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An extracellular matrix (ECM) model at high malignant colorectal tumor increases chondroitin sulfate chains to promote epithelial-mesenchymal transition and chemoresistance acquisition

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ABSTRACT

Chemoresistance is one of the major barriers for tumor chemotherapy. It is clinically known that chemoresistance increases during tumor progression. Additionally, the extracellular matrix (ECM) is also remodeled during tumor progression. However, it remains unclear how ECM remodeling contributes to chemoresistance acquisition. Recently, it has been reported that epithelial-mesenchymal transition (EMT) contributes to chemoresistance acquisition. Here, how ECM remodeling contributes to 5-fluorouracil (5-FU) resistance acquisition was investigated from the viewpoints of EMT using in vitro ECM models mimicking native ECM in colorectal tumor tissue at three different malignant levels. 5-FU partially induced EMT and increased *ABCB1* in colorectal HT-29 cells via TGF-β signaling (an invasive tumor cell model). When HT-29 cells were cultured on an ECM model (high malignant matrices) mimicking native ECM in highly malignant tumor tissues, the cells facilitated TGF-β-induced EMT and increased *ABCB1* upregulation compared with that of other ECM models mimicking the low malignant level and normal tissues. High malignant matrices contained more chondroitin sulfate (CS) chains than those of other ECM models. Finally, CS chain-reduced high malignant matrices could not facilitate *ABCB1* upregulation and TGF-β-induced EMT. These results demonstrated that ECM remodeling during tumor progression increased CS chains to facilitate EMT and *ABCB1* upregulation, contributing to chemoresistance acquisition.

1. Introduction

Malignant tumors are one of the leading causes of death in the developed nations [1]. Although chemotherapy is an important malignant tumor therapy, it is clinically well-known that chemotherapeutic effects decrease during the progression of tumor malignancy [2]. There are many studies that have addressed the mechanisms of tumor chemoresistance acquisition. Many of these studies have been focusing on genetic mutations to modulate the expression and activity of intracellular molecules (e.g., survival signal molecules, efflux transporters) [3,4]. Additionally, it has also been reported that the extracellular microenvironment influences tumor chemoresistance. In particular, the extracellular matrix (ECM) has been well-studied because the ECM is a key component of the extracellular microenvironment [5–10]. For example, solid stress which is led by high stiffness of extracellular matrix (ECM) increases to compress blood vessels according to tumor progression and the compression of blood vessels induces hypoxia which leads tumor chemoresistance [9,10]. In addition to the stiffness of ECM, the biological signals activated by ECM have been also studied [5–8].

ECM plays pivotal roles in the regulation of cell functions such as cell survival, proliferation, differentiation, migration, and tumor chemoresistance [11,12]. ECM is composed of many ECM proteins and carbohydrates, and their compositions differ depending on tissue types, developmental stages, and pathological states to regulate cell function precisely [13–17]. In tumor tissues, ECM compositions are remodeled during the progression of tumor malignancy [15–17]. Although it is well-known that ECM can influence tumor chemoresistance, it remains unclear how ECM remodeling comprehensively contributes to the acquisition of tumor chemoresistance during the progression of tumor malignancy.

Epithelial-mesenchymal transition (EMT) is a process in which the cells lose their polarity and cell-cell adhesions and gain mesenchymal phenotypes [18,19]. EMT decreases epithelial cell-cell adhesion molecules (e.g., E-cadherin) and increases mesenchymal cytoskeleton

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molecules (e.g., vimentin) and EMT transcription factors (e.g., Snail and Twist) [18,19]. It has been pointed that EMT contributes to the progression of tumor malignancy [18–23]. Recently, it has been reported that EMT contributes to chemoresistance acquisition [20–23]. It is well-known that TGF-β is a strong EMT inducer and that anticancer drugs activate the TGF-β signaling pathway and can induce EMT [18,19]. Additionally, it has been reported that ECM can also induce EMT [18,24,25]. However, it is not clear whether and how ECM remodeling contributes to EMT to acquire tumor chemoresistance during tumor progression.

ECM models have been formed by using cultured cells and have been used for the analyses of ECM roles after decellularization [26,27]. Recently, I reported the development of staged tumorigenesis-mimicking matrices as in vitro ECM models that mimic native ECM in tumor tissues at different malignant levels [28–30]. Staged tumorigenesis-mimicking matrices were prepared by in vitro ECM formation by cultured tumor cells with different degrees of malignancy followed by decellularization [28–30]. I have also demonstrated that 5-fluorouracil (5-FU) resistance of colorectal tumor cells increased via the upregulation of *ABCB1*, a drug efflux transporter, when grown on matrices mimicking native ECM in a highly malignant colorectal tumor (high malignant matrices) [30]. Thus, it is expected that staged tumorigenesis-mimicking matrices are useful in vitro ECM models to examine the effects of ECM remodeling comprehensively.

In this study, the effects of ECM remodeling on EMT and chemoresistance were investigated using staged tumorigenesis-mimicking matrices. First, I checked whether ECM remodeling contributed to EMT by the measurement of the expression of EMT-related genes (e.g., *VIM*, *SNAI1*, and *CDH1*) on staged tumorigenesis-mimicking matrices. It was also examined whether EMT contributed to *ABCB1* upregulation on highly malignant matrices. Finally, it was investigated how ECM remodeling contributes to EMT and the following *ABCB1* upregulation.

2. Materials and methods

2.1. Cell culture

The colorectal tumor cell lines, HT-29 (invasive tumor cells: a highly malignant tumor cell model) and SW480 (non-invasive tumor cells: a minimally malignant tumor cell model) and a normal colorectal cell line, CCD-841-CoN, were obtained from the American Type Culture Collection (ATCC: Manassas, VA). These 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) with 10% fetal bovine serum (FBS, Equitech-Bio, Kerrville, TX) (serum medium). The cells were subcultured after treatment with 0.25% trypsin/EDTA (Sigma, St. Louis, MO).

2.2. Preparation of staged tumorigenesis-mimicking matrices

The cells were seeded at a density of 3×10^4 cells/cm^2 and were cultured in serum medium for two weeks. After the culture, the cells were removed from the culture samples by incubation with phosphate-buffered saline (PBS) containing 0.5% Triton X-100 and 20 mM NH_4OH for 5 min at 37 °C, followed by incubation with PBS containing 100 μg/ml DNase I (Roche Diagnostics, Mannheim, Germany) and 100 μg/ml RNase A (Nacalai tesque, Kyoto, Japan) for 1 h at 37 °C. After removal of the cells, the samples were incubated in PBS containing 0.1% glutaraldehyde for 6 h at 4 °C for stabilization of the matrices. After the stabilization, the samples were incubated in PBS containing 0.1 M glycine at 4 °C overnight to quench unreacted aldehyde groups. The samples were stored at − 80 °C until use. In this study, HT-29, SW480, and CCD-841-CoN cell-derived matrices were referred to as high malignant, low malignant, and normal matrices, respectively.

For the preparation of the high malignant matrices with reduced chondroitin sulfate (CS) chains, high malignant matrices were treated with 0.5 U of chondroitinase ABC (CHase, Sigma) in a buffer containing 50 mM Tris-HCl, pH 8.0, 60 mM sodium acetate and 0.02% bovine serum albumin (BSA, Sigma) for 36 h at 37 °C before the glutaraldehyde treatment [31].

2.3. Cell culture on staged tumorigenesis-mimicking matrices

HT-29 cells were seeded on staged tumorigenesis-mimicking matrices and were cultured in serum medium for 1 day. After 1 day of culture, the medium was changed to serum medium containing 0.01 mM of 5-FU (Sigma) or 10 ng of TGF-β (Aviscera Bioscience, Santa Clara, CA). The cells were seeded at a density of 3 or 2×10^4 cells/cm^2 to examine their response to 5-FU or TGF-β, respectively. For inhibition of intracellular signaling from the TGF-β receptor, the cells were co-cultured with 10 μM of SB431542 (Wako, Osaka, Japan) in serum medium containing 0.01 mM 5-FU. The cells were additionally cultured for 1 or 3 days in serum medium containing 5-FU or TGF-β.

2.4. cDNA synthesis

After the culture, the cells were collected with Sepasol-RNA I Super G reagent (Nacali Tesque) and total RNA was extracted according to the manufacturer's instruction. Total RNA (1 or 0.4 μg) was used for the first-strand reaction with oligo(dT) and ReverTra Ace-α reverse transcriptase (TOYOBO, Osaka, Japan).

2.5. Real-time polymerase chain reaction (PCR)

Real-time PCR was amplified for *GAPDH*, *VIM*, *SNAI1*, *CDH1*, and *ABCB1* using specific primers and probe sets or TaqMan Gene Expression Assays (Table 1). The reaction was performed with 10 ng of cDNA, 300 nM PCR primers, 150 nM PCR probe (or TaqMan Gene Expression Assays), and Premix Ex Taq (Probe qPCR) (TaKaRa, Shiga, Japan) according to the manufacturer's instruction. The gene expression levels relative to *GAPDH* were calculated using the comparative Ct method.

2.6. Semi-quantitative reverse transcription-PCR (RT-PCR)

To check the ECM gene expression patterns in HT-29, SW480, and CCD-841-CoN cells, the cells were cultured on TCPS for 4 days in serum medium. cDNA was synthesized by the method described above. Semi-quantitative RT-PCR was performed using rTaq DNA polymerase (Bioline, London, UK) with specific primer sets (Table 2). For each experiment, *GAPDH* was amplified to normalize the expression of other genes in the sample. The PCR products were analyzed by 1% agarose gel electrophoresis.

2.7. Enzyme-linked lectin assay (ELLA)

The matrices were treated with Blocking-one (Nacalai tesque) for

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′
CDH1	Hs01023895_m1
VIM	Hs00958111_m1
SNAI1	Hs00195591_m1
ABCB1	Hs00184500_m1

GAPDH was designed according to Hoshiba et al. [32] and was obtained from Eurofins Genomics (Tokyo, Japan). Other genes were analyzed using TaqMan Expression Assays (Applied Biosystems, Waltham, MA).