The Seoul Journal of Medicine
Vol. 27, No. 2:169-172, June 1986

Comparison of Agar and Agarose Culture Methods for Human Tumor Stem Cell Assay of Gynecologic Tumors

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= Abstract =The human tumor stem cell assay (HTSCA) has broad research applications in the field of cell biology as well as in clinical chemotherapy. A prospective clinical trial for improvement of the *in vitro* growth rate of gynecologic cancers was done between agar and agarose culture methods. Specimens from 27 gynecologic tumors were disaggregated by mechanical and enzymatic methods and assayed in soft agar and agarose culture matrix. 9 of 27 tumors grew in the agar system yielding a cloning success rate of 33.3%. Of 27 specimens, 17 have shown colony growth in the agarose culture matrix showing a cloning success rate of 63.0%. Cloning efficiency in agarose improved approximately 2-fold as compared with that in agar. The development of a soft agarose assay for gynecologic tumor cells will provide a possibility of an *in vitro* technique for predicting *in vivo* response to anticancer drugs.

Key Words: ***Agar, Agarose, Clone Cells, Gynecologic Tumor***

INTRODUCTION

Since the late 1970's, many laboratories across the world have begun performing the Human Tumor Stem Cell Assay (Hamburger and Salmon 1977; Hamburger *et al.* 1978). Considerable variation in rates of successful growth *in vitro* have been published.

But this assay has not been totally successful for growing tumors of gynecological field though Welander *et al.* (1983) have successfully grown 183 of 207 (88.4% growth rate) of gynecologic tumors from primary lesions and metastases. Schiff and Shugar (1984) reported that growth of human head and neck squamous cell carcinoma stem cells in agarose media was better than that in agar media. Until now, there has been no comparison report of agar and agarose in the Human Tumor Stem Cell Assay of gynecologic field.

Our objective in this study was to compare the cloning success rate and the cloning efficiency in agar and agarose culture methods for human tumor stem cell assay of gynecologic tumors and to apply these findings to provide a basis for investigating therapeutic modalities used in the clinical management of gynecologic cancer patients, as well as for studying the fundamental biologic behavior of gynecologic cancers.

MATERIALS AND METHODS

1. **Tumor procurement**: Between May, 1985 and December, 1985, specimens from 27 patients having primary carcinomas of either ovary, cervix, or endometrium were obtained at the department of obstetrics and gynecology of the Seoul National University Hospital for study with the HTSCA. These tumors included 7 ovarian, 15 cervical, and 5 endometrial carcinomas. The distribution of solid tumor specimens is shown in Table 1. Surgical specimens were placed in sterile container with approximately 20 ml of Rosewell Park Memorial Institute (RPMI) 1640 medium (Gibco laboratory, Grand Island, NY) with 100 mg/ml of streptomycin (Calbiochem, La Jolla, California), 100 mg/ml of penicillin G (Sigma chemical Co., St. Louis, Missouri), and kept at 4°C. Malignant effusions were collected in sterile bottles containing 1 unit/ml of preservative free heparin. All these specimens were processed the same day as obtained from the patient, with a maximum delay of 4 hours.

2. **Preparation of single cell suspensions**: Solid tumor specimens were washed 3 to 5 times

Table 1. Type of tumors from 27 patients

Tumor type	No. of specimens
Ovary	7
Cervix	15
Endometrium	5
Total	27

with RPMI medium and then minced with scalpels into small fragments. These tissue fragments were then incubated at 37°C in DNAse 0.03% (Calbiochem, La Jolla, Chalifornia). This suspension is then worked with progressively small needles, beginning with 18 gauge down to 26 gauge in order to break up clusters of cells. The cells are then layered onto a Ficoll–Paque gradient (Pharmacia, Piscataway, New Jersey) to separate dead cells and cellular debris from viable cells (Gaines *et al.* 1983). The gradient interface is collected and cells are counted. The cell viability for each isolated cell populaton was determined using the Trypan Blue dye exclusion method (Phillips 1973).

3. Assay for colony-forming cells: The two-layer agar procedure described by Hamburger and Salmon(1977) was used. Following dispersion, 5×10^6 cells per ml were suspended in 0.3% agar in enriched RPMI 1640 medium (× 1.5 strength) supplemented with 15% fetal calf serum (Gibco, Grand Island, NY), 100 mg/ml penicillin G, 100 mg/ml streptomycin, 2 mM glutamine (Gibco, Grand Island, NY), and 1 unit/ml insulin. This medium-agar mixture was added to a feeder layer consisting of enriched RPMI 1640 (× 1.5 strength) containing 15% fetal calf serum, 30 ng/ml of epidermal growth factor (Collaborative Research, Lexington, MA) and agar at a final concentration of 0.5%. Agarose was also evaluated in the standard Salmon technique, as has been reported. The cells were also suspended in the same concentration of agarose (Seaplaque agarose, M.A. Bioproducts, Walkersville, MD) 0.3%, with an underlayer of 0.5% agarose. Once the top and bottom layer agar/agarose layers were added to the 35 × 10-mm dishes, and permitted to solidify, the plates were inspected for cell clumps. Only plates with single cell suspension were used in this study. The cultures were incubated at 37°C in a 7.5% CO_2 and air atmosphere. After 10 to 21 days in culture, colonies were visible and the plate could be counted with an inverted phase microscope. Groups of approximately 20 or more cells were considered colonies and 5 to 20 cells were clusters. For a specimen to be considered as growing, there must be a minimum of 30 colonies in the plate.

4. Histologic verification for morphologic studies: Permanent slides for histologic analysis of agar/agarose cultures were prepared using the dried slide method described by Salmon and Buick(1979). In this method, the dishes are first washed with balanced salt solution to remove background staining, then fixed with 3% glutaraldehyde for 10 minutes. The agar/agarose was then removed from the culture dish and air dried on a microscopic slide overnight. Staining was done with the Papanicolaou stain (Salmon and Buick 1979).

RESULTS

The percentage of tumor specimens which grew in the agar and agarose media is shown in Table 2. Of the 27 gynecological tumors studied, 9 (33.3% growth rate) grew in the agar culture medium and 17 (63.0% growth rate) grew in the agarose culture medium. Of the 7 ovarian tumors studied, 5 (71.4% growth rate) in agarose and 4 (57.1% growth rate) in agar grew. Of the 15 cervical cancers tested in the laboratory, 9 grew (60.0%) in agarose and 3 grew (20.0%) in agar. Of the 5 endometrial cancers, 3 (60.0%) in agarose and 2 (40.0%) in agar grew. The results of the colony counts are shown in Table 3, reported as an average of triplicate plate in each group. The value was calculated by X^2 and paired t test. Although the end point of tumor cell colony formation was not an all or nothing result, colony growth seemed to be enhanced by the agarose culture matrix. Fig. 1 illustrates ovarian colony formation after 21 day culture using inverted microscopic examination. After 3 days in culture, clusters (5-20 cells) of irregularly shaped cells appeared. After 7 days, the colonies appeared as tightly packed cells without a

Table 2. Growth of gynecologic cancer stem cells

Tumor type	No. of specimens		Growth rate(%)
	Rec'd	Grew	
		Agarose/Agar	Agarose/Agar
Ovary	7	5/4	71.4/57.1
Cervix	15	9*/3	60.0/20.0
Endometrium	5	3/2	60.0/40.0
Total	27	17*/9	63.0/33.3

*$p<0.05$ *v.s.* agar

Table 3. Effect of agarose on tumor cell colony growth as compared with agar

Tumor type		No. of colonies formed*		Growth
		Agar	Agarose**	increase (%)
Ovary	1	127	316	249
	2	85	153	180
	3	174	337	194
	4	223	512	240
Cervix	5	26	45	173
	6	33	89	270
	7	41	74	229
Endometrium	8	124	321	259
	9	79	133	168
Mean±S.E.M.		101±21	222±52	214

*No. of colonies formed at 21st day is an average of three plates in each group ($p<0.01$).
**$p<0.05$ *v.s.* agar

free cell layer. The number of cells per colony increased at 14 days and reached peak size at 21 days. The morphology of the colonies in agar or agarose did not vary in histologic examination.

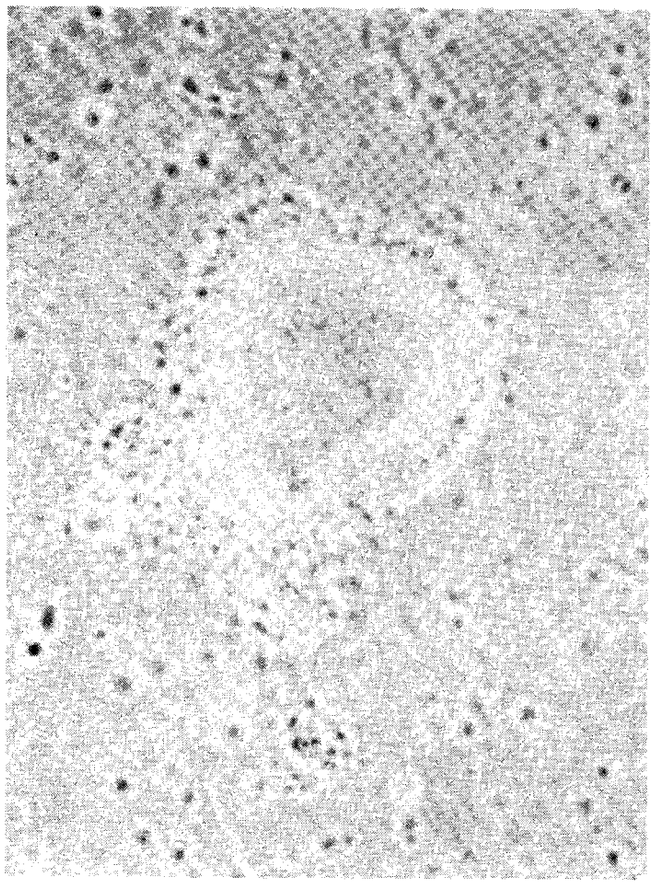

Fig. 1. Photomicrograph of ovarian cancer cells growing as a colony in agarose at 21st day of incubation using inverted microscope (× 200).

DISCUSSION

The current study was initiated with the hope of improving the success rate of growing gynecologic cancers in a clonal assay. We examined the effect of an agarose culture matrix on colonial growth. In this study, tumor cells from patients with gynecologic cancers showed the ability to grow in semisolid medium. Colony growth seemed to be increased in agarose comparing with agar. Cells plated in agarose also exhibited a 2-fold increase in cloning efficiency. We chose to use agarose based on the report of Neugut and Weinstein (1979) and Schiff and Shugar (1984) who showed that agarose was a more sensitive marker for tumorigenicity. When morphologically transformed foci of rat embryo cells were cloned, early passages grew but not in agar. During later passages, colony formation in agar increased. It was suggested that growth in agar requires more extensive changes in gene control, *i.e.*, suppressor genes that prevent expression of transformation, than growth in agarose. A similar genetic mechanism may be responsible for the differences in growth of gynecologic cancers demonstrated in agar and agarose.

There are a number of nutritional factors and culture conditions having influence on HTSCA. Our laboratory has had a 63.0% growth rate processing these 27 gynecologic tumors. This is lower than Welander *et al.*'s report and may be related to the difference of growth enhancing agent between our epidermal growth factor and Welander *et al.*'s xenogeneic peritoneal macrophage (Welander *et al.* 1983). Improvement in cell disaggregation techniques, requirement of a feeder layer, and addition of growth factors should help increase the plating efficiency in this system (Soehnlen *et al.* 1980; Kubota *et al.* 1982; Welander *et al.* 1982). Our results, utilizing agarose in the HTSCA will provide a basis for investigating various therapeutic modalities used in the clinical management of gynecologic cancer patients, as well as for studying the basic biologic behavior of gynecologic cancers.

REFERENCES

Gaines CE, Homesley HD, Jobson VW. *In vitro* chemotherapy testing of gynecologic tumors: Basis for planning therapy? Am. J. Obstet. Gynecol. 1983, 147:188-191

Hamburger, AW, Salmon SE. Primary bioassay of human tumor stem cells. Science 1977. 197:461-463

Hamburger AW, Salmon SE, Kim MB, Direct cloning of human ovarian carcinoma cells in agar. Cancer Res. 1978, 38:3438-3443

Kubota K, *et al.* Comparison of agar and methylcellulose culture methods for human erythroid colony formation. Exp. Hematol. 1982, 10(3):292-296

Neugut AI, Weinstein IB. Use of agarose in the determination of anchorage-independent growth. *In Vitro* 1979, 15:351-354

Phillips HJ. Dye exclusion tests for cell viability. In: Kruse PF, Patterson MK(Ed) Tissue culture methods application. New York Academic Press, 1973:pp. 406

Salmon SE, Buick RN. Preparation of permanent slides of intact soft-agar colony clusters of hematopoietic and tumor stem cells. Cancer Res. 1979, 38:1133-1136

Schiff LJ, Shugar MA. Growth of human head and neck squamous cell carcinoma stem cells in agarose. Cancer 1984, 53:286-288

Soehnlen B, Young L, Liu R. Standard laboratory procedures for *in vitro* assay of human tumor stem cells. Cloning of human tumor stem cells. Alan R Liss Inc. 1980:pp. 331

Welander CE, Homesley HD, Jobson VW. *In vitro* chemotherapy testing of gynecologic tumors: Basis for planning therapy? Am. J. Obstet. Gynecol. 1983, 147:188-195

Welander CE, Natale RB, Lewis JL Jr. *In vitro* growth stimulation of human ovarian cancer cells by xenogeneic peritoneal macrophages. J. Natl. Cancer. Inst. 1982, 69:1039-1044

= 국문초록 =

부인과 영역의 악성종양에 있어서 human tumor stem cell assay를 위한 agar배지와 agarose배지의 비교

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이효표 · 차상헌

Human tumor stem cell assay (HTSCA)는 종양세포생물학의 연구뿐만아니라 항암제의 감수성을 측정하는 데 가장 유용한 방법의 하나로 알려져 있다. 저자들은 agar와 agarose배지를 이용하여 27례의 부인과 악성종양의 세포집락형성을 비교 연구하였다.

Agar 배지에서는 27례중 9례에서 세포집락형성을 보여서 33.3%의 성장율을 나타내었고 agarose 배지에서는 27례중 17례에서 세포집락형성을 보여 63.0%의 성장율를 보였다. 그리고 agarose배지에서의 세포집락 형성율은 agar 배지에 비해 약 2배의 성적을 나타내었다.