

Acta Medica Okayama

Volume 37, Issue 2

1983

Article 6

APRIL 1983

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Abstract

We applied a tumor stem cell assay using an enriched double-layered soft agar system for the detection of metastatic sites of lung cancer. Lung cancer colonies grew from 7 of 10 effusions cytologically positive for tumor cells and 7 of 10 bone marrow aspirates cytologically and histologically positive for tumor cells. Twenty-six of 29 bone marrow aspirates cytologically and histologically negative for tumor cells showed no colony growth. However, the remaining three bone marrow aspirates, which were obtained from patients with small cell lung cancer, formed colonies in soft agar. These results indicate that the tumor stem cell assay is useful for detecting metastatic sites of lung cancer.

KEYWORDS: tumor stem cell assay, colony growth, human lung cancer

*PMID: 6869064 [PubMed - indexed for MEDLINE]

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Acta Med. Okayama 37, (2), 141—146 (1983)

TUMOR STEM CELL ASSAY FOR DETECTING METASTASES OF HUMAN LUNG CANCER

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Received November 4, 1982

Abstract. We applied a tumor stem cell assay using an enriched double-layered soft agar system for the detection of metastatic sites of lung cancer. Lung cancer colonies grew from 7 of 10 effusions cytologically positive for tumor cells and 7 of 10 bone marrow aspirates cytologically and histologically positive for tumor cells. Twenty-six of 29 bone marrow aspirates cytologically and histologically negative for tumor cells showed no colony growth. However, the remaining three bone marrow aspirates, which were obtained from patients with small cell lung cancer, formed colonies in soft agar. These results indicate that the tumor stem cell assay is useful for detecting metastatic sites of lung cancer.

Key words: tumor stem cell assay, colony growth, human lung cancer.

Unfortunately, in many instances, malignancies of patients with lung cancer are not resectable at the time of diagnosis. Detection of metastases at the time of diagnosis has a great influence on the therapeutic approach. Accurate staging provides valuable information about which treatment is most appropriate.

Since Hamburger and Salmon (1, 2) reported a soft agar culture system for the assay of human tumor "stem cells" (self-renewing cells), several investigators (3-10) have assessed colony growth in soft agar of cells from a variety of human tumors. The purpose of the present study was to evaluate a tumor stem cell assay for the detection of metastatic sites of lung cancer. We describe in this paper the successful colony growth in soft agar of tumor cells from specimens taken directly from patients with lung cancer.

MATERIALS AND METHODS

Patients and specimens. Patients with well-documented lung cancer were selected for this study. The histological classification of lung cancer was performed according to WP-L classification. Specimens were obtained by aspiration of pleural effusions and bone marrows and by biopsy of lymph nodes and the primary tumor. A part of each specimen was processed for cytological and histological examination. Bone marrow biopsies were done for routine histological study in all patients with a Jamshidi needle from bilateral posterior iliac crests. The biopsied specimens were cut into small pieces with scissors and single-cell suspensions

were prepared by treatment with 0.8 % collagenase II (Sigma Chemical Co.) and 0.002 % DNase I (Sigma Chemical Co.) in RPMI 1640 medium (Grand Island Biological Co.) at 37°C in a humidified atmosphere of 5 % CO₂ in air for 2 h (7). Pleural effusions and bone marrow aspirates were collected in a syringe containing preservative-free heparin (100 units/ml), and red blood cells were eliminated by the Ficoll-Conray specific gravity method.

Tumor stem cell assay. Cells were cultured by a modification of the method described by Hamburger and Salmon (1, 2). Single cells were suspended in 0.3 % agar in RPMI 1640 medium supplemented with 15 % fetal bovine serum (FBS, Flow Laboratories). One ml of the mixture was pipetted onto triplicate 1 ml feeder layers that had hardened in 35-mm plastic Petri dishes. The feeder layers consisted of 0.5 % agar and RPMI 1640 medium supplemented with 15 % FBS. The final concentration of cells in each plate was $1-5 \times 10^5$ viable nucleated cells. The plates were then incubated at 37°C in a humidified atmosphere of 5 % CO₂ in air. Colony counts were made 14 days after plating. Aggregates of 30 or more cells were considered colonies. Colonies were picked up with a Pasteur pipet, placed on a glass slide and stained with May-Giemsa.

RESULTS

Cell aggregates consisting of from several to 20 round cells appeared 3 to 5 days after plating. These aggregates gradually grew to colonies after 7 to 16 days of culture. The colonies were observed under an inverted microscope as spherical aggregates of numerous tightly packed round cells (Fig. 1). The cytological characteristics of the lung cancer colonies after May-Giemsa staining were similar to

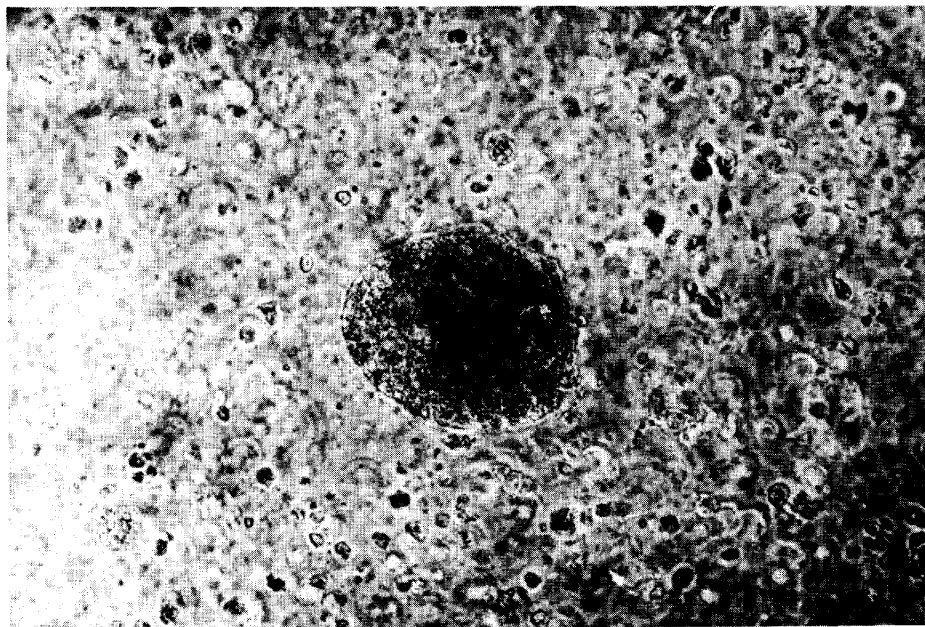


Fig. 1. A lung cancer colony in soft agar 14 days after plating a bone marrow aspirate of a patient with small cell lung cancer. The cells are tightly packed in a spherical aggregate.

those observed in the original specimens. Fig. 2 is an example of dispersed cells from a single small cell lung cancer colony that show the cytological appearance of small cell lung cancer cells, *i.e.*, scant cytoplasm, finely granular chromatin and inconspicuous nucleoli. The number of colonies per plate ranged from 2 to 167. The minimum and maximum plating efficiency (number of lung cancer colonies per number of plated viable nucleated cells times 100 %) were 0.005 and 0.028 % respectively.

The growth of lung cancer stem cell colonies are summarized in Table 1.

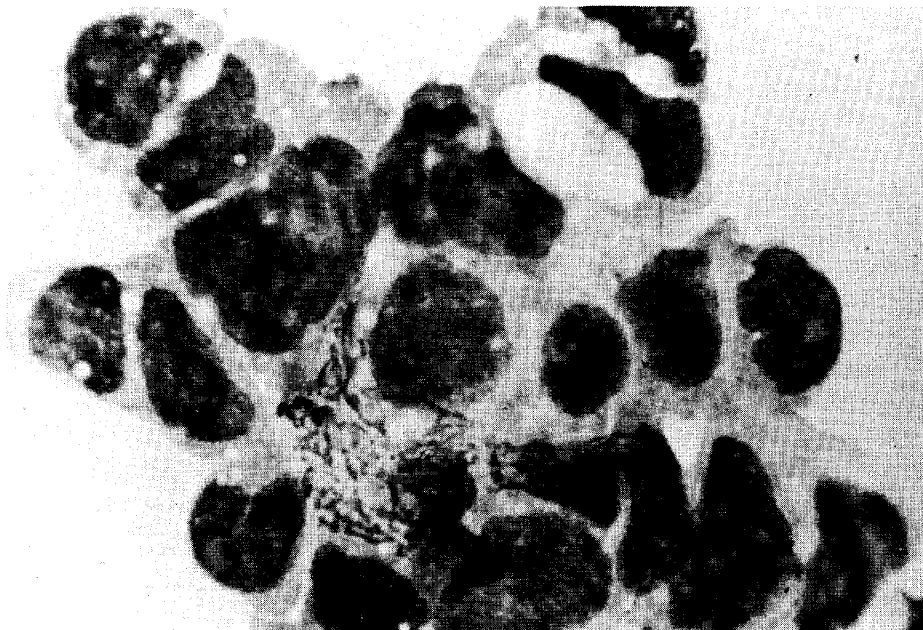


Fig. 2. Dispersed cells from the single colony illustrated in Fig. 1. The cells have the cytological appearance of small cell lung cancer cells.

TABLE 1. GROWTH OF LUNG CANCER STEM CELL COLONIES

Source	Positive for tumor cells				Total	Negative for tumor cells
	Adenoca.	Epidermoid ca.	Small cell ca.			
Pleural effusion	7/9 ^a	—	0/1	7/10	0/7	
Bone marrow	1/3	—	6/7	7/10	3 ^b /29	
Lymph node	1/1	—	—	1/1	—	
Primary tumor	1/2	1/2	—	2/4	—	
Total	10/15	1/2	6/8	17/25	3/36	

^a No. of specimens with colony growth/No. of specimens tested

^b Small cell carcinoma

Lung cancer colonies grew from 68 % of 25 specimens cytologically and histologically positive for tumor cells. Colony growth was observed in 7 of 10 cytologically positive pleural effusions, all 7 of which were cytologically adenocarcinoma. Seven of 10 cytologically and histologically positive bone marrow aspirates showed colonies, and 6 of these were cytologically or histologically small cell carcinoma. Twenty-six of 29 bone marrow aspirates cytologically and histologically negative for tumor cells including 14 small cell carcinoma cases yielded no colonies. However, the remaining three, which were obtained from patients with small cell lung cancer, formed colonies in soft agar. The cells in these colonies had features characteristic of small cell carcinoma. None of 7 cytologically negative pleural effusions produced colonies. Colony formation was found in 3 of 5 tumor specimens obtained surgically.

DISCUSSION

Since Hamburger and Salmon (1, 2) developed a soft agar culture system suitable for direct bioassay of tumor stem cells from biopsy specimens of a variety of human cancers, a few investigators (4, 8, 9) have reported that this technique was suitable for the culture of lung cancer. Carney *et al.* (4) and Pollard *et al.* (9) have shown an excellent correlation between colony growth and histologically confirmed malignancy in fresh tumor specimens of small cell lung cancer using a tumor stem cell assay.

The present study demonstrated that tumor cells from patients with lung cancer formed colonies in soft agar in 68 % of the cases. Growth of colonies was easily achievable from pleural effusions and bone marrow aspirates cytologically and histologically positive for tumor cells. The cytological characteristics of cells which formed colonies in soft agar were identical to those of the tumor cells in the original specimens. The plating efficiencies in the present study ranged from 0.005 to 0.028 %, which were low but comparable to those of tumor cells of fresh specimens by several investigators (2-8, 11).

It should be noted that 3 of 14 cytologically and histologically negative bone marrow aspirates obtained from patients with small cell lung cancer yielded colonies in soft agar. Pollard *et al.* (9) reported that 3 of 37 bone marrow aspirates which were histologically negative for small cell lung cancer formed colonies, and that injection of the colonies into athymic mice cause tumor-induction at the site of inoculation. These findings suggest that the tumor stem cell assay may be more sensitive than routine bone marrow aspirates and biopsies. Several investigators (11-13) have reported on high dose combination chemotherapy with autologous bone marrow transplantation in adults with solid tumors including small cell lung cancer. In such therapeutic trials as these, it is important that the infused autologous bone marrow be free of tumor cells, and a tumor stem cell assay before bone marrow harvesting could aid in avoiding injection of bone marrow containing tumor cells.

Recent reports (14-16) have shown the usefulness of a tumor stem cell assay to determine the sensitivity of an individual patient's tumor to a variety of chemotherapeutic agents in patients with multiple myeloma and ovarian cancer. However, further studies are necessary to determine whether a tumor stem cell assay will be useful for the *in vitro* screening of new chemotherapeutic agents and selecting of the most effective drugs for individual lung cancer patients.

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