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RESEARCH ARTICLE

TISSUE PROCESSING AND ISOLATION OF TUMOR CELLS FROM HUMAN COLORECTAL CANCER TISSUE

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ABSTRACT

Purpose: To investigate of cellular biology and physiology of colon cancer tissues in in vitro requires viable dissociation of single cells. The amount of tissue and dissociation methods can affect the amount of single cell viability. Inadequate initial tissue has negative effects on experiment quality by resulting in insufficient quality and the number of cells.
Material and Methods: In the context of this study different-weigh and different-textured colon tumor tissues have been evaluated to emphasize the importance of initial tissue properties during the operation of tissue processing and cell isolation success. Effect of the necrotic areas is also evaluated with the isolated viable cells number and the success of three-dimensional (3D) primary culture.
Results: Elevated weight of the tissue resulted with more total isolated cells. Necrotic tissues caused low percentage of viable cells. Since resected tissues were bigger than biopsy samples, resected tissues derived primary 3D culture were succesfully maintained the culture.
Conclusion: To conclude, isolated cells from the bigger and non-necrotic tumor tissues showed better growth pattern for 3D cultures. On the other hand, size was found as a crucial parameter for obtaining more viable cancer cells.

Keywords: colorectal cancer, chemical dissociation, cancer cell isolation

INTRODUCTION

Many cell culture methods have been developed for enabling new research for decades. Therefore, the methods to be chosen in cancer research are to provide a better understanding of cancer biology, the best chemotherapy selection, and the development of new treatment strategies (1). Immortalized cancer cell lines have been used to cancer research by

conventional two-dimensional (2D) models due to the cost-effectiveness, low-maintain and allowing to genetic manipulation (2). However, they cannot mimic the three-dimensional (3D) *in vivo* structure because of their monolayer growing pattern on the surface (3). Therefore, many *in vitro* 3D culture models have been developed to better explain and understand the cancer biology. Especially, 3D cell culture models created from primary cells isolated from tumor tissue were defined as *ex vivo* culture models such as patient-derived spheroids and organoids, represent of tumor tissue better than 2D cell cultures. Primary cell-based culture models, created from primary cells isolated from tumor tissue with stem cell-like properties, allow personalized and more effective drug screening due to the better explaining capacity of molecular mechanisms. (4). In order to obtain a primary cell-based culture, it is necessary to complete the steps such as ethical approval, surgical biopsy, pathological evaluation of tumor, tissue processing, cell isolation and culturing (5).

Tissue dissociation process to create primary cell culture is highly important to obtain sufficient viable cells. Enzymatic, mechanical, and chemical methods can be used to obtain for the tumor tissue processing (6). Enzymes such as trypsin, papain, elastase, hyaluronidase, and collagenase can be used in the enzymatic method, depending on the type of tissue (7). Mechanical dissociation involves applying physical force to tissue using scissors, scalpel, or sharp blade. Although mechanical dissociation is faster than enzymatic dissociation, cellular damage is higher than enzymatic dissociation (5).

Ca+2 and Mg+2 ions are needed for the stabilization of cell surface integrity and intracellular matrix (7). In chemical dissociation, EDTA or ethylene glycol tetraacetic acid chelates these ions and disrupts cell connections (8). Although chemical separation is an expensive method, it preserves cell membrane integrity and provides easier isolation. Besides, concentration, temperature, incubation time and tissue type are important parameters to achieve high-yield and cell viability (5)

In this study, cell isolation for primary cell-based culture models was performed with the help of EDTA from different-sized colorectal cancer (CRC) tissues. Then, organoid formation in MatriGel from the viable obtained primary cancer cells was examined. Tissue size and structural phenotype plays an important role for successfull chemical isolations of cancer cells from human colon tumor tissue.

MATERIAL AND METHODS

Obtaining Human Colorectal Cancer Tissue and Handling

This study has been approved by the ethics committee of Dokuz Eylul University, Non-Invasive Research Ethical Committee (Approval date: 01.06.2017, Decision number: 2017/14-35). In this study, tumor samples taken by surgery or colonoscopy from patients diagnosed with CRC between the ages of 60-80 were used. Informed consents were taken from all patients. In total, 5 resection material and 3 biopsy material were included. Tissue samples have been provided after the pathological evaluation and diagnosed as CRC. Cancer tissues were transferred to the laboratory in a transfer medium that includes ADMEM-F12 supplemented with %10 FBS, 1%Penicilin-Streptomicin at 4°C.

Tissue Processing for Cell Isolation

The cancer cell isolation process from human colorectal cancer tissue and culturing steps are as illustrated in Figure 1. These steps are highly important for quality isolation.

Tissues were cut out with sterile scalpel and tissue fragments and kept in cold 1X PBS (Corning, USA) on ice. During this step, necrotic parts of the tissue were scored from 0 (no necrotic part) to 5 (highly necrotic). The tissue fragments were washed with cold 1X PBS until obtaining a clear supernatant in a 50ml falcon tube, and then taken in a falcon tube filled up with 50ml of cold 10mM EDTA (Promega, USA) for the EDTA incubation for 60 minutes in a cold room on a shaker. At the end of the incubation, EDTA was

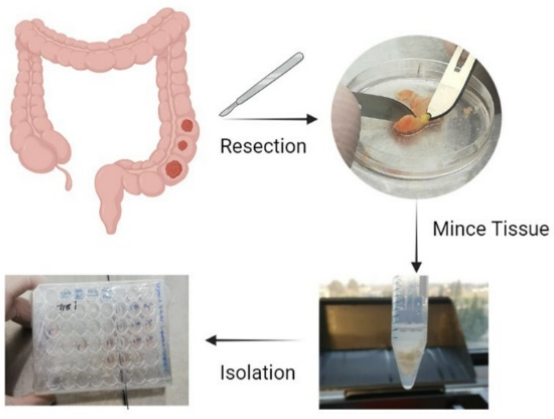

Figure 1. CRC tissue processes during isolation of cancer cells and culturing

removed, and the tissue was washed once with cold PBS. Then, tissues were shaken vigorously for 25 seconds in a tube filled with 30 ml of cold 1X PBS. After the completion of isolation step, supernatant was filtered through 70µm mesh. Filtered cells were checked under a microscope in terms of the cellular density and viability. Cells were precipitated by centrifugation at 300g for 5 min and the pellet was resuspended in organoid medium and counted under the microscope.

Cell Viability Assay

Trypan blue dye exclusion method was used to count total/viable cell numbers. 50µl of cell suspension was mixed with the same amount of trypan blue dye (Thermo Fisher, USA) and counted over a hemacytometer slide under a microscope for each tissue.

Patient-derived Ex Vivo Culture

Three thousand isolated cells were mixed with 30µl organoid culture medium includes Advanced DMEM basal medium supplemented with 1X Glutamax (Life Technologies, USA), 40ng/ml EGF (Peprotech, UK), 10µM Y27632 (Apexbio, Taiwan), 100ng/ml Noggin (Peprotech, UK). Cell suspension was mixed with MatriGel (Corning, USA) with 1:2 cell suspension-Matrigel ratio. Cell suspension-MatriGel mix was plated in a flat bottom 48-well plate drop wise. Plating step was completed on ice to keep Matrigel liquid. Pre-heated complete medium is added to Matrigel dome after 30 minutes of incubation for jellifying of Matrigel. Organoid generation was monitored under the microscope continuously.

Statistical Analysis

Graph Pad Prism 9 (GraphPad Software, Inc.) was used for data analysis. Non-parametric Mann-Whitney u test was performed for statistical comparison. Results were shown as means with standard error (SE). P-value showed significance at $p < 0.05$. The number of tissue samples was as N.

RESULTS

Before isolation step, tumor tissues from CRC patients were weighed on a sensitive scale, and necrotic areas were determined if any (Figure 2). Weigh and tissue properties were given in Table 1. After that, tumor tissues were dissociated with EDTA and isolated cells, as total and viable, were counted under a microscope.

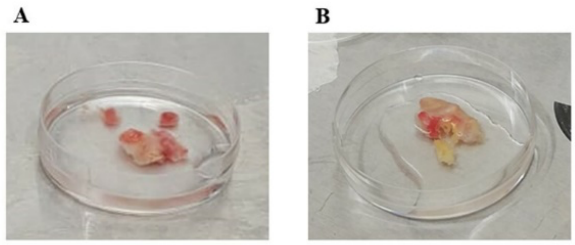

Figure 2. Different CRC patient's tissues. A) Pink-red colored tissue from Patient-1, has more live cells. B) Yellow-white colored tissue from Patient-3 had necrotic parts, and less viable cells

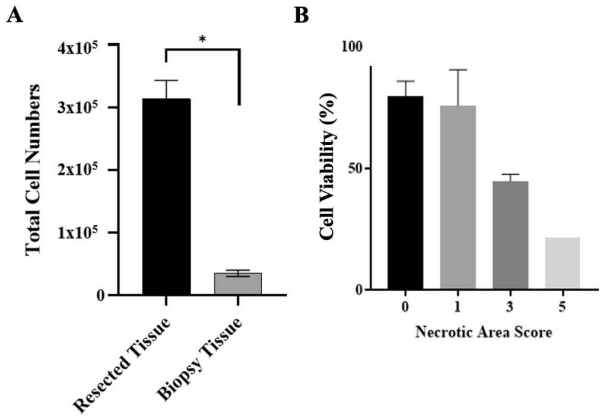

Figure 3. A) Comparison of total isolated cell number between all resected and biopsy tissue samples (p value: 0.0357). B) Bar graphs show correlation between cell viability percentages and necrotic area scores

As it is illustrated in Figure 3 (A), total number of isolated cells from resected tissues were higher than biopsy samples due to their size difference (*p* value: 0.0357). Resected tissues sizes were approximately 10-fold more than biopsy samples. Thus, total isolated cell numbers from resected tissues and biopsy tissues were found to correlate with the initial tissue weight and averaged 313.330 (278.320-356.544) and 35.432 (30.100-39.964) respectively. The count of viable cells and percentages were also shown in Table 1.

Necrotic scores were given in table 1 for each individual tissues. These scores were found correlated with the percentages of isolated viable cells. In patient 3 tissue with a high necrotic area score, the percentage of viable cell was found as 21.5% which was the lowest percentage overall. The highest percentage of viable cell was obtained as

Table 1. Isolated viable cells affect cancer organoid generation

Samples	Weigh of Tissues (mg)	Necrotic Area	Cell Viability (%)	Viable Cell Number	Organoid Generation
Tissue 1	226	0	86.4	256,058	Successful
Tissue 2	253	3	42.8	132,825	Successful
Tissue 3	228	5	21.5	59,736	Unsuccessful
Tissue 4	313	1	86.1	307,053	Successful
Tissue 5	294	3	46.7	151,704	Successful
Biopsy 1	21	0	77.8	23,415	Unseccessful
Biopsy 2	25	1	65.3	23,650	Unseccessful
Biopsy 3	31	0	74.0	29,574	Unseccessful

86.4% in patient 1 tissue, without a necrotic area, it is shown in Figure 2. As shown in Figure 3(B), the percentages of viable cells decrease with an increase on number of necrotic areas.

Isolated cells from eight tumor tissues were plated and organoid generation was observed. Four cultures out of five with cells isolated from resected tissues could generate organoid forms and maintain growth for seven days of culture. The only resected tissue with no organoid generation, had the most necrotic areas, and lowest viable cell percentage. On the other hand, cultures with isolated cells from biopsy samples were observed as having no organoid generation over seven days.

DISCUSSION

This study focused on the tissue properties (such as weight and necrosis) and processing of isolation of tumor cells from human CRC tissue. Chemical isolation with EDTA to dissociate cells from tumor tissue, was chosen due to the low-cost and easy to handle. After dissociation, viable cell numbers and organoid culture conditions were checked.

As it is predictable, isolated cell number generally is found to be correlated with tissue weigh. Results showed that the higher tissue weigh means that higher number of isolated cells. Although bigger tissue represents a higher capacity of viable cells, the data was controversial for one tissue sample. To evaluate this opposing data, the percentages of isolated viable cells and other properties of tumor tissues were evaluated. As it has been observed in all collected tissues, only one tissue sample from Patient 3 (named as Tissue 3 on Table 1), had less viable cell percentage.

As an important *in vitro* tool primary tumor tissue-based culture has several limitations in culturing and maintainence of the culture. As a novel technology, 3D organoid cultures are now considering for better mimicking of original tumor tissue in terms of structure and function. This novel *in vitro* culture system has recently started to be used for cancer models. It is possible starting from individual tissue and create *in vitro* cancer organoid culture indefinitely (9). In this study**,** in order to evaluate the organoid culturing success of isolated cancer cells in *in vitro* culture, cells were seeded in Matrigel and supplied several crucial niche factors to support their survival and expansions such as EGF, Noggin etc. Generally, cultures originated from resected tumor tissues present organoid generation capacity compared to biopsy tissues. Although there is less necrotic area in biopsy samples, it was concluded that not having organoid formation linked with low number of cells obtained. It shows that, initial tissue size matters for organoid culture, or in other words, the success of obtaining organoids from biopsy materials is quite difficult for this procedure. As it is observed, the more viable isolated cells show more success to create organoid culture.

CONCLUSION

To conclude, for primary tumor cell derived cultures, the initial tissue properties such as weight and necrotic areas are very important and has a direct impact on 3D culture quality. Tumor tissues without

necrotic areas and stiff has more capacity to obtain more viable cancer cells that have possible stemness phenotype and potentiate *in vitro* organoid generation (10). For primary tumor cell derived cultures, it is necessary to characterize the cells other than tissue properties. The stemness reservoir of cancer cells in the tissue should be determined by using the stemness marker panel. With this kind of detailed information, results can be associated with the stemness capacity of cells.

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Conflict of interests: None.

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