Cultivation of cancer cells in malignant pleural effusion and their use in a vascular endothelial growth factor study

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Abstract

Background and Aims: Cancer cells can be isolated from malignant pleural effusion (MPE). They may provide an experimental system to explore the cell biology of lung cancer. Anti-VEGF antibody has been reported to efficiently control MPE, which contains a high concentration of VEGF; this suggests the important role of VEGF acting on MPE. We aimed to develop a method for culturing cancer cells from MPE and investigate the role of VEGF in MPE.

Methods: Cancer cells in MPE were obtained from six patients and cultured using three different types of media, including (1) supernatant of MPE, (2) 1:1 mixture of supernatant and common culture medium, and (3) common culture medium only. We further co-cultured cancer cells with γ-irradiated mouse 3T3-J2 embryonic fibroblasts. Using co-cultured cells, we investigated the effect of VEGF on cancer cells and measured the amount of VEGF secreted from them.

Results: Cancer cells were poorly maintained in three types of media, while well proliferated when co-cultured with 3T3-J2 feeder cells. VEGF didn’t affect cell proliferation. VEGF secreted from cancer cells didn’t reach to a concentration that may show an apparent biological effect.

Conclusion: Proliferation of cancer cells in MPE requires a specific factor(s) in addition to those residing in the supernatant of MPE or conventional culture medium. Co-cultured cancer cells suggested a lack of proliferative effect of VEGF. The concentration of VEGF produced by cancer cells was not sufficient to exert a biological effect. Co-cultured cancer cells may provide a valuable experimental system for further cancer studies.

Keywords: Malignant pleural effusion, Cancer cells, Co-culture, 3T3-J2 feeder cells, Vascular endothelial growth factor

(Received September 28, 2020; Accepted October 13, 2020)

Introduction

Malignant pleuritis is caused by cancer cells metastatically or locally invading the pleural space and manifested as the emergence of malignant pleural effusion (MPE)1,2,3. Cancer cells in MPE often form a colony-like structure, suggesting that the cells floating in the effusion have proliferative capacity. Consequently, many cell lines have been established from MPE. Culture of cancer cells in MPE may be considered easy; however, this is not always the case.

Anti-vascular endothelial growth factor (VEGF) antibody has been shown to effectively control MPE in a clinical trial4. Moreover, a high concentration of VEGF has been reported in MPE5-6. These results suggest that VEGF plays a role in the progression of MPE. VEGF may stimulate cancer cells by an autocrine mechanism, as has been reported in mesothelioma7, or may increase the permeability of intrathoracic vessels, providing nutrients to cancer cells. However, their exact roles have not yet been clarified.

MPE displaces the heart and lungs and compromises their functions. In clinical management, reducing the volume of MPE by drainage is the first step, and a large amount of sterile, waste effusion containing cancer cells is usually obtained. In the current study, we investigated the proliferation of cancer cells and the role of VEGF by examining the waste effusion obtained by drainage.

Materials and Methods

Patient

Malignant pleural effusion was collected from 6 patients with lung adenocarcinoma complicated by MPE. These patients visited our hospital between December, 2017 and July, 2019. All participants provided written informed consent.
Isolation of MPE components

An aliquot of MPE (20 mL) was overlaid onto an equal volume of fetal bovine serum (FBS) placed in a 50 mL conical tube and allowed to stand for 1 h at room temperature. Cancer cells have a greater sedimentation velocity and settle in the bottom of the tube, while blood cells remain at the halfway point (Miyazawa H, personal communication). Cells at the bottom of the tube were collected and washed three times with pre-warmed phosphate buffered saline (Sigma-Aldrich, St. Louis, MO, USA). This procedure condensed the cancer cells without physical damage to the cells (Fig. 1). The other aliquot of MPE was centrifuged (900 × g for 5 min) and the supernatant (MPE supernatant hereafter) was isolated.

Primary culture of cancer cells

Cancer cells were seeded in quintuplicate in a 96-well microplate (REF353072; Falcon, Tewksbury, MA, USA) at 20,000 cells/mL. Cells were cultured in one of three types of media: (1) RPMI-1640 (Sigma-Aldrich) containing 10% FBS, (2) MPE supernatant, or (3) a mixture of (1) and (2). The lung adenocarcinoma cell line, NCI-H1975 (CRL-5908™; ATCC, Manassas, VA, USA), was cultured in RPMI-1640 containing 10% FBS and served as a control for cell growth. All cultures were grown at 37°C in an incubator supplied with 5% CO₂. At 72 h, 10 μL of Cell Counting Kit 8 reagent (Dojindo, Osaka, Japan) was added to each well and the absorbance at 450 nm was measured using a multiple microplate reader (Epoch2; Biotek, Winooski, VT, USA). Significant differences were determined by the Student’s t-test.

Co-culture with feeder cells

Mouse 3T3-J2 embryonic fibroblasts (Kerafast, Boston, MA, USA) were cultured in DMEM (Sigma-Aldrich) containing 10% FBS. Cells were collected at 80% confluency, irradiated by γ-rays using a Gammacell 40 Exactor (Best Theratronics, Ottawa, Ontario, CA) to deprive the proliferative capacity, and seeded on the surface of a flask to form a single cell layer (feeder cell layer). Co-culture of cancer cells with feeder cells was performed by seeding cancer cells on the feeder cell layer in the co-culture medium, which was a 3:1 mixture of Ham’s F-12 medium and DMEM (Sigma-Aldrich); FBS, 5%; hydrocortisone (Sigma-Aldrich), 0.4 μg/mL; insulin (FUJIFILM, Osaka, Japan), 5 μg/mL; cholera toxin (FUJIFILM), 8.4 ng/mL; rH-EGF (SHENANDOAH Biotechnology, Warwick, PA, USA), 10 ng/mL; Adenine (Sigma-Aldrich), 24 μg/mL; and Y-27632 dihydrochloride (Enzo Lifesciences, Farmingdale, NY, USA), 10 μM. Subculture was performed by collecting cancer cells by pipetting and transferred to a new flask without a feeder cell layer. Cell numbers were monitored in triplicate by seeding the cells at 20,000 cells/mL. RealTime-Glo Cell Viability Assay reagent (Promega Corporation, Madison, WI, USA) was added, and the fluorescence was observed at 0, 24, 48, and 72 h. Significant differences were determined using two-way ANOVA.

Cell morphology

Cell morphology was observed under a phase-contrast microscope or after Diff-Quik staining.

Effect of VEGF and anti-VEGF antibody on cell proliferation

Cancer cells of more than three passages after co-culturing were used. Cells were seeded in triplicate in a 96-well microplate at 20,000 cells/mL. VEGF [Recombinant Human VEGF165 Protein, R&D Systems, Minneapolis, MN, USA; Effective dose 50% (ED₅₀) 1–6 ng/mL] at 1000 ng/mL and anti-VEGF antibody [Human VEGF165 Antibody (neutralizing antibody), R&D Systems; Neutralizing dose 50% (ND₅₀) 20–120 ng/mL] at 1000 ng/mL were added to the medium. The cell number was monitored using the RealTime-Glo Cell Viability Assay (Promega, Madison, WI, USA). Human

![Fig. 1 Isolation of cancer cells.](image-url)
umbilical vein endothelial cells (HUVEC; Promocell, Heidelberg, Germany) that respond to VEGF were cultured in Endothelial Growth Medium 2 (EGM2; Promocell) were used as a control. The difference in fluorescence intensity (Envision multi-detection microplate reader; PerkinElmer, Waltham, MA, USA) between the cells to which VEGF was added and the cells to which anti-VEGF-antibody was added was investigated by the Student’s t-test.

**VEGF concentration in the culture supernatant**

Cancer cells of more than three passages after co-culturing were used. Cells were seeded in a 24-well microplate (3820-024; Iwaki, Shizuoka, Japan) at $2 \times 10^5$ cells/well and cultured for 1 week. VEGF concentration in the medium was then tested using the DuoSet ELISA Development System Human VEGF (R&D Systems, USA). VEGF concentration was calculated using 4-parameter logistic (4PL) regression after establishing the standard curve.

**Statistical analysis**

All statistical analyses were performed using the GraphPad Prism (Version 5.01) software package.

**Ethics**

This study was approved by the Ethics Committee of Jichi Medical University (Iden 19-037).

**Results**

**Patient**

Patient characteristics are summarized in Table 1. Adenocarcinoma of the lung was the most frequent cause of MPE.

**Primary culture of cancer cells**

The cancer cells just isolated from MPE is shown (Fig. 2). The cells in MPE often form a colony-like structure. This suggests that cells proliferate in the pleural effusion in situ. We hypothesized that the MPE supernatant would be a good culture medium for the cells because it is the milieu in which they reside. We thus cultured the cells using (1) MPE supernatant, (2) MPE supernatant plus DMEM with 10% FBS, expecting to supply additional nutrients, and (3) DMEM with 10% FBS for 72 h. Unexpectedly, the cells did not proliferate in any of the media (Fig. 3). This suggests that MPE supernatant as well as DMEM with 10% FBS does not provide an adequate environment for cell proliferation.

**Co-culture with feeder cells**

Feeder cells have been reported to help cancer cells proliferate *ex vivo*, probably through cell reprogramming. We thus decided to co-culture with feeder cells. Cells from all patients proliferated (Fig. 4). Once the cells began to proliferate, the feeder cells were no longer required and they continued to proliferate in the co-culture medium. These results suggest that the cells are reprogrammed, at least, in their proliferative capacity.

**Cell Morphology**

We compared the morphology of the cancer cells immediately after isolation from MPE and after co-culturing (Fig. 5A and B). The shape of each cell did not change. However, after co-culturing, the cells formed a colony-like, spheroidal structure (Fig. 5C).

**Effect of VEGF on cancer cells**

A favorable prognosis after treatment with anti-VEGF antibody and a high concentration of VEGF contained in MPE suggest important roles of VEGF in MPE. Although co-culturing may have changed the responsiveness to VEGF, information regarding the cells after co-culturing may be important for speculating on the cells’ behavior in the patient. We added a saturating concentration of VEGF (1,000 ng/μL) or anti-VEGF antibody (1,000 ng/μL) to the medium and compared the results. None of the cancer cells showed a significant difference in proliferation (Fig. 6). We concluded that cancer cells do not respond to VEGF.

**VEGF concentration in the culture supernatant**

A high concentration of VEGF has been observed in MPE, but its origin has not yet been clarified. We thus investigated whether cancer cells produce VEGF after co-culturing. Cells from only two patients secreted detectable concentrations of VEGF (Fig. 7). Considering that the EC50 of VEGF is 20 ng/mL, the VEGF detected in our culture supernatant was not likely to show significant biological effects (Fig. 6).

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**Table 1**

| Patient | Age | Gender | Primary Site | TNM Classification | STAGE at 1st visit | Histopathology |
|---------|-----|--------|--------------|--------------------|--------------------|----------------|
| 1       | 62  | Male   | Right lung   | cT4N1M1c           | IVB                | Adenocarcinoma |
| 2       | 76  | Male   | Left lung    | cT4N3M1c           | IV                | Adenocarcinoma |
| 3       | 78  | Male   | Right lung   | pT2aN0M0 (at surgery) | IB (at surgery) | Adenocarcinoma |
| 4       | 73  | Male   | Right lung   | cT4N0M1a           | IVA               | Adenocarcinoma |
| 5       | 72  | Male   | Right lung   | cT3N3M1c           | IV                | Adenocarcinoma |
| 6       | 63  | Male   | Right lung   | cT2bN3M1c          | IVB               | Adenocarcinoma |
Fig. 2  
A colony-like congregate of cancer cells just after isolation from MPE.

Fig. 3  
Proliferation of cancer cells isolated from MPE. Cells from six patients were cultured in MPE supernatant, an equal mixture of the MPE supernatant plus RPMI 1640 containing 10% FBS, or RPMI-1640 containing 10% FBS, for 72 h. Cell number was measured using a cell counting kit. The NCI-1975 cell line cultured in RPMI-1640 containing 10% FBS was used as a proliferation control. Error bars indicate the standard error.
Discussion

In the current study, we attempted to cultivate cancer cells in MPE either solely or on a feeder cell composed of mouse embryonic fibroblasts. We also investigated the effect of VEGF on co-cultured cells and their production of VEGF.

When initiating the current study, we believed that the cultivation of cancer cells in MPE supernatant would be easy because this is their natural environment. However, the cells did not proliferate even with the addition of RPMI-1640 and FBS. This suggests several possibilities: (1) cells may require a supply of rapidly degrading nutrients from the thoracic wall, (2) cells may require interaction with the thoracic wall, or (3) cells may be senescent and unable to proliferate under any condition. However, successful cultivation on the feeder
layer indicates that possibility (3) is not probable. These results have important clinical implications in that both cancer cells in MPE and those residing on the thoracic wall could be targets for cancer treatment.

Our results that the proliferation of cancer cells was significant at 72 h after exposure to the feeder cell layer indicates that the cells changed their behavior over a short period of time. The cells continued to proliferate in RPMI-1640 with 10% FBS thereafter, while the cells without exposure to a feeder cell layer failed to proliferate in the same medium. These results indicate that the cells were likely reprogrammed to proliferate. Such changes are likely to accompany a change in the gene expression profile. This warrants a study comparing gene expression profiles before and after exposure to a feeder cell layer, which might provide important insights into the proliferation of cancer cells.

We aimed to investigate the role of VEGF in cancer
cells in MPE. However, such a study was difficult to perform because cancer cells in MPE were difficult to cultivate. Exposure to a feeder cell layer may change the responsiveness of the cells to VEGF; however, investigation of the responsiveness to VEGF may still provide important information. We thus decided to examine the effect of VEGF using co-cultured cells. The cells were not responsive to VEGF and did not produce VEGF, which may exert a biological effect.

In the current study, we investigated cancer cells in MPE. Difficulty in cultivating them suggests that the proliferation of cancer cells in patients may be affected by many factors provided by the body. Exposure to a feeder cell layer may change some characteristics of the cell but may maintain many of them. Studies using co-cultured cells or those that compare cells before and after coculture may provide important information regarding the biology of cancer.

Acknowledgements and Funding:

We would like to thank Editage (www.editage.com) for English language editing. The research is funded by the internal grant for medical research from the Jichi Medical University.

Conflicts of Interest Statement:

The authors declare no conflicts of interests.

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