 12th day implanted in the subrenal capsule of a mouse after freezing for 7 days (× 100).

Fig. 3. Microscopic picture of human squamous cell carcinoma of the uterine cervix on the 6th day implanted in the subrenal capsule of a mouse after freezing for 7 days (× 100).
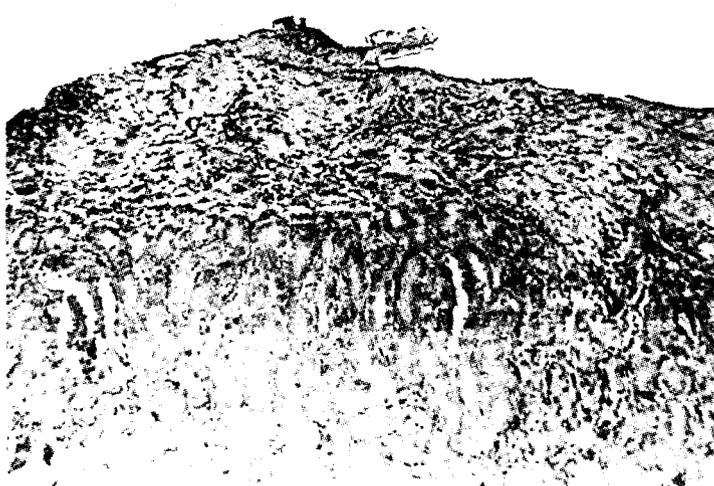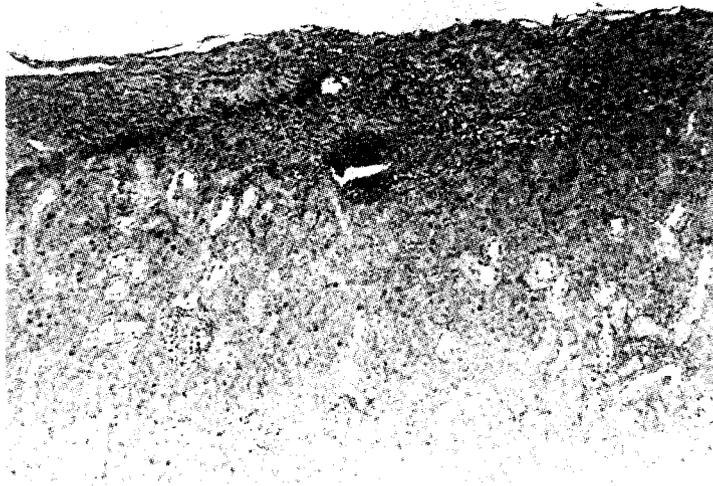

Fig. 4. Microscopic picture of human squamous cell carcinoma of the uterine cervix on the 12th day implanted in the subrenal capsule of a mouse after freezing for 7 days (× 100).

sule with a No. 11 surgical blade. A piece of tumor fragment was inserted into the subcapsular region using a 20-gauge needle with trocar. The implanted tumor fragment was measured in two dimensions with ocular microunits (OMU; 10 OMU = 1.0mm) under a stereomicroscope (Nikon, Type 102). After measuring the size of the implant, the kidney was returned into the abdominal cavity and the wound was closed using a stapler. Animals were allowed to recover from surgery in a cage under a heat lamp.

In the case of human uterine cervical cancer, the immumosuppressive agent, cyclosporin-A(SIGMA Chemical Co., St.Louis, USA) was injected subcutaneously at a dose of 30 mg/kg daily to suppress the immune reaction to the xenografts.

The experimental mice were divided into four groups; the 6th day-group of mouse sarcoma, the 12th day-group of mouse sarcoma, the 6th day-group of cervix cancer and the 12th day-group of cervix cancer according to the day of

evaluating tumor growth and the kind of tumor specimen. Each tumor specimen was implanted in 6 to 7 experimental mice per group, so in total 146 SRCA were performed. The mice were sacrificed by cervical dislocation on the 6th or 12th day according to the group. Then the tumor-bearing kidneys were exposed and the tumor implants measured once again in situ. The average change in tumor size in width and length measurements was calculated for each animal. A theoretical volume of the tumor can be calculated using the formula for the volume of an ellipsoid, $(L \times W^2)/2$. We compared the means of width and length of implants to evaluate growth of implants and the growth of implanted tumor was histologically confirmed by a pathologist under hematoxylin-eosin staining.

The evaluable assays were confined to the cases where the mean of width and length of the implant had increased and the area of necrosis did not exceed 50 percent. Paired t-test was used for statistical analysis.

RESULTS

Average viabilities before freezing and after thawing

The average viabilities before freezing and after thawing were 86.7 ± 9.0% (range; 70~95%) and 10.5 ± 2.7% (range; 6~15%) in mouse sarcoma-180 respectively. And those of human uterine cervix cancer were 73.3 ± 9.5% (range; 60~90%) and 9.5 ± 2.4% (range; 5~12%) respectively(Table 1).

Evaluability of implanted tumor

The evaluability of implanted tumor was 83.3%(30/36) on the 6th day and 73.7% (28/38) on the 12th day in mouse sarcoma-180, and in human uterine cervical cancer, 75.0%(27/36) and 66.7%(24/36) respectively. The unevaluable implants included the missing ones(11 implants) or those showing regression(26 implants)(Table 2).

Growth of implants

The growth of previously frozen and thawed

Table 1. Cell viabilities of tumor specimens before freezing and after thawing

	Before Freezing	After Thawing
Mouse sarcoma-180	86.7 ± 9.0% (70–95%)	10.5 ± 2.7% (6–15%)
Uterine cervix cancer	73.3 ± 11.0% (60–90%)	9.5 ± 2.4% (5–12%)

*Mean ± Standard deviation (Range)

Table 2. Evaluabilities of implants

	Day 6	Day 12
Mouse sarcoma-180	83.3%(30/36)	73.7%(28/38)
Uterine cervix cancer	75.0%(27/36)	66.7%(24/38)

*Unevaluable: Missing or necrosis of implants

implants of mouse sarcoma-180 was significant. The increment in volume was 2.1 times on the 6th day (from 10.2 ± 1.2 OMU to 13.5 1 ± 1.2 OMU in mean of width and length) and 73.9 times on the 12th day (from 10.6 ± 1.2 OMU to 45.5 ± 12.9 OMU in mean of width and length). In the case of uterine cervical cancer, the previously frozen and thawed tumor implants also grew significantly, 1.7 times on the 6th day (from 10.0 ± 0.8 OMU to 11.9 ± 0.9 OMU in mean of width and length)and 2.1 times on the 12th day (from 10.2 ± 0.6 OMU to 13.5 ± 1.8 OMU in mean of width and length)(Table 3,4).

DISCUSSION

For over 10 years, many clinical investigators have made considerable efforts to develop an in vivo test that could accurately predict the chemosensitivity of a malignant tumor such as microbial culture and sensitivity assays. A number of assay techniques have evolved including "clonogenic assay". The preliminary clinical experience with the stem cell clonogenic assay was encouraging. The overall accuracy of the test was reported to be greater than 75% with

Table 3. Cell viabilities of tumor specimens before freezing and after thawing

	Initial OMU	Final OMU	P-value	Increment in volume
Day 6	10.3 ± 1.2	13.5 ± 1.2	<0.01	2.1 times
Day 12	10.6 ± 1.2	45.5 ± 12.9	<0.01	73.9 times

*Mean ± Standard deviation

Table 4. Growth of human uterine cervical cancer implants

	Initial OMU	Final OMU	P-value	Increment in volume
Day 6	10.3 ± 0.8	11.9 ± 0.9	<0.01	21.7 times
Day 12	10.2 ± 0.2	13.5 ± 1.8	<0.01	2.1 times

*Mean ± Standard deviation

85-92% for the prediction of clinical resistance to chemotherapy. But the current problems in the assay methods include low plating efficiencies, nonstandardized drug concentrations and methods of exposure, existence of nonstandardized criteria for in vitro sensitivity, and methods of colony counting. The major disadvantages of the clonogenic assay are the dismal cloning efficiency of about 0.001%, evaluable growth rates of 23% to 36%, the high incidence of bacterial and fungal contamination, the long time necessary for evaluable clones to develop, and the technical difficulties of obtaining a single cell suspension.

But there are several merits of the SRC assay. The use of fresh tumor fragments provides well preserved tissue permeability barriers for chemotherapeutic drug testing. Also, drugs that need in vivo activation such as cyclophosphamide are evaluable in this in vivo assay. Conceptually intact cell-to-cell contact and spatial relationships considered to be important in predicting true drug activity can be afforded by SRC assay. The SRC assay is technically simple and the results are available within a week allowing use of the results in the treatment of cancer patients.

In our study, the viabilities of tumor tissue

after thawing might have been dramatically improved if we had adopted procedures for single cell suspension before freezing. But as mentioned earlier, procedures for single cell suspension are technically difficult, need much time, are expensive and through these procedures tumor tissue may lose its own stromal microstructures. So we selected the method of direct freezing and thawing of intact tumor tissue.

Tumors have the potential to grow in the subrenal capsule assay because of the rich blood supply to the subcapsular region. The small size of the tumor graft allows for diffusion of nutrients as well as test drugs into the implant without the lag time that would be required to develop a vascular supply. Many studies have shown that the increase in tumor size of untreated tumors is related to the mitotic activity as well as to the degree of tumor necrosis present in the surgical specimen. Recent histologic studies by Reale et al. showed that (a) tumor histologic architecture is preserved in the implants up to day 6 in both immunocompetent and athymic nude mice; (b) host infiltrate of inflammatory cells begins as early as day 3 and peaks at day 10; (c) the extent of infiltration does not significantly affect tumor size up to and through day 6.

Levi et al. (1984) found that fresh implants of

human tumor retain their proliferative and metabolic capacity at least 4 days after implantation in the renal capsule of immunocompetent mice. He also found that the mean tumor size from any tumor specimen did not differ between days 4 and 6. These observations suggest that the cellular infiltration is not artifactual to the extent that it precludes the validity of a simple tumor size parameter for evaluating drug effects. Furthermore, the immune system of the mice cannot reject the implant of the tumor within such a short 6-day time frame.

But frozen and thawed tumor specimens in our study had only a small portion(approximately 10%) of viable cells. So if we should perform the chemosensitivity test by injecting chemotherapeutic agents at the time of implantation, almost all viable tumor cells might be sacrificed and interpretation of SRCA be impossible. To solve this problem, we planned the assay to continue till the 12th day of implantation for sufficient growth of implants, and injection of the chemotherapeutic agent could be started from the 7th day of implantation. In other words, we tested the possibility of the growth of the frozen and thawed tumor tissues in subrenal capsular space in the 6-day groups and then in the control groups of chemosensitivity by SRCA with those tissues in the 12-day groups.

Hunter *et al.* (1982) reported that an average of 60% showed positive growth and 11% demonstrated no measurable change in size of untreated tumors implanted. In our previous study, we obtained similiar positive growth(73. 8%) and no measurable growth (14.1%). In the present study, the evaluabilities mean the proportions of positive growth as defined and ranged from 66.7% to 83.3%. This showed that the growth of frozen and thawed tissue was comparable to that of fresh tissue.

In the case of human uterine cervix cancer implants, immunocompetent ICR mice show infiltration of inflammatory cells as an immune reaction to xenograft. This had not been a problem in 4 -day or 6-day SRCA using fresh tumor tissue because the immune system of the mice cannot reject the implant of the tumor within such a short 6-day time frame. But in the present study, because we continued the assay till the 12th day of implantation, immunosuppression against xenograft rejection was required. Kusuyama *et al.* reported succesful immunosuppression by alternate daily subcutaneous injection of bredinin at a dose of 200mg/Kg or by daily subcutaneous injection of cyclosporin-A at dose of 60mg/Kg. We obtained excellent immunosuppression by daily subcutaneous injection of cyclosporin-A at a dose of 60mg/Kg to the dorsa of the mice.

We adopted the rapid-cooling freezing method of 1 hour-freezing at -20°C followed by 6 hour-freezing at -70°C to simplify the procedure. However because it is known that slow-cooling freezing provides better viabilities of cells, more studies are required of the cooling rate in SRCA with frozen tissue.

In the present study, we selected dimethyl sulfoxide as a cryoprotectant according to the method of Lovelock and Bishop, but other cryoprotectants such as glycerol or propylene could be applied in further studies.

In conclusion, this preliminary laboratory study showed that frozen and thawed tumor tissues could grow in subrenal capsular space till the 12th day of implantation and so SRCA could be applied to frozen tumor tissue as well as fresh tissue for chemosensitivity test. From now on, we can perform SRCA at anytime we need by freezing of the tumor tissue at the time of surgery or biopsy.

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