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Decellularized matrices as *in vitro* models of extracellular matrix in tumor tissues at different malignant levels: Mechanism of 5-fluorouracil resistance in colorectal tumor cells

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ABSTRACT

Chemoresistance is a major barrier for tumor chemotherapy. It is well-known that chemoresistance increases with tumor progression. Chemoresistance is altered by both genetic mutations and the alteration of extracellular microenvironment. Particularly, the extracellular matrix (ECM) is remodeled during tumor progression. Therefore, ECM remodeling is expected to cause the acquisition of chemoresistance in highly malignant tumor tissue. Here, we prepared cultured cell-derived decellularized matrices that mimic native ECM in tumor tissues at different stages of malignancy, and 5-fluorouracil (5-FU) resistance was compared among these matrices. 5-FU resistance of colorectal tumor cells increased on the matrices derived from highly malignant tumor HT-29 cells, although the resistance did not increase on the matrices derived from low malignant tumor SW480 cells and normal CCD-841-CoN cells. The resistance on HT-29 cell-derived matrices increased through the activation of Akt and the upregulation of *ABCB1* and *ABCC1* without cell growth promotion, suggesting that ECM remodeling plays important roles in the acquisition of chemoresistance during tumor progression. It is expected that our decellularized matrices, or "staged tumorigenesis-mimicking matrices", will become preferred cell culture substrates for *in vitro* analysis of comprehensive ECM roles in chemoresistance and the screening and pharmacokinetic analysis of anti-cancer drugs.

1. Introduction

Malignant tumors are one of the leading causes of death in the world, especially in developed nations [1]. Although chemotherapy is an important cancer therapy, it is clinically well-known that the chemotherapeutic effect decreases with the increase of tumor malignancy [2]. There are many studies that reveal a chemoresistance mechanism in highly malignant tumors [3,4]. These studies have mainly focused on the genetic mutations that alter the expression and activity of intracellular molecules (*e.g.*, survival signal molecules and efflux transporters). In addition to genetic mutations of intracellular molecules, it has been observed that extracellular microenvironment influences the chemoresistance [5].

The extracellular microenvironment changes in tumor tissues at different stages of malignancy. In particular, the extracellular matrix (ECM) is dynamically remodeled to regulate various functions of tumor cells (*e.g.*, cell survival and growth) according to the tumor malignancy [6,7]. It has been reported that the interaction between ECM proteins and integrins on the cell membrane activates survival signal molecules, such as Akt and extracellular signal-regulated kinase (ERK), to protect from apoptotic cell death and to increase chemoresistance [8,9]. However, it is not clear how ECM remodeling influences the chemoresistance during tumor progression comprehensively. The effects of ECM on chemoresistance during tumor progression should be unveiled using *in vitro* ECM models mimicking native ECM in tumor tissues at different stages of malignancy.

Decellularized matrices are one of important *in vitro* ECM models mimicking native ECM because of its composition [10,11]. In particular, decellularized matrices derived from cells cultured *in vitro* are superior to tissue-derived decellularized matrices for large-scale *in vitro* analysis because the matrices derived from cells are easy to obtain [11]. We have reported that the matrices derived from cells are useful to analyze comprehensive roles of ECM in stem cell differentiation [12–14]. More recently, we have prepared "staged tumorigenesis-mimicking matrices" which are *in vitro* ECM models that mimic native ECM in tumor tissue at different stages of malignancy [15,16]. The tumor cells exhibited higher chemoresistance against 5-fluorouracil (5-FU) on the matrices derived from highly malignant (*i.e.*, invasive) tumor cells than those derived from minimally malignant (*i.e.*, non-invasive) and normal/benign tumor cells [15,16]. Also, the tumor cells exhibited higher 5-FU

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resistance on invasive tumor cell-derived matrices than a conventional cell culture polystyrene substrate [15]. The results indicated that ECM might play an important role in the acquisition of chemoresistance in highly malignant tumors. Additionally, staged tumorigenesis-mimicking matrices are useful to analyze the comprehensive roles of ECM in the acquisition of chemoresistance in highly malignant tumors.

There are various mechanisms to acquire 5-FU resistance: cell growth promotion [17], survival signal activation [18,19], and over-expression of a 5-FU target molecule (*thymidylate synthase*: *TS*) [20], a 5-FU metabolic molecule (*dihydropyrimidine dehydrogenase*: *DPYD*) [21], DNA repair enzymes (*single-strand-selective monofunctional uracil-DNA glycosylase 1*: *SMUG1* and *O^6-methylguanine-DNA methyltransferase*: *MGMT*) [22,23], *ATP-binding cassette B*1 (*ABCB1*, also known as P-glycoprotein/multidrug resistance 1), and *ATP-binding cassette C*1 (*ABCC1*, also known as multidrug resistance-associate protein 1) [24,25]. In this study, we examined the acquisition mechanisms of colorectal tumor cell chemoresistance against 5-FU on staged tumorigenesis-mimicking matrices, especially focusing on the above mechanisms.

2. Materials and methods

2.1. Cell culture

Colorectal tumor cell lines, HT-29 (invasive: a highly malignant tumor cell model), and SW480 (non-invasive: a minimally malignant tumor cell model) and normal colorectal cell line, CCD-841-CoN, were obtained from the American Type Culture Collection (ATCC: Manassas, VA). The cells were maintained on tissue culture polystyrene (TCPS: Iwaki, Tokyo, Japan) in Dulbecco's modified Eagle's medium/Ham F-12 medium mixture (1:1) (DMEM/F-12: Gibco, Carlsbad, CA) containing 10% fetal bovine serum (FBS: Equitech-Bio, Kerrville, TX) (serum medium). The cells were subcultured with the treatment of 0.25% of trypsin/EDTA (Sigma, St Louis, MO) when the cells had reached 80% confluency.

2.2. Preparation of staged tumorigenesis-mimicking matrices

Staged tumorigenesis-mimicking matrices were prepared by a method similar to that reported previously [15,16]. Briefly, the cells were seeded at a density of 3×10^4 cells/cm^2 and were cultured in serum medium for two weeks. Then, cellular components were removed from the matrices by incubation with phosphate-buffered saline (PBS) containing 0.5% Triton X-100 and 20 mM NH_4OH for 5 min at 37 °C. Subsequently, the samples were treated with 100 μg/ml DNase I and 100 μg/ml RNase A containing PBS for 1 h at 37 °C. After the removal of cellular components, the samples were treated with 0.1% glutaraldehyde containing PBS for 6 h at 4 °C to stabilize the matrices. After the stabilization, the samples were treated with 0.1 M glycine containing PBS at 4 °C overnight. The samples were stored at −20 °C until use.

2.3. Chemoresistance assay

HT-29 or SW480 cells were seeded on staged tumorigenesis-mimicking matrices at a density of 3×10^4 cells/cm^2 and were cultured in serum medium for 1 day. After 1 day of culture, the medium was changed to serum medium containing 0.1 mM of 5-FU (Sigma). After an additional three days of culture, the viable cells were evaluated by the WST-8 assay (Dojindo Laboratories, Kumamoto, Japan).

For the chemoresistance assay with inhibitor, the cells were cultured on TCPS at a density of 3×10^4 cells/cm^2 for 1 day in serum medium. The cells were then exposed to 0.1 mM of 5-FU with Akt inhibitor VIII (Adooq Bioscience, Irvine, CA) or U0126 (LC Laboratories, Woburn, MA) at indicated concentrations for three days. After the culture, the viable cells were evaluated by the WST-8 assay. The data are expressed as the percentage of viable cells relative to those without 5-FU in both experiments.

2.4. Cell growth assay

HT-29 cells were seeded on the matrices at a density of 5×10^3 cells/cm^2 and were cultured in serum medium without 5-FU for the indicated time. After the culture, the cell number was quantified by WST-8 assay with a standard curve.

2.5. Western blot analysis

For the analysis of phosphorylated Akt and ERK, the cells were cultured in serum-free medium for 24 h prior to detachment. Serum-starved cells were seeded on the matrices at a density of 3×10^4 cells/cm^2 and were cultured for 24 h in serum-free medium. For β-catenin analysis, the cells were seeded on the matrices at a density of 3×10^4 cells/cm^2 and were cultured for 24 h in serum medium. After the culture, the medium was changed to serum medium containing 0.01 mM 5-FU and were further cultured for 24 h. After the culture, the cells were lysed by a 1-h incubation in RIPA solution (Wako, Osaka, Japan) at 4 °C and then the cell lysate was collected as a supernatant after centrifugation at 15,000 rpm for 30 min at 4 °C. Protein concentrations of cell lysates were determined with *DC* Protein Assay kit (BioRad, Hercules, CA). Loading samples were prepared with Laemmli sample buffer. Same amount of proteins were loaded and then were separated on 7.5% SDS-PAGE. After being transferred to a PVDF membrane (Millipore, Bedford, MA), the proteins were treated with antibodies and detected with an Immobilon Western System (Millipore) and Ez-Capture MG (Atto, Tokyo, Japan) following treatment with Blocking-one (Nacalai tesque, Kyoto, Japan) to prevent non-specific reactions. The specific antibodies for these experiments were anti-phosphorylated Akt (S473), anti-Akt, anti-phosphorylated ERK1/2, anti-ERK-1/2, and anti-β-catenin. They were obtained from Cell Signaling Technology (Beverly, MA). Additionally, GAPDH was detected with anti-GAPDH (Medical & Biological Laboratories, Nagoya, Japan) as an internal control.

2.6. Real-time PCR

The cells were seeded on the matrices at a density of 3×10^4 cells/cm^2 and were cultured for 24 h in serum medium. After the culture, the medium was changed to serum medium containing 0.01 mM 5-FU and were further cultured for 24 h. After the culture, total RNA was extracted from the cells using Sepasol-RNA I Super G reagent according to the manufacturer's instructions (Nacalai Tesque). One microgram of total RNA was used as a first-strand reaction that included random hexamer primers and ReverTra Ace-α reverse transcriptase (TOYOBO, Osaka, Japan). Real-time PCR was amplified for *GAPDH*, *TS*, *DPYD*, *SMUG1*, *MGMT*, *ABCB1*, *ABCC1*, and *JUN* using specific primers and

Table 1
Primers and probes for real-time PCR analysis.

Gene	Oligonucleotide sequence
GAPDH	(Forward) 5′-ATGGGGAAGGTGAAGGTCG-3′ (Reverse) 5′-TAAAAGCAGCCCTGGTGACC-3′ (Probe) 5′-CGCCCAATACGACCAAATCCGTTGAC-3′
TS	Hs00426586_m1
DPYD	Hs00559279_m1
SMUG1	Hs04274951_m1
MGMT	Hs01037698_m1
ABCB1	Hs00184500_m1
ABCC1	Hs01561502_m1
JUN	Hs01103582_s1

GAPDH was designed according to Hoshiba et al. [12–14] and was obtained from Eurofins Genomics (Tokyo, Japan). Other genes were analyzed using TaqMan Expression Assays (Applied Biosystems, Waltham, MA).