Spheroid Culture of Primary Lung Cancer Cells with Neuregulin 1/HER3 Pathway Activation

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Introduction: Primary culture of cancer cells is expected to be useful for investigating the biology of cancer and predicting chemosensitivity for individual patients, yet has been hampered by technical difficulties. We recently developed the cancer tissue–originated spheroid (CTOS) method for the primary culture of colorectal cancer cells. In the present study, we applied this system to the primary culture of non–small-cell lung cancer.

Methods: We used 125 surgical specimens and 18 pleural effusions for CTOS preparation. Partially digested tumor fragments were cultured in a medium for embryonic stem cells. CTOSs were subjected to sensitivity assay and signal transduction assay for the epidermal growth factor receptor (*EGFR*) tyrosine kinase inhibitor (TKI) erlotinib. We also investigated the effects of growth factors in culturing lung cancer CTOS.

Results: The success rate of CTOS preparation from surgical specimens was 80.0%. The CTOS method was also suitable for culturing tumor spheroids from pleural effusions. CTOSs from lung cancer consisted mostly of pure cancer cells. CTOSs and CTOS-derived xenografts retained the characteristics of the original tumors. In vitro assay results showed that *EGFR* mutation status and expression levels corresponded with erlotinib sensitivity, confirming previous clinical findings. Furthermore, we found that neuregulin 1, a ligand of HER3, potently induced CTOS growth.

Conclusions: The CTOS method enables us to obtain primary lung tumor cells of high viability and purity. CTOS could be a new platform for studying lung cancer biology.

Key Words: Primary culture, Non–small-cell lung cancer, EGFR tyrosine kinase inhibitor, Neuregulin 1, HER3.

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Despite constant efforts to improve diagnostics and therapeutics, cancer remains a leading cause of death in developed countries, and pulmonary malignancies are the leading cause of cancer-related death worldwide. Studies have shown that some lung adenocarcinomas are addicted to epidermal growth factor receptor (*EGFR*) signaling, and EGFR tyrosine kinase inhibitors (TKIs) are effective treatment in such cases.[1,2] However, these therapies are challenged by primary and acquired resistance, including additional point mutations[3–5] and alternative pathways that bypass the targets of therapeutic reagents.[6–8] Other ErbB family tyrosine kinase receptors are also involved in non–small-cell lung carcinoma (NSCLC);[9] human epidermal growth factor receptor 3 (HER3) is reportedly involved in the acquired resistance to EGFR TKI, whereas HER2 is overexpressed in 10% to 20% of NSCLC and carrying mutations in approximately 2%.[10,11] Bypassing EGFR signaling can be achieved through HER3 phosphorylation by overexpressed hepatocyte growth factor receptor (MET),[6] or by the induction of HER3 ligand as an autocrine loop.[12] Targeting of HER2 is already in clinical use and that of HER3 is under clinical trial.

Much of the above described data have been obtained through preclinical cancer therapy research performed using cancer cell lines. Although cancer cell lines are originally established from patient tumors, they have adapted to the culture conditions, that is, serum-supplemented medium.[13] Long-term cultivation in serum-containing medium reportedly leads to the accumulation of genetic alterations;[14,15] therefore, it must be considered whether cancer cell lines continue to accurately represent the parental cancer cells.

Nevertheless, primary culture of cancer cells is expected to be useful for investigating cancer biology and predicting chemosensitivity for individual patients. However, it has been hampered by technical difficulties, including poor cell viability, weak growth, and contamination by host cells, especially fibroblasts. We recently reported a novel primary culture system for colorectal cancer.[16] The principle of this method is to retain cell–cell contact during preparation. Tumor biopsy specimens are partially digested into fragments and cultured in a serum-free medium for embryonic stem (ES) cells; within a few hours, the irregularly shaped tumor fragments spontaneously form spheroids, termed cancer tissue–originated spheroids (CTOSs). This CTOS method

enables preparation and culture of multiple spheroids consisting of highly pure and viable colorectal cancer cells.

In the present study, we applied the CTOS method to the primary culture of non–small cell lung cancer (NSCLC). We were able to prepare and culture CTOSs from surgical specimens and pleural effusion. CTOSs from NSCLC showed individual responses to EGFR tyrosine kinase inhibitor in vitro and in vivo. CTOSs were also applicable in a signal transduction assay. Moreover, we identified neuregulin 1 (NRG1, also known as heregulin β1), a ligand for HER3, as a prominent growth factor for CTOS growth.

MATERIALS AND METHODS

CTOS Preparation

Surgical specimens and pleural effusion samples from lung cancer patients were obtained from Osaka Medical Center for Cancer and Cardiovascular Diseases, with the patients' informed consent. Surgically resected tissues were minced with a scalpel into approximately 1-mm^3 pieces, and washed with Hank's balanced salt solution (HBSS, Invitrogen, Carlsbad, CA). Specimens were transferred to a 100-ml glass flask and digested in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 0.26 U/ml Liberase DH (5401089; Roche, Basel, Switzerland) and 1% PenStrep (Invitrogen), at 37°C for 1 to 2 hours with gentle stirring by a magnetic bar. Digested tissue suspensions were passed through 500-μm and 250-μm metal mesh filters to remove large masses of undigested fragments. Suspensions were further filtered through 100-μm and 40-μm cell strainers (BD FALCON, Franklin Lakes, NJ). Fragments on the cell strainer and cells in the flow-through fractions were collected separately, and were each washed with HBSS and cultured in StemPro hESC medium (GIBCO, Carlsbad, CA) in a non-treated dish (EIKEN, Tokyo, Japan). Pleural effusions were transferred to 50-ml tubes and centrifuged at 200 *g*. Pellets were resuspended in HBSS, filtered through 40-μm cell strainers, and collected and cultured in the same manner as surgical specimens.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tumors and CTOSs. CTOSs were embedded in Matrigel growth factor reduced (GFR) (BD Biosciences, Bedford, MA) before fixation in formalin. Sections were dewaxed, rehydrated, and subjected to antigen retrieval by autoclave incubation in citrate buffer (pH 6.0). The primary antibodies used are listed in the Supplementary text (Supplemental Digital Content, http://links.lww.com/JTO/A386). After secondary antibody incubation, sections were visualized with a fluorescence method or biotin-amplified method (Nova RED, VECTOR, Burlingame, CA).

CTOS Culture

For assessing the effects of growth factors, CTOSs were embedded in Matrigel GFR as described above, and cultured in 100 μl of basal medium containing 10 ng/ml or 100 ng/ml of the following growth factors: NRG1 (Peprotech, Rocky Hill, NJ), Long-IGF1 (Gropep, Thebarton SA, Australia), bFGF (Invitrogen), Activin A (R&D Systems, Minneapolis, MN), or EGF (Invitrogen). Basal medium consisted of DMEM/F12, 2% BSA fraction V, 1× nonessential amino acids, 50 U/ml penicillin, 50 μg/ml streptomycin (all from Invitrogen), 50 μg/ml ascorbic acid (SIGMA, St. Louis, MO), 10 μg/ml human transferrin (SIGMA), 0.1 mM β-mercaptoethanol (Wako, Osaka, Japan), and 1× trace elements A, B, C (Mediatech, Manassas, VA).

For assessment of the inhibitory effect of anti-HER3 antibodies on CTOS growth, CTOSs were embedded in Matrigel GFR as described above, cultured in medium containing 10 ng/ml NRG1 or in StemPro hESC, and treated with anti-HER3 antibody (cloneH3.105.5; Calbiochem, La Jolla, CA) at the indicated doses.

CTOS Sensitivity Assay In Vitro

For the erlotinib sensitivity assay, each CTOS was embedded in a gel droplet of Matrigel GFR, and cultured in StemPro hESC containing erlotinib (Toronto Research Chemicals, North York, ON, Canada) at the indicated doses. CTOSs were exposed to erlotinib for 7 days. CTOS viability was evaluated based on CTOS size at day 7, corrected for the CTOS size at day 0. CTOS size was measured using image analysis software (Image J; National Institutes of Health, Bethesda, MD). Half maximal (50%) inhibitory concentration values were calculated with GraphPad Prism 4 software, using the sigmoidal dose-response function.

Animal Studies

Animal studies were performed in compliance with the guidelines of the institutional animal study committee of Osaka Medical Center for Cancer and Cardiovascular Diseases. Primary xenograft tumors were generated by inoculating small pieces of patient tumors into non-obese diabetic/severe combined immunodeficient (NOD/Scid) mice. One hundred CTOSs were suspended in 50 μl of Matrigel GFR, and transplanted subcutaneously into the flanks of NOD/scid mice. CTOSs prepared from mouse xenografts are designated with the postfix "m" in the figures. Treatment was started when the tumor volume reached approximately 65 mm^3. Erlotinib (Tarceva, CHUGAI Pharmaceutical, Tokyo, Japan) was suspended in 0.5% methyl cellulose, and administered at a dose of 100 mg/kg orally once a day, ×5, for 2 weeks.

Western Blot

CTOSs were cultured overnight in growth factor-free DMEM-F12, and pulsed with StemPro hESC supplement or 10 ng/ml of the indicated growth factors for 15 minutes. CTOSs were lysed with cell lysis buffer (10 mM Tris (pH 7.4), 0.15 M NaCl, 1% NP40, 0.25% sodium deoxycholate, 0.05 M NaF, 2 mM EDTA, 0.1% SDS, 2 mM $NaVO_4$, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF). Western blot was performed as previously described.[17] For signal transduction assays under erlotinib treatment, CTOSs were cultured overnight under growth-factor–starved conditions, incubated for 1 hour with erlotinib, and pulsed with 10 ng/