The American Journal of Pathology, Vol. 187, No. 7, July 2017

ELSEVIER

The American Journal of PATHOLOGY

ajp.amjpathol.org

TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Differentiation Affects the Release of Exosomes from Colon Cancer Cells and Their Ability to Modulate the Behavior of Recipient Cells

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Accepted for publication March 4, 2017.

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Exosomes are involved in intercellular communication. We previously reported that sodium butyrate—induced differentiation of HT29 colon cancer cells is associated with a reduced CD133 expression. Herein, we analyzed the role of exosomes in the differentiation of HT29 cells. Exosomes were prepared using ultracentrifugation. Gene expression levels were evaluated by real-time PCR. The cell proliferation rate was assessed by MTT assay and with the electric cell-substrate impedance sensing system, whereas cell motility was assessed using the scratch test and confocal microscopy. Sodium butyrate—induced differentiation of HT29 and Caco-2 cells increased the levels of released exosomes and their expression of CD133. Cell differentiation and the decrease of cellular CD133 expression levels were prevented by blocking multivesicular body maturation. Exosomes released by HT29 differentiating cells carried increased levels of miRNAs, induced an increased proliferation and motility of both colon cancer cells and normal fibroblasts, increased the colony-forming efficiency of cancer cells, and reduced the sodium butyrate—induced differentiation of HT29 cells. Such effects were associated with an increased phosphorylation level of both Src and extracellular signal regulated kinase proteins and with an increased expression of epithelial-to-mesenchymal transition—related genes. Release of exosomes is affected by differentiation of colon cancer cells; exosomes might be used by differentiating cells to get rid of components that are no longer necessary but might continue to exert their effects on recipient cells. *(Am J Pathol 2017, 187: 1633—1647; http://dx.doi.org/10.1016/j.ajpath.2017.03.015)*

Colorectal cancer is the third most common malignancy in males and the second in females worldwide. Unfavorable trends of this cancer are thought to reflect a combination of factors, including changes in dietary patterns, obesity, and an increased prevalence of smoking.[1] Alimentary fibers exert protective effects against colon carcinogenesis,[2] and it has been suggested that sodium butyrate (NaBu), a short-chain fatty acid produced by anaerobic bacteria fermentation of dietary fibers preferentially used as an energy source by gut epithelial cells, may inhibit the development of colon cancer.[3] NaBu has been shown to inhibit the growth of colonic cancer cells, and to stimulate their apoptosis and differentiation.[4–6]

In 1997, Weigman et al[7] identified a novel cholesterol-interacting pentaspan-transmembrane glycoprotein, prominin-1 or CD133, as a surface protein marker of a subset of hematopoietic stem cells and progenitor cells. Ricci-Vitiani et al[8] and O'Brien et al[9] subsequently identified CD133 as a marker of colon cancer stem cells. CD133 is specifically concentrated in plasma membrane protrusions containing

Supported by a Università Cattolica del Sacro Cuore grant (A.S.) and an Italian Association for Cancer Research Loredana Gualandi Sabotti fellowship (D.L.). Dynamic light-scattering and microscopic measurements were performed at the facility Laboratorio Centralizzato di Microscopia Ottica ed Elettronica, Università Cattolica del Sacro Cuore (Rome, Italy).

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Disclosures: None declared.

Data were presented in part as an Abstract (C30) at the Italian 3rd Joint Meeting of Pathology and Laboratory Medicine, October 4–6, 2016, Montesilvano, Pescara, Italy.

http://dx.doi.org/10.1016/j.ajpath.2017.03.015

lipid rafts and, more recently, it has been demonstrated that CD133-containing lipid rafts play a pivotal role in the maintenance of stem cell features.[10] Moreover, CD133 is released from neural progenitor cells, epithelial cells, and hematopoietic stem cells by extracellular vesicles on differentiation.[11] It has been suggested that the release of CD133-containing vesicles may contribute to cell differentiation by reducing and/or modifying the composition of stem cell–characteristic membrane microdomains within the apical plasma membrane. Bauer et al[12] showed that hematopoietic stem cells and progenitor cells, which have the ability to differentiate into mature blood cells, release CD133-containing exosomes concomitant with cellular differentiation. Moreover, Rappa et al[13] isolated, from a whole fraction of exosomes released by melanoma cancer cells, only the exosomes positive for CD133: the exposure of bone marrow–derived stromal cells to the exosomes positive for CD133 increased the invasion ability of these cells compared with mock-treated bone marrow–derived stromal cells. These findings support the concept of cancer stem cell–specific lipid rafts holding molecular determinants needed to maintain cancer stem cell/prometastatic properties. We previously reported that NaBu-induced differentiation of HT29 colon cancer cells is associated with loss of CD133 expression.[14] Thus, it was of interest to verify whether exosome release is affected by colon cell differentiation and whether the release of CD133-containing vesicles might contribute to this phenomenon.

Materials and Methods

Exosome Purification

This study largely followed the protocol previously described by Théry et al,[15] with some modifications. The exosome purification process can be summarized as follows: cell culture media were centrifuged at 750 × *g* for 15 minutes, and then at 1500 × *g* for 5 minutes. Supernatants were saved and centrifuged at 14,000 × *g* for 45 minutes. Supernatants were transferred to fresh tubes and centrifuged at 100,000 × *g* for exosome purification. Exosome pellets were resuspended in phosphate-buffered saline and used for the treatment of cells or to prepare cell extracts for Western blot analysis. The Bradford assay and dynamic light scattering were used for the quantitative evaluation of exosome, as previously described.[15,16]

Cell Culture

Rat-1 (normal rodent fibroblasts), HT29, Caco-2, and HCT116 (human colorectal adenocarcinoma) cell lines were used for *in vitro* studies and were cultured in Eagle's minimum essential medium and in Dulbecco's modified Eagle's medium, respectively, supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 2 mmol/L L-glutamine at 37°C in a humid 5% CO_2 atmosphere.

Dynamic Light Scattering and Transmission Electron Microscopy

Dynamic light-scattering measurements were performed using a Zetasizer Nano ZS apparatus (Malvern Instruments Ltd, Worcestershire, UK). Data for each sample were collected on a continuous basis for 12 minutes in sets of four measurements for each sample. Peak-intensity analyses were used to determine hydrodynamic radius by number distribution, described elsewhere.[16]

For morphological study, we used transmission electron microscopy: isolated exosomes were fixed, adding Karnovsky EM fixative (2% formaldehyde and 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.4.) to the suspension in 1:1 ratio for 1 hour. Samples were then placed on Formvar-carbon–coated grids and air dried for 10 minutes. After being rinsed with distilled water, the specimens were post fixed in 1.5% osmium tetroxide in 0.1 mol/L cacodylate buffer (pH 7.3), were allowed to dry, and were observed with a Zeiss Libra 120 (Zeiss NTS GmbH, Oberkochen, Germany).

Western Blot Analysis

For Western blot analysis, cells or exosomal preparations were lysed using lysis buffer (50 mmol/L Tris-HCl, pH 7.2, 150 mmol/L NaCl, 100 mmol/L NaF, 100 mmol/L sodium pyruvate, and 1% Triton X-100) containing protease inhibitors, 2 mmol/L phenyl methyl sulfonyl fluoride, 10 mg/mL aprotinin, and 2 mmol/L Na_3VO_4. Extracted protein (10 μg) was separated by SDS-PAGE, transferred to immobilon-P membranes, and analyzed using the enhanced chemiluminescence kit for Western blotting detection (Amersham Pharmacia Biotech, Milan, Italy), as previously described.[15] Primary monoclonal antibodies were used following suppliers' instructions and included the following: mouse anti-human monoclonal CD63 (dilution, 1:1000; Santa Cruz Biotechnology, Inc., Dallas, TX), mouse monoclonal anti-human CD133 (dilution, 1:200; Miltenyi Biotec S.r.l., Bologna, Italy), rabbit polyclonal anti-human caveolin-1 (Cav-1; dilution, 1.500; Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-human Src (dilution, 1:1000; Cell Signaling Technology, Milan, Italy), rabbit polyclonal anti-human pSrc (dilution, 1:1000; Cell Signaling Technology), rabbit polyclonal anti-human extracellular signal regulated kinase (ERK; dilution, 1:1000; Cell Signaling Technology), and rabbit polyclonal anti-human pERK (dilution, 1:1000; Cell Signaling Technology).

Cell Viability Assay

Cell viability was evaluated by using an MTT assay, as previously described.[17] Briefly, 1×10^4 cells were plated in 100 μL of medium in 96-well microtiter plates and incubated for 24 hours. Medium was then replaced, and cells were incubated for 48 hours with exosomes or phosphate-buffered