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Development of different organ derived decellularized tissue gels that support *in vitro* tumor cell behavior in a tumor type-specific manner

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SUMMARY

Tumor biology can no longer be understood simply by focusing on the characteristics of the tumor cells, but instead must encompass the contributions of the “tumor microenvironment”. Although the tumor microenvironment contains multiple natural extracellular matrices (ECMs), only single or specific ECMs are presently used to assess the behavioral characteristics of tumor cells *in vitro*. Recently, the use of decellularized tissue gels (DTGs) has gained attention, as they represent a new platform to provide multiple ECM components of the cellular microenvironment. However, as tissues contain unique components of ECMs, it is essential to reproduce the tissue-specific landscape when evaluating tumor functions *in vitro*. We hypothesized that DTGs affect the *in vitro* tumor cell behavior and that their protein composition varies between tissues. To test this, we created lung and liver DTGs via freeze-thawing and investigated the effects of DTGs on tumor cell migration. DTGs regulated the migration of tumor cells when these were cultured on DTG-coated plates. The proteins present in the DTGs were separated via SDS-PAGE, and multiple bands were observed using silver staining. Noteworthy, the band pattern varied depending on the tissue from which the DTGs were derived. In summary, this study shows that certain DTGs modulate the behavioral characteristics of tumor cells, which may have potential applications in cancer studies and therapy development. Hence, DTGs are worth investigating as useful tools for tumor cell culture and *in vitro* assays.

Key words: decellularized tissue gels, extracellular matrix, tumor cell behavior, SDS-PAGE

INTRODUCTION

Extracellular matrices (ECMs) consist of structural and functional proteins secreted by cancer associated fibroblast¹⁻⁴. The structure and biochemical composition of ECMs vary between tumor types⁵. ECMs regulate and support several cellular functions, including excessive growth, invasion of surrounding tissues, distant metastasis^{5,6}, and resistance to antitumor drugs⁷. ECMs activate multiple intracellular signaling pathways that are coordinated within the cells to exhibit specific cellular behaviors⁴. Thus, it is important to explore ECMs for the better understanding of the mechanisms underlying malignant cellular characteristics.

ECMs are used in cell cultures and in assays to investigate

tumor behavior, such as invasion⁸ and migration assays⁹. Although ECMs are mainly composed of macromolecules such as collagen, laminin, elastin, and fibronectin¹⁰, *in vitro* tumor assays currently use only single or specific ECM components. In addition, as tissues have unique structures and ECM components, it is essential to reproduce the tissue-specific ECM components when evaluating tumor functions *in vitro* in order to improve the assessment of the behavioral characteristics of tumor cells.

As an alternative to conventional *in vitro* tumor models, to address the complex challenges in tumor *in vitro* assays, “decellularization” technology has recently emerged as a new platform for mimicking the tumor microenvironment¹¹. Decellularization involves the removal of cellular components from tissues to retain the native ECMs. Whole-organ

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scaffolds have been created via decellularization for application in regenerative medicine¹²⁾. Preservation of the intact vascular networks and other essential organ structures provides an ideal foundation for cellular reprogramming^{13, 14)}. Whole-organ-decellularized scaffolds can be used for *in vitro* cultures to provide natural organ microenvironments, as well as to understand the mechanisms underlying cell-ECM interactions^{14, 15)}. The discovery that decellularized tissues can be solubilized and subsequently manipulated to form decellularized tissue gels (DTGs) has expanded the potential *in vitro* utility of ECMs to act as scaffolds^{16, 17)}. In our previous report¹⁸⁾, we analyzed the ability of DTGs to support *in vitro* cell proliferation. After 48 h of culture with lung- and liver- derived DTGs, MG63 and A549 cells showed an enhanced proliferation as compared with cells cultured without DTGs. Moreover, lung DTGs had a more pronounced effect on the proliferation of A549 cells than liver DTGs.

Here, we hypothesized that DTGs contain multiple proteins that affect the *in vitro* tumor behavior in addition to cell proliferation and that the composition of DTGs varies between tissues. To test this hypothesis, we created lung and liver DTGs and investigated their impact on tumor cell migration. Furthermore, the protein components present in the DTGs were analyzed via SDS-PAGE to investigate potential differences between lung- and liver-derived DTGs.

MATERIALS AND METHODS

Cell culture

This study used human lung tumor cell line A549 (Japanese Collection of Research Bioresources, Ibaraki, Osaka, Japan), human hepatoma cell line HuH-7 (Japanese Collection of Research Bioresources), and human osteosarcoma cell line MG63 (RIKEN BioResource Research Center, Tsukuba, Ibaraki, Japan). A549 cells were maintained in DMEM (Thermo Fisher Scientific Inc., Waltham, MA, USA), containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific Inc.) and 1% penicillin-streptomycin solution (Nacalai Tesque Inc., Nakagyo-ku, Kyoto, Japan). HuH-7 and MG63 cells were maintained in Minimum Essential Media (Thermo Fisher Scientific Inc.) containing 10% FBS and 1% penicillin-streptomycin solution. These cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂.

Decellularization of mice liver and lung followed by gelation

The liver and lungs of C57/BL6 male mice (8 weeks old) (CLEA Japan Inc., Meguro-ku, Tokyo, Japan) were rinsed with PBS (Nacalai Tesque Inc.), frozen in liquid nitrogen, and then crushed and ground with liquid nitrogen using a mortar. Decellularization was then performed by freeze-thawing the tissue powder. Briefly, PBS was added to a tube containing tissue powder that was centrifuged for

Fig. 1. Preparation of decellularized tissue gels (DTGs).

Schematic representation of the preparation of DTGs from mouse liver and lung tissues. Native tissues were frozen, ground, and decellularized via freeze-thawing. The decellularized tissues were digested using pepsin/HCl to create the DTGs, which were neutralized using NaOH.

2 min at 8,000×g using a High Speed Refrigerated Micro Centrifuge (TOMY Co. Ltd., Nerima-ku, Tokyo, Japan). This process was repeated thrice. Next, the decellularized tissues were placed in a freeze dryer (EYELA Co. Ltd., Bunkyo-ku, Tokyo, Japan) and decompressed using a vacuum pump (EYELA Co. Ltd.) to evaporate the liquid content. After freeze-thawing, pepsin (FUJIFILM Wako Pure Chemical Corp., Chuo-ku, Osaka, Japan) that was activated using HCl (FUJIFILM Wako Pure Chemical Corp.) (1 mg/mL pepsin in 0.1 M HCl) was used to digest the decellularized tissues for 24 h at 37°C; 1 mL of pepsin solution was added to 40 mg dry weight of decellularized tissue. Subsequently, pepsin was inactivated via neutralization using 0.1 M NaOH (FUJIFILM Wako Pure Chemical Corp.) (Fig. 1). DTGs (50 µL/well) were added to a 96-well polystyrene plate using a micropipette with cold chips and incubated overnight at 37°C. All animal procedures performed in this study were conducted in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute.

Silver staining

Total proteins were extracted from the DTGs using the Mammalian Protein Extraction Reagent (Thermo Fisher Scientific Inc.) and protein concentrations were determined using the Protein Assay Dye Reagent Concentrate (Bio-Rad

Laboratories Inc., Hercules, CA, USA) as previously reported¹⁸⁾. Extracted proteins (5 ng/lane) were applied in each lane and separated on 12.5% SDS-polyacrylamide gels (ATTO Corp., Taito-ku, Tokyo, Japan). Each band were detected using silver staining (Kanto Chemical Co. Inc., Chuo-ku, Tokyo, Japan) according to the manufacturer's instructions. Images of the stained gel were acquired with the Amersham Imager 600 (GE Healthcare, Chicago, IL, USA).

Cell migration assay

Migration assays were performed by plating 2.5×10^4 cells on 96-well Image-lock plates (Essen Bioscience Inc., Ann Arbor, MI, USA) coated with DTGs and commercially available Matrigel (Corning, Corning Inc., Corning, NY, USA). Cell monolayers were scratched using the IncuCyte 96-well WoundMaker Tool (Essen Bioscience Inc.) to create a wound. Cell migration was observed every 4 h for 46 h using the IncuCyte Zoom system (Essen BioScience Inc.). The ratio of migrating cells occupying the scratched portion was measured.

RESULTS

Lung- and liver-derived DTGs differently affected tumor cell migration

In our previous report¹⁸⁾, we found that DTGs supported *in vitro* cell proliferation. Moreover, this study newly

showed the ability of DTGs to support *in vitro* cell migration. We found that the cells showed a different migration potential depending on the organ used to obtain the DTGs (Fig. 2). Lung DTGs promoted the migration of HuH-7 cells more than liver DTGs after 48 h, whereas liver DTGs promoted the migration of A549 cells more than lung DTGs. In turn, lung and liver DTGs showed similar positive effects on the migration of MG63 cells.

Lung- and liver-derived DTGs are composed by different proteins

Silver staining of the electrophoresis gels showed that the supernatants and DTGs contained multiple protein bands. Notably, the pattern of bands for both supernatants and DTGs (silver staining of DTGs was also reported in our previous research¹⁸⁾) differed depending on the tissue (Fig. 3). In particular, the supernatant of both the lung and liver showed predominance of proteins of approximately 25 kDa, whereas no prominent bands were observed in the DTGs of both tissues. Three independent experiments revealed consistent protein band profiles for the supernatant of each organ.

DISCUSSION

Herein, we showed that the presence of DTGs in the culture of tumor cells affected their migration, in addition

Fig. 2. Changes in cell migration via decellularized tissue gels (DTGs) treatment.

Bar graph showing the effect of DTGs on cell migration. The Y-axis represents the ratio of migrating cells and the X-axis represents the time elapsed after cell seeding.

Fig. 3. Silver staining analysis of proteins obtained in the process of decellularization.

Extracted proteins from tissues were separated using SDS-PAGE and visualized using silver staining. 1) Protein molecular weight markers, 2–4) lung supernatant obtained from the first, second, and third freeze-thaw process, respectively, 5–7) liver supernatant obtained from the first, second, and third freeze-thaw process, respectively, 8) protein molecular weight markers, 9) lung decellularized tissue gels (DTGs), and 10) liver DTGs. This result shows the electrophoresis performed on the same gel and under the same conditions. A part of these data has already been shown in our previously published paper¹⁸.

to proliferation¹⁸). This work extends our understanding of the two elements that are important for the use of DTGs: the organs/tissues used for decellularization and the types of cells cultured with each DTG. The use of DTGs suitable for certain cells resulted in enhanced cell proliferation¹⁸) and migration. However, when the DTGs used were not suitable for the targeted cells, a decrease in cell proliferation was observed¹⁸). Hence, DTGs can modulate the behavior of cells.

A previous report explored the effects of DTGs on colony formation and compared DTGs to Matrigel and collagen, showing that primary rat pre-adipocytes cultured on the surface of adipose tissue-derived DTGs could form significantly larger colonies than those obtained using Matrigel after 7 days of incubation¹⁹). In agreement with this, we also found that DTGs affected cell proliferation and migration assays of different tumor cell lines. Moreover, we found that the organ used to generate the DTGs is an important factor that determines the effects of DTGs on tumor cells. The ECM composition has been reported to vary between normal and tumor tissues²⁰); therefore, DTGs derived from tumor tissues are expected to be more suitable for examining the role of cancerous ECMs in the regulation of tumor cell behavior at primary sites. Considering the importance of the source tissue of DTGs, we believe that the *in vitro* use of Matrigel extracted from Engelbreth-Holm Swarm mouse sarcoma for normal cells is not suitable for research on normal cell characteristics.

A previous study using mass spectrometry analysis of Matrigel has confirmed that Matrigel contains as much intracellular proteins as extracellular proteins¹⁵). Fur-

thermore, electrophoresis²¹) and mass spectrometry^{15, 22}) showed that most of the ECM components of Matrigel were laminin. Our analysis of the protein components contained in lung- and liver-derived DTGs revealed that they were different. A more detailed analysis of the ECM components in each DTGs using mass spectrometry and SDS-PAGE is still warranted.

In this study, we focused on liver and lung as sources for DTGs, but we plan to expand the application of DTGs to other organs in future studies. Furthermore, differences in the components of DTGs among organs were also revealed in this study, which highlights the possibility that such differences may also expand to different species. It is questionable whether using collagen derived from rats or pigs for culturing human tumor cells is sufficient to adequately reproduce the cellular microenvironment. By engineering DTGs tailored to the purpose of the experiment and using them in *in vitro* assays, we hope to clarify the contributions of the “tumor microenvironment” to tumorigenesis and find new therapeutic targets.

CONCLUSION

The effects of DTGs on cell migration differed greatly depending on the sourced organ of DTGs and cultured cell type. In addition, the composition of DTGs differed depending on the source tissue. Nevertheless, decellularized tissues hold components that affected the behavior of tumor cells. Hence, DTGs are worth investigating as useful tools for tumor cell culture and *in vitro* assays. As an alternative to conventional *in vitro* tumor models, DTGs have the potential to address the complex challenges for mimicking the

tumor microenvironment *in vitro*. In future studies, we will investigate the microenvironmental molecules essential for tumor cell proliferation by evaluating DTGs via proteomic analysis.

ABBREVIATIONS

DTG, decellularized tissue gel

ECM, extracellular matrix

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

- 1) Valkenburg KC, de Groot AE, Pienta KJ. Targeting the tumour stroma to improve cancer therapy. *Nat Rev Clin Oncol*. 2018;15:366–381.
- 2) Kalluri R. The biology and function of fibroblasts in cancer. *Nat Rev Cancer*. 2016;16:582–598.
- 3) Cox TR. The matrix in cancer. *Nat Rev Cancer*. 2021;21:217–238.
- 4) Bonnans C, Chou J, Werb Z. Remodelling the extracellular matrix in development and disease. *Nat Rev Mol Cell Biol*. 2014;15:786–801.
- 5) Lu P, Weaver VM, Werb Z. The extracellular matrix: a dynamic niche in cancer progression. *J Cell Biol*. 2012;196:395–406.
- 6) Walker C, Mojares E, Del Río Hernández A. Role of extracellular matrix in development and cancer progression. *Int J Mol Sci*. 2018;19:3028.
- 7) Meads MB, Gatenby RA, Dalton WS. Environment-mediated drug resistance: a major contributor to minimal residual disease. *Nat Rev Cancer*. 2009;9:665–674.
- 8) Liu H, Lu T, Kremers G-J, *et al*. A microcarrier-based spheroid 3D invasion assay to monitor dynamic cell movement in extracellular matrix. *Biol Proced Online*. 2020;22(3).
- 9) Pillar N, Polsky AL, Weissglas-Volkov D, *et al*. Comparison of breast cancer metastasis models reveals a possible mechanism of tumor aggressiveness. *Cell Death Dis*. 2018;9:1040.
- 10) Kular JK, Basu S, Sharma RI. The extracellular matrix: structure, composition, age-related differences, tools for analysis and applications for tissue engineering. *J Tissue Eng*. 2014;5:1–17.
- 11) Choudhury D, Tun HW, Wang T, *et al*. Organ-derived decellularized extracellular matrix: a game changer for bioink manufacturing? *Trends Biotechnol*. 2018;36:787–805.
- 12) Yagi H, Soto-Gutierrez A, Kitagawa Y. Whole-organ re-engineering: a regenerative medicine approach to digestive organ replacement. *Surg Today*. 2013;43:587–594.
- 13) Ott HC, Matthiesen TS, Goh S-K, *et al*. Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. *Nat Med*. 2008;14:213–221.
- 14) Uygun BE, Soto-Gutierrez A, Yagi H, *et al*. Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. *Nat Med*. 2010;16:814–820.
- 15) Bi H, Ye K, Jin S. Proteomic analysis of decellularized pancreatic matrix identifies collagen V as a critical regulator for islet organogenesis from human pluripotent stem cells. *Biomaterials*. 2020;233:119673.
- 16) Saldin LT, Cramer MC, Velankar SS, *et al*. Extracellular matrix hydrogels from decellularized tissues: structure and function. *Acta Biomater*. 2017;49:1–15.
- 17) Giobbe GG, Crowley C, Luni C, *et al*. Extracellular matrix hydrogel derived from decellularized tissues enables endodermal organoid culture. *Nat Commun*. 2019;10:5658.
- 18) Ono T, Noguchi R, Yoshimatsu Y, *et al*. Development of an in vitro culture system using decellularized tissue gels. *Denkieido*. 2021;65:75–78. (Japanese).
- 19) Uriel S, Huang J-J, Moya ML, *et al*. The role of adipose protein derived hydrogels in adipogenesis. *Biomaterials*. 2008;29:3712–3719.
- 20) Cho A, Howell VM, Colvin EK. The extracellular matrix in epithelial ovarian cancer - a piece of a puzzle. *Front Oncol*. 2015;5:245–245.
- 21) Sternlicht MD, Safarians S, Calcaterra TC, *et al*. Establishment and characterization of a novel human myo-epithelial cell line and matrix-producing xenograft from a parotid basal cell adenocarcinoma. *In Vitro Cell Dev Biol Anim*. 1996;32:550–563.
- 22) Kohen NT, Little LE, Healy KE. Characterization of matrigel interfaces during defined human embryonic stem cell culture. *Biointerphases*. 2009;4:69–79.