The American Journal of Pathology, Vol. 186, No. 4, April 2016

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The American Journal of PATHOLOGY

ajp.amjpathol.org

GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Dynamic Change of Polarity in Primary Cultured Spheroids of Human Colorectal Adenocarcinoma and Its Role in Metastasis

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Accepted for publication December 3, 2015.

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Intestinal epithelial cells possess apical-basal polarity, which governs the exchange of nutrients and waste. Perturbation of cell polarity appears to be a general feature of cancers, although most colorectal cancers are differentiated adenocarcinomas, in which polarity is maintained to some extent. Little is known about the role of dysregulated polarity in cancer. The cancer tissue—originated spheroid method was applied to the preparation and culture of spheroids. Spheroids were cultured in suspension or in type I collagen gel. Polarity was assessed by IHC of apical markers and electron microscopy. Two types of polarity status in spheroids were observed: apical-in, with apical membrane located at cavities inside the spheroids in type I collagen gel; and apical-out, with apical membrane located at the outermost layer of spheroids in suspension. These polarities were highly interchangeable. Inhibitors of Src and dynamin attenuated the polarity switch. In patients, clusters of cancer cells that invaded vessels had both apical-in and apical-out morphologic features, whereas primary and metastatic tumors had apical-in features. In a mouse liver metastasis model, apical-out spheroids injected into the portal vein became apical-in spheroids in the liver within a few days. Inhibitors of Src and dynamin significantly decreased liver metastasis. Polarity switching was observed in spheroids and human cancer. The polarity switch was critical in an experimental liver metastasis model. *(Am J Pathol 2016, 186: 899—911; http://dx.doi.org/10.1016/j.ajpath.2015.12.011)*

Intestinal epithelial cells are organized into adherent groups of cells that separate and protect the organism from the external environment. A closed epithelium regulates the exchange of nutrients and waste between the internal and external environments. The direction of the exchange is determined by the epithelial cells' apical-basal polarity. The molecular mechanism of forming cell polarity is based on evolutionarily conserved partition-defective (PAR)/atypical protein kinase C (aPKC) system.[1,2] Asymmetrically localized components of the PAR-aPKC system are linked with other cellular machinery, such as junctional structure formation and actin polymerization. The perturbation of cell polarity is a general feature in cancer. It is becoming evident that Par proteins are directly involved in the loss of cell and tissue architecture in carcinoma.[3,4] For example, the loss of Par3 is directly linked to tumor invasion and metastasis in breast cancer.[5,6]

Because >90% of colorectal cancers (CRCs) are differentiated adenocarcinoma, polarity in most CRCs is disorganized but not completely lost. For most histologic types of CRC, the pathologic grades are independent of disease progression; even differentiated CRCs have malignant features.[7] The status and role of disorganized polarity in

Supported by Japan Society for the Promotion of Science Grant-in-Aid for Scientific Research (C) 26430138 (H.O., M.I.) and the Japan Advanced Molecular Imaging Program of the Ministry of Education, Culture, Sports, Science, and Technology of Japan (M.I., H.O., H.E., J.M.P.).

Disclosures: None declared.

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http://dx.doi.org/10.1016/j.ajpath.2015.12.011

differentiated cancer are poorly understood because most established CRC cell lines barely retain polarity as clusters of cells *in vitro*, with the exception of a few lines.[8,9]

We recently developed a method of preparing and culturing cancer cells from patient tumor samples or mouse xenografts: the cancer tissue–originated spheroid (CTOS) method.[10–12] The principle of the CTOS method is to maintain cell-cell contact throughout the process of preparation and culture. A CTOS is a spheroid that consists of pure cancer cells; it retains characteristics of original tumors and mouse xenograft tumors *in vitro*, particularly the formation of gland-like structures.

It has been widely accepted that metastasis originates from a single cell. In theory, multiple distinct steps are involved: a cancer cell detaches from the original tumor, migrates to the blood or lymphatic vessels, penetrates the lumen, circulates to distant organs, attaches to the vessels of distant organs, invades into the parenchyma, and finally forms tumors.[13] However, it was recently proposed that cancer cells that originate from solid tumors migrate not only as single cells but also as cell clusters in a process referred to as collective migration.[14] Clusters that maintain E-cadherin–based adhesions migrate in Matrigel synthetic matrix *in vitro*.[5] In clinical pathology, microvascular invasion in a solid tumor is diagnosed as a tumor mass surrounded by blood or lymphatic vessels. Microvascular invasion correlates strongly with the risk of metastasis.[15] In addition, circulating tumor clusters in the bloodstream of patients with breast cancer have been linked with disease progression.[16] Thus, clusters of cancer cells may also be an origin of metastasis.

We found that a dynamic change of polarity status occurs in CRC CTOS *in vitro* and *in vivo*. We also investigated the role of polarity in the metastasis of CTOS as clusters of cancer cells.

Materials and Methods

Ethics Statement

The institutional ethics committees at the Osaka Medical Center for Cancer and Cardiovascular Diseases (OMCCCD) approved this study. CRC specimens were collected from patients treated at the Department of Surgery, OMCCCD. Animal studies were approved by the OMCCCD Institutional Animal Care and Use Committee and performed in compliance with the institutional guidelines.

Isolation and Culture of CTOS from Xenograft and Primary Tumors

Primary cultures of human CRC cells were prepared according to the CTOS method.[10] Briefly, surgically resected tumor samples or xenograft tumors were mechanically dissociated, partially digested with liberase DH (Roche, Mannheim, Germany), and filtered through cell strainers. Cell clusters were collected on 100-μm or 40-μm cell strainers (BD Falcon, Franklin Lakes, NJ). CTOSs were cultured in StemPro hESC (Invitrogen, Carlsbad, CA). Institutional pathologists examined the colorectal tumors. CTOSs in gel were embedded in CellMatrix I-A (Nitta Gelatin, Osaka, Japan) on 35-mm dishes and cultured in StemPro medium. CTOS in suspension were cultured with the CTOS method in noncoated 10-cm dishes in StemPro medium.

Immunostaining

CTOSs in suspension were fixed for 5 minutes with 10% formalin, embedded in CellMatrix I-A (Nitta Gelatin) to condense the CTOSs and fixed for 1 additional hour. CTOSs in gel were also fixed for 1 hour. Next, gel droplets that contained CTOSs were stained with Sirius red to facilitate further manipulation. Then, the gel was embedded in paraffin. The paraffin blocks were cut into 4-μm sections and subjected to immunostaining. Immunohistochemistry (IHC) was performed as previously described.[10] The following primary antibodies were used: E-cadherin (BD Biosciences, San Jose, CA), PARD3 (Sigma-Aldrich, St Louis, MO), D2-40 (Nichirei Biosciences, Tokyo, Japan), CD31 (Dako, Glostrup, Denmark), villin and cleaved caspase-3 (Cell Signaling Technology, Danvers, MA), and ezrin and ZO-1 (Invitrogen). LMO7 was a gift from Dr. Jun Miyoshi (OMCCCD, Osaka, Japan). For whole-mount staining of CTOSs, the CTOSs in gel were extracted by digesting the gel with 2 mg/mL of collagenase IV (Worthington Biochemical Corporation, Lakewood, NJ) for 10 minutes. The extracted CTOSs in gel or in suspension were fixed and permealized with 4% paraformaldehyde/phosphate-buffered saline containing 1% Triton X-100 at 4°C for 1 hour. After blocking with 5% bovine serum albumin (Sigma-Aldrich) for 1 hour, CTOSs were incubated with anti–E-cadherin antibody (BD Biosciences) overnight at 4°C. Next, they were incubated with secondary antibodies conjugated with Alexa-488 or Alexa-555 (Life Technologies, Carlsbad, CA) or Rhodamine Phalloidin with Hoechst33342 (Life Technologies) overnight. Cells were mounted with FluorSave Reagent (Calbiochem, San Diego, CA). Fluorescence images were obtained using confocal microscopy (Leica Microsystems, Wetzlar, Germany).

Quantitative Analysis of Polarity Status

The CTOSs were immunostained with villin. The polarity status of the CTOSs was classified into 4 groups: apical-out (apical proteins located only at the outermost membrane of a CTOS), apical-in (apical proteins located only at multiple lumens inside a CTOS), mixed (apical proteins located both at the outermost membrane and at multiple lumens inside a CTOS), and none (apical proteins not detected). Multiple CTOSs were analyzed and categorized, and the proportion of each polarity status was calculated. The number of CTOSs