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Optimization of the tissue source, malignancy, and initial substrate of tumor cell-derived matrices to increase cancer cell chemoresistance against 5-fluorouracil

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ABSTRACT

The low chemoresistance of *in vitro* cancer cells inhibits the development of new anti-cancer drugs. Thus, development of a new *in vitro* culture system is required to increase the chemoresistance of *in vitro* cancer cells. Tumor cell-derived matrices have been reported to increase the chemoresistance of *in vitro* cancer cells. However, it remains unclear how tissue sources and the malignancy of cells used for the preparation of matrices affect the chemoresistance of tumor cell-derived matrices. Moreover, it remains unclear how the initial substrates used for the preparation of matrices affect the chemoresistance. In this study, we compared the effects of tissue sources and the malignancy of tumor cells, as well as the effect of the initial substrates on chemoresistance against 5-fluorouracil (5-FU). The chemoresistance of breast and colon cancer cells against 5-FU increased on matrices prepared with cells derived from the corresponding original tissues with higher malignancy. Moreover, the chemoresistance against 5-FU was altered on matrices prepared using different initial substrates that exhibited different characteristics of protein adsorption. Taken together, these results indicated that the appropriate selection of tissue sources, malignancy of tumor cells, and initial substrates used for matrix preparation is important for the preparation of tumor cell-derived matrices for chemoresistance assays.

1. Introduction

The development of effective anti-cancer drugs is crucial for cancer therapy; as a result, trials to develop new anti-cancer drugs have been extensively performed [1]. Despite extensive efforts, the development of a new anti-cancer drug remains difficult due to the lack of an effective development process [2]. The developmental process of an anti-cancer drug generally involves *in vitro* screening, *in vivo* testing using animal models, and clinical trials. Although a cell culture system is widely used for *in vitro* screening, *in vitro* cancer cells exhibit considerably lower chemoresistance compared to *in vivo* cancer cells, which results in faulty results and causes inadequate screening. Thus, a new cell culture system that increases the chemoresistance of cancer cells has been proposed to enable reliable *in vitro* anti-cancer drug screening [2–4].

Focusing on the extracellular matrix (ECM) is one of the important approaches used to increase the chemoresistance of *in vitro* cancer cells because the ECM regulates many cell functions, including cell survival, proliferation, and chemoresistance [5]. In particular, several reports have indicated that cell-derived matrices, which consist of ECM proteins deposited by cultured cells and are prepared as new cell culture substrates by the removal of cellular components from the culture (decellularization), can increase the chemoresistance of cancer cells *in vitro* [6–8]. We previously prepared "staged tumorigenesis-mimicking matrices" that mimic *in vivo* ECM surrounding tumor cells at various stages of malignancy [9]. We also compared the chemoresistance of a breast cancer cell line, MDA-MB-231, against 5-fluorouracil (5-FU) on staged tumorigenesis-mimicking matrices. The chemoresistance of MDA-MB-231 cells against 5-FU changed on matrices derived from cells with different degrees of malignancy [9] indicated that the malignancy of tumor cells for matrix preparation can affect the chemoresistance on tumor cell-derived matrices. However, it still remains unclear how matrices derived from cells with different degrees of malignancy affect chemoresistance. Moreover, it is not

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clear how matrices prepared with cells derived from different tumor tissue sources affect the chemoresistance of tumor cell-derived matrices, although the tissue sources of the cells for the preparation of matrices are important for the regulation of normal cell functions on cell-derived matrices [10,11].

In addition to the cell sources for matrix preparation, the initial substrates used for matrix preparation could affect the ability of cell-derived matrices because ECM deposition is strongly affected by protein adsorption, which is regulated by the surface properties of the initial substrates [12]. We have previously reported that blood compatible polymers of poly (2-methoxyethyl acrylate) (PMEA) and poly (tetrahydrofurfuryl acrylate) (PTHFA) enable tumor cells to attach, although protein adsorption characteristics, such as the amount of protein adsorption and adsorption-induced conformational change, are different [13]. This difference in protein adsorption characteristics can lead the cells to form ECMs of different compositions. Thus, it may be possible to increase the chemoresistance of cancer cells on cell-derived matrices prepared on these polymer substrates with different characteristics of protein adsorption.

In this study, we compared the chemoresistance of cancer cells on tumor cell-derived matrices prepared with cells obtained from different tumor tissues at various degrees of malignancy to determine the effects of the malignancy and tissue sources of the cells on the preparation of the matrices. In addition, we compared the chemoresistance on cell-derived matrices prepared with substrates that exhibit different protein adsorption characteristics to determine the effects of the characteristics of the initial substrates.

2. Materials and methods

2.1. Preparation of cell culture substrates

PMEA and PTHFA were synthesized according to previous reports [14,15]. PMEA and PTHFA were dissolved in methanol and methanol/chloroform (5:1) at a concentration of 0.2 wt%, respectively. Twelve microliters of each polymer solution was added to a 96-well tissue culture polystyrene (TCPS) plate, which was subsequently air-dried for seven days. The prepared substrates were sterilized via exposure to UV light on a clean bench for 2 h.

2.2. Cell culture for ECM formation

The human invasive breast cancer cell line, MDA-MB-231; the human mammary gland benign cell line, MCF-10A; the human invasive colon cancer cell line, HT-29; the human non-invasive colon cancer cell line, SW480; and the normal colonic epithelial cell line, CCD-841-CoN, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The human non-invasive breast cancer cell line, MCF-7, was obtained from the Health Science Research Resources Bank (Osaka, Japan). MDA-MB-231 and MCF-10A cells were seeded on bare TCPS, PMEA-, and PTHFA-coated substrates at densities of 1×10^4 cells/cm^2 and were cultured for 2 weeks in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (Ham) (1:1) (DMEM/F-12, Gibco, Carlsbad, CA) containing 10% fetal bovine serum (FBS, Equitech-Bio, Kerrville, TX). MCF-7, HT-29, SW480, and CCD-841-CoN cells were seeded at densities of 3×10^4 cells/cm^2 and were cultured for 2 weeks in DMEM/F-12 containing 10% FBS.

2.3. Preparation of tumor cell-derived matrices

Six types of tumor cell-derived matrices were prepared as new culture substrates using a method similar to that previously reported [9]. Briefly, after being cultured for two weeks on bare TCPS, PMEA, and PTHFA substrates in DMEM/F-12 containing 10% FBS, the cellular components were removed from the matrices through incubation with phosphate-buffered saline (PBS) containing 0.5% Triton X-100 and 20 mM of NH_4OH for 5 min at 37 °C. Subsequently, the samples were treated with 100 μg/ml of DNase I (Roche Applied Science, Penzberg, Germany) and 100 μg/ml of RNase A (Nacalai Tesque, Kyoto, Japan) for 1 h at 37 °C. After the cellular components were removed, the matrices were treated with 0.1% glutaraldehyde in PBS for 6 h at 4 °C to stabilize the matrices and were subsequently treated with 0.1 M glycine in PBS.

2.4. Chemoresistance test against 5-fluorouracil (5-FU)

The cancer cells were seeded on the tumor cell-derived matrices and bare TCPS at a density of 3×10^4 cells/cm^2. After one day of culture in DMEM/F-12 containing 10% FBS, the media were changed to DMEM/F-12 containing 10% FBS supplemented with 5-fluorouracil (5-FU, Sigma) at the indicated concentrations. After an additional three days of culture, the viable cells were evaluated using the WST-8 assay. The data were expressed as the percentage of the number of cells exhibiting growth inhibition relative to the number of the cells cultured without anti-cancer drugs.

2.5. Statistical analysis

All data were presented as the mean ± SD ($n = 3$). All statistical analyses were performed using Microsoft Excel 2010. Significant differences were detected using Student's t test. P-values < 0.05 were considered to be statistically significant.

3. Results

3.1. Effects of the tissue source of the cells used for the preparation of matrices on 5-FU resistance

We examined whether the tissue source of the cells used for the preparation matrices affected the chemoresistance against 5-FU. The percentages of growth inhibition of MDA-MB-231 and HT-29 cells were evaluated on tumor cell-derived matrices using the WST-8 assay (Fig. 1). Chemoresistance of MDA-MB-231 cells against 5-FU increased only on MDA-MB-231 cell-derived matrices (Fig. 1A). Similar chemoresistance of MDA-MB-231 cells against 5-FU was observed on other matrices. Furthermore, we also examined the chemoresistance of HT-29 cells against 5-FU to examine whether MDA-MB-231 cell-derived matrices specifically increased the chemoresistance. Chemoresistance of HT-29 cells against 5-FU increased on only HT-29 cell-derived matrices and not on MDA-MB-231 cell-derived matrices. These results indicated that only matrices derived from cells that had a similar tissue source as the target cells exhibited an increase in the chemoresistance against 5-FU in the chemoresistance assay.

3.2. Malignancy effects of the cells used for the preparation of matrices on 5-FU resistance

The chemoresistance of the invasive cancer cell lines of MDA-MB-231 and HT-29 increased on the corresponding cell-derived matrices. However, it is still not clear whether the chemoresistance of the target cells at lower malignancy increases on matrices derived from cells that were similar to the target cells in the chemoresistance assay (*i.e.*, the effect of the malignancy of the cells used to prepare the matrices is unknown). Next, we examined the chemoresistance of the non-invasive cancer cell lines of MDF-7 and SW480, which exhibit lower malignancy than MDA-MB-231 and HT-29 on matrices prepared with cells derived from the

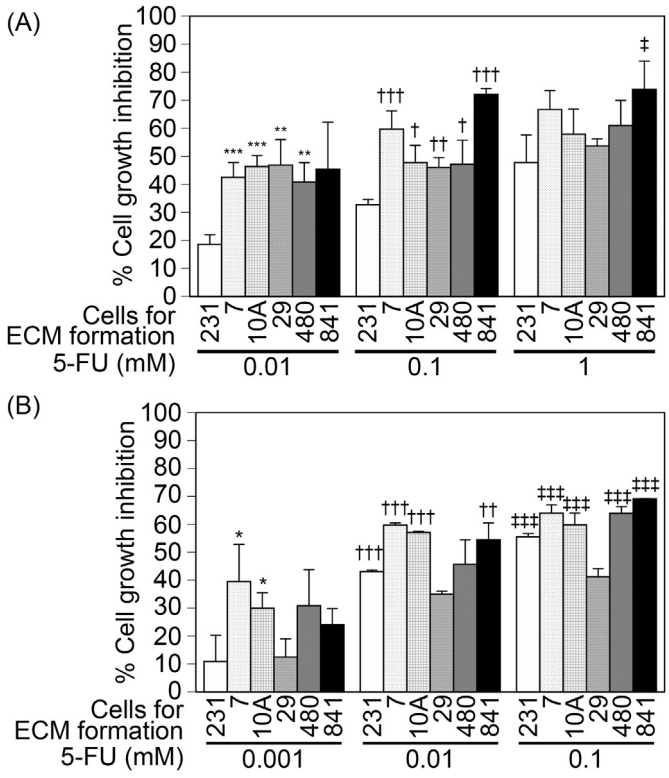

Fig. 1. Chemoresistance of cancer cells on matrices prepared using cells from different tissue sources. 231, 7, 10A, 29, 480, and 841 indicate MDA-MB-231, MCF-7, MCF-10A, HT-29, SW480, and CCD-841-CoN cells. (A) Chemoresistance of MDA-MB-231 cells. Data represent the mean ± SD (n = 3). **, ***: $P < 0.01$, <0.005 *vs.* MDA-MB-231 cell-derived matrices at a concentration of 0.01 mM of 5-FU. †, ††, †††: $P < 0.05$, <0.01, <0.005 *vs.* MDA-MB-231 cell-derived matrices at a concentration of 0.1 mM ‡‡: $P < 0.01$ *vs.* MDA-MB-231 cell-derived matrices at a concentration of 1 mM. (B) Chemoresistance of HT-29 cells. Data represent the mean ± SD (n = 3). *: $P < 0.05$ *vs.* HT-29 cell-derived matrices at a concentration of 0.001 mM of 5-FU. ††, †††: $P < 0.01$, <0.005 *vs.* HT-29 cell-derived matrices at a concentration of 0.01 mM ‡‡‡: $P < 0.005$ *vs.* HT-29 cell-derived matrices at a concentration of 0.1 mM.

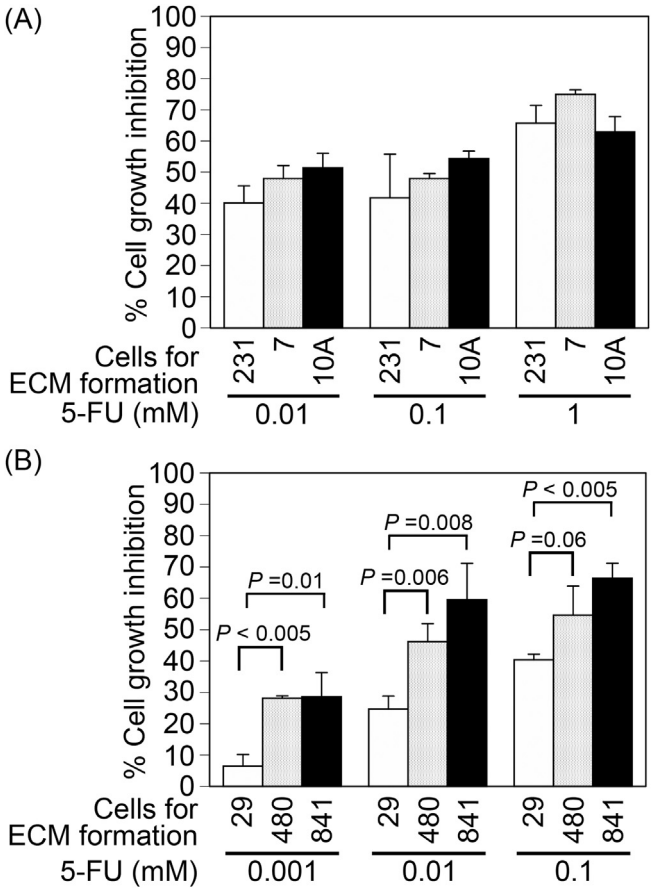

Fig. 2. Chemoresistance of cancer cells with lower malignancy on tumor cell-derived matrices. (A) Chemoresistance of MCF-7 cells. Data represent the mean ± SD (n = 3). 231, 7, and 10A indicate MDA-MB-231, MCF-7, and MCF-10A cells, respectively. (B) Chemoresistance of SW480 cells. Data represent the mean ± SD (n = 3). 29, 480, and 841 indicate HT-29, SW480, and CCD-841-CoN cells, respectively.

corresponding tissues (Fig. 2). No significant differences were detected in the chemoresistance of MCF-7 against 5-FU on matrices derived from cells with a different malignancy. However, the chemoresistance exhibited was higher on MDA-MB-231 cell-derived matrices compared to MCF-7- and MCF-10A-dervied matrices (Fig. 2A). In addition, the chemoresistance of SW480 against 5-FU was statistically higher on HT-29-derived matrices than on SW480- and CCD-841-CoN-derived matrices (Fig. 2B). In addition to MCF-7 and SW480 cells, MDA-MB-231 and HT-29 cells also exhibited higher chemoresistance against 5-FU on matrices prepared from cells derived from corresponding tissues with higher malignancy (Fig. 1). Taken together, these results indicated that matrices prepared from cells with higher malignancy increased the chemoresistance of cancer cells against 5-FU.

3.3. *Effect of the initial substrates used for the preparation of tumor cell-derived matrices*

Finally, we examined the effect of the initial substrates used for the preparation of matrices on chemoresistance against 5-FU. We compared the chemoresistance of cancer cells on MDA-MB-231- and HT29-derived matrices prepared on PMEA, PTHFA, and bare TCPS. The chemoresistance of MDA-MB-231 cells was determined to be the highest on MDA-MB-231-derived matrices prepared on PTHFA compared to those prepared on PMEA and bare TCPS (Fig. 3A). Similarly, the chemoresistance of HT-29 cells was the highest on HT-29-derived matrices prepared on PTHFA compared to those prepared on PMEA and bare TCPS (Fig. 3B). Taken together, these results indicated that the initial substrates affected the chemoresistance of cancer cells on 5-FU and PTHFA is suitable for the preparation of tumor cell-derived matrices for a chemoresistance assay.

4. Discussion

4.1. *Effect of the tissue source used for the preparation of matrices on chemoresistance*

The chemoresistance of cancer cells is determined by the activation of many intracellular signal pathways, such as cell survival and proliferation. It has been well-reported that the cell survival and proliferation of normal cells are strongly promoted on decellularized matrices prepared by cells derived from original tissues because decellularized matrices exhibit similar ECM components compared with original tissues and because tissue-specific ECM components strongly promote cell functions [10,11]. Our study revealed that the function of cancer cells (*i.e.*, chemoresistance) was also promoted on an ECM prepared with cells derived from original tissues, which is similar to the normal cell case. The compositions of ECMs are different between tumor and normal tissues [16]. However, it is possible that tissue-specific ECM components remained, even in the ECM, in tumor tissues. Thus, these tissue-specific ECM

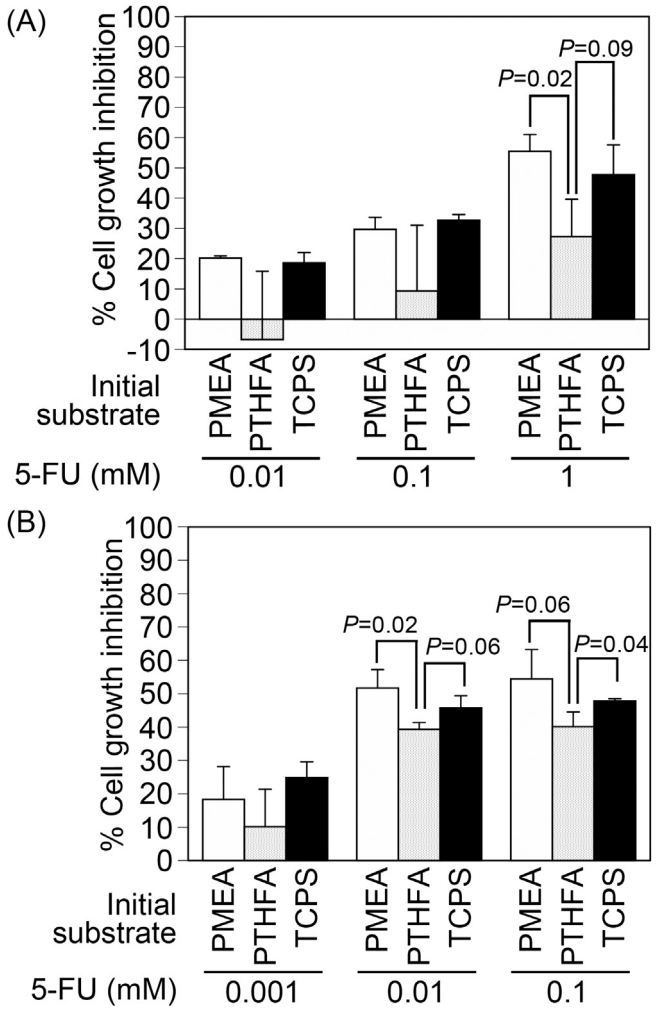

Fig. 3. Chemoresistance of cancer cells on the matrices prepared on different initial substrates. (A) Chemoresistance of MDA-MB-231 cells on MDA-MB-231 cell derived matrices prepared on different initial substrates. (B) Chemoresistance of HT-29 cells on HT-29 cell derived matrices prepared on different initial substrates. Data represent the mean ± SD (n = 3).

components could promote tumor cell survival and proliferation and increase chemoresistance.

4.2. Effects of the malignancy of the cells used for the preparation of matrices

In this study, we examined the effects of the malignancy of cells used for the preparation of matrices. We found that the chemoresistance increased on matrices prepared with cells of a higher malignancy, which indicated that tumor cells of high malignancy are suitable for the preparation of tumor cell-derived matrices for chemoresistance assays. It has been previously reported that the chemoresistance increased according to the progression of the tumor malignancy via the activation of intracellular signaling with epithelial–mesenchymal transition (EMT) and the expression of transporters [5,17]. Our study demonstrated that ECM remodeling is one of the mechanisms used to acquire chemoresistance with the progression of tumor malignancy. In this study, we cannot explain the details of the chemoresistance acquired by tumor–progression-dependent ECM remodeling; thus, future studies are required to investigate such questions.

4.3. Effects of the initial substrates used for the preparation of matrices

Previously, we reported that ECM protein deposition was strongly affected by protein adsorption on initial substrates [12]. As expected, different ECM proteins were deposited onto the substrates, and different ECM deposition induces different chemoresistance of cancer cells when tumor cell-derived matrices are prepared on initial substrates using different characteristics of protein adsorption. Our results suggested that the matrices prepared on PTHFA exhibited the highest chemoresistance compared with the matrices prepared on PMEA and TCPS (Fig. 3). There are two potential mechanisms, which can account for this result. One possibility is that strong integrin signaling is activated to promote cell survival and proliferation in the cells on matrices prepared on PTHFA. We previously reported that the availability of cell attachment sites in fibronectin is higher on PTHFA than PMEA and bare TCPS [13]. Thus, it is possible that fibronectin with a high availability of cell attachment sites is assembled in tumor cell-derived matrices prepared on PTHFA, thereby activating integrin-dependent signaling more strongly on matrices prepared on PTHFA compared to those prepared on PMEA and bare TCPS. Another mechanism is the difference in ECM protein deposition on PTHFA. We previously reported that integrin signaling is suppressed on PMEA compared with PTHFA [13]. The activation of integrin signaling, particularly focal adhesion kinase (FAK), promotes the expression of genes encoding fibrotic ECM proteins [18]. ECM deposition was more abundant on PTHFA compared to PMEA; the abundant ECM proteins deposited on PTHFA induced the activation of intracellular signaling, which increased chemoresistance. In this study, we could not specify the mechanism that increased the chemoresistance on the matrices prepared on PTHFA; this mechanism will be determined in future studies.

4.4. Optimal conditions of tumor cell-derived matrices for the chemoresistance assay

It has been reported that cell-derived matrices prepared with cells from the original tissue strongly induce the cellular function (e.g. proliferation and differentiation) of normal cells [10,11]. In this study, we highlighted the importance of tissue sources and malignancy to regulate the functions of cancer and normal cells. Tumor cells with original tissue sources and higher malignancy are superior for the preparation of tumor cell-derived matrices used to assess the chemoresistance of cancer cells. Moreover, we investigated the initial substrates and demonstrated that tumor cell-derived matrices prepared on PTHFA exhibited an increased chemoresistance compared with cell-derived matrices prepared on other substrates. To date, initial substrates have not been the focus of studies performed to improve cell-derived matrix functions because the initial substrates are covered after preparation of the matrices. Our study clearly demonstrated that the initial substrates affected the induction ability of cell-derived matrices, suggesting that the selection of appropriate initial substrates is one of the methods used to improve the functionality of cell-derived matrices.

Conflict of Interest

There are no conflict of interest.

Acknowledgments

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