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Original Article

Investigation of the origin of stromal and endothelial cells at the desmoplastic interface in xenograft tumor in mice

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ABSTRACT

Carcinoma-associated fibroblasts found at the interface between a tumor and the normal stroma play several roles in the development of cancer, including cancer initiation, growth, and progression, thereby also affecting patient prognosis. Although recent studies have focused on carcinoma-associated fibroblasts as potential treatment targets, the origin of these fibroblasts remains unclear. One theory suggests that these cells arise from tumor cells undergoing the epithelial–mesenchymal transition, i.e., tumor cells transform into carcinoma-associated fibroblasts. Therefore, in this study, we aimed to elucidate the cellular origin of carcinoma-associated fibroblasts in a mouse xenograft model. Mice were transplanted with human lung cancer cells (H226 and A549 cells). After sacrifice, tumor masses and surrounding tissues were excised. Interestingly, the excised xenograft tissues contained a significant proportion of desmoplastic fibroblasts that exhibited strong expression of α-smooth muscle actin (SMA). Immunohistochemical staining with pan-cytokeratin, vimentin, β-catenin, E-cadherin, and CD34 showed no evidence of the epithelial–mesenchymal transition. Additional evaluation using dual-color silver in situ hybridization with dinitrophenyl-labeled human epidermal growth factor receptor 2 (HER2) and digoxigenin-labeled chromosome 17 centromere probes also showed similar results. In conclusion, our results revealed that the epithelial–mesenchymal transition may not occur in tumor xenograft models, regardless of evidence supporting this phenomenon in humans.

1. Introduction

Desmoplasia is a pathological term referring to the fibrotic reaction in the peritumoral and intratumoral stroma [1]. The fibrotic reaction has a protective function during wound healing, wherein fibroblasts are activated in response to tissue injury and lead to fibrosis of the surrounding tissue by differentiating into myofibroblasts [2]. Myofibroblasts are contractile and therefore contribute to tissue contraction during wound healing. Moreover, myofibroblasts may produce excessive amounts of extracellular matrix proteins, which can cause normal organs to become fibrotic [3].

By disruption of normal tissue characteristics, cancer tissues can function as wounds and recruit constitutively activated fibroblasts, resulting in excessive desmoplasia [2]; these cells are specifically termed carcinoma-associated fibroblasts (CAFs) and express α-smooth muscle actin (α-SMA) as a distinctive feature [2]. Interestingly, many studies have reported that CAFs are involved in various cancer-related functions, including cancer initiation, growth, migration, invasion, metastasis, microenvironment formation, and angiogenesis, and can predict patient prognosis [2,4]. However, the source of CAFs is not fully understood. Fibroblasts are a group of many heterogeneous subpopulations of cells that can show genomic and phenotypic diversity, even in the same organ [5]. Likewise, the origin of CAFs is also under debate, with previous reports suggesting that these cells stem from resident tissue fibroblasts [6], adipose tissue-derived stem cells [7,8], cancer-associated adipocytes [9], mesenchymal or hematopoietic stem cells from the bone marrow [10–12], the endothelial–mesenchymal transition [13,14], and the epithelial–mesenchymal transition (EMT) [2,15–18].

The EMT, which is thought to be a source of fibroblasts in benign fibrosis and cancer [2,15–19], is a transitional process in which epithelial cells lose their epithelial traits and obtains mesenchymal characteristics through accumulation of protein modifications and transcriptional events, resulting in motile spindle-shaped cells [20,21]. However, the EMT is also associated with more pathologic fibrotic conditions, such as wound healing and carcinoma [19–21]. To date, immunohistochemical (IHC) staining is the primary method for investigation of the EMT and prediction of cancer invasion [20]; indeed, IHC analysis of various marker proteins (e.g.,

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E-cadherin and cytokeratin as epithelial markers and vimentin as a mesenchymal marker) is typically used to identify the EMT in tissues and cells. Moreover, nuclear expression of β-catenin is associated with the EMT in epithelial cancer [20–22]. However, IHC staining is limited in that the EMT can occur incompletely or partially, making it difficult to detect the EMT in the clinical setting [20]. Additionally, IHC staining is unable to distinguish EMT-derived cells from normal mesenchymal cells [22]. Although the use of a combination of many markers may provide more accurate results, the specificity of conventional IHC markers remains questionable [20,22].

Therefore, we aimed to define the source of peritumoral fibroblast using a cellular/chromosomal-level analysis by adopting a species-specific technique with which to identify human and mouse cells in a xenograft model. Human lung cancer cells were transplanted into mice and analyzed using species-specific chromosome in situ hybridization (ISH). Our data provide important insights into the mechanisms of carcinogenesis, particularly the EMT, and describe a novel animal model with which to study various cancer-related processes.

2. Materials and methods

2.1. Ethical considerations

All experiments were performed with the approval of the Institutional Review Board of Kangwon National University.

2.2. Cell line preparation and innate characteristics

H226 (human lung squamous cell carcinoma; ATCC CRL5826) and A549 (human lung adenocarcinoma; ATCC CCL185) cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Ham's F-12/DME (1:1) medium (Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum at 37 °C in an atmosphere containing 5% CO_2 for 1 month. The innate morphological and IHC staining characteristics of both cell lines are described elsewhere [23–25]. Briefly, H226 and A549 cells have a typical epithelioid appearance with a high N/C ratio and express epithelial markers, including pan-cytokeratin, but do not express mesenchymal markers, including vimentin.

2.3. Xenograft and microscopic examination

Cells were collected using trypsin, resuspended in phosphate-buffered saline, and injected subcutaneously using 1 mL BD slip-tip syringes into the flanks of 5-week-old male NOD/SCID mice (NOD/mrkBOMTac-Prkdcscid, $n = 6$ for each group; Taconic, Ry, Denmark). The mice were maintained under specific pathogen-free conditions for 6 weeks with autoclaved food and water available ad libitum. After 2 months, tumors and surrounding tissues were surgically excised from the mice under enflurane anesthesia. The excised specimens were fixed in 10% neutral buffered formaldehyde for 24 h at room temperature and embedded in paraffin. Paraffin-embedded blocks were serially sectioned using a microtome at 4 μm thickness and subjected to routine hematoxylin and eosin (H&E) staining. We evaluated and compared the structures and IHC staining patterns of xenografted tumor cells, peritumoral and intratumoral blood vessels, and fibroblasts with dual-color silver in situ hybridization (SISH) in order to identify the origin of desmoplastic fibroblasts.

2.4. IHC staining and evaluation

IHC staining of formalin-fixed, paraffin-embedded tissue sections was performed using an automatic IHC staining device (BenchMarkXT; Ventana Medical Systems, Tucson, AZ, USA). Briefly, 4 μm thick tissue sections were mounted on poly-L-lysine-coated adhesive slides and dried at 74 °C for 30 min. After heat-induced antigen recovery for 1 h in ethylenediaminetetraacetic acid (pH 8.0) in an autostainer, the slides were incubated with primary antibodies targeting pan-cytokeratin (1:50 dilution; AE1/AE3; Zymed, South San Francisco, CA, USA), vimentin (1:200 dilution; V9; Zymed), α-SMA (1:500 dilution; 1A4; Sigma, St. Louis, MO, USA), E-cadherin (1:1000 dilution; Clone 36; Transduction Laboratories, Lexington, KY, USA), and β-catenin (1:50 dilution; E5; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). IHC staining for CD34 (1:100 dilution; QBEnd/10; Thermo Scientific, Fremont, CA, USA) was also performed without special pretreatment based on the manufacturer's suggestion. The slides were subsequently incubated with secondary antibodies and visualized using an ultraView Universal DAB Detection kit (Ventana Medical Systems).

2.5. SISH

Dual-color SISH (Ventana INFORM HER2 Dual ISH DNA Probe Cocktail Assay; Ventana Medical Systems) was performed on an automated Ventana slide stainer (BenchMarkXT) according to the manufacturer's protocols. Briefly, the tissue cores were deparaffinized with EZPrep (Ventana Medical Systems) at 75 °C and then subjected to three 12 min cycles of heat pretreatment at 90 °C in EZ Prep-diluted Cell Conditioning 2 (Ventana Medical Systems) followed by protease digestion with ISH Protease 3 (Ventana Medical Systems) for 16 min at 37 °C to unmask DNA targets. Dinitrophenyl (DNP)-labeled human epidermal growth factor receptor 2 (HER2) and digoxigenin (DIG)-labeled chromosome 17 centromere (CEN17) probes were co-denatured with the tissue sections by heat treatment for 20 min at 80 °C and then hybridized for 6 h at 44 °C. After three 8 min stringent washes in 2× SSC (Ventana Medical Systems) at 72 °C, the signals for DNP and DIG were visualized using ultraView SISH DNP and ultraView Red ISH DIG Detection Kits (Ventana Medical Systems), respectively. For *HER2* gene detection, the slides were incubated with rabbit monoclonal anti-DNP antibodies for 20 min and then with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibodies for 16 min at 37 °C. The HER2 signal was visualized as deposition of metallic silver-black dots, driven by the sequential addition of silver acetate, hydroquinone, and hydrogen peroxide, for 4 min at 37 °C. For CEN17 detection, the tissue sections were incubated with mouse monoclonal anti-DIG antibodies for 20 min and then with an alkaline phosphatase-conjugated goat anti-mouse antibody for 32 min at 37 °C. CEN17 staining appeared as red dots following reaction with fast red and naphthol phosphate for 16 min. Finally, the slides were counterstained with Hematoxylin II (Ventana Medical Systems) for 8 min and with Bluing reagent (Ventana Medical Systems) for 4 min.

2.6. Microscopic analysis of SISH results

Human cells showed two discrete red dots representing CEN17 and two black dots representing the *HER2* gene in the nuclei (Fig. 1A). However, no red dots were noted in mouse cells, and some mouse cells were partially reactive to the HER2 probe with 0–2 black dots found in the nuclei (Fig. 1B). In SISH analysis, we focused on cancer cells, desmoplastic fibroblasts, and vascular cells.