Differentiated Human Colorectal Cancer Cells Protect Tumor-Initiating Cells From Irinotecan

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BACKGROUND & AIMS: Stem cells of normal tissues have resistance mechanisms that allow them to survive genotoxic insults. The stem cell–like cells of tumors are defined by their tumor-initiating capacity and may have retained these resistance mechanisms, making them resistant to chemotherapy. We studied the relationship between resistance to the topoisomerase I inhibitor irinotecan and tumor-initiating potential in human colonosphere cultures and in mice with colorectal xenograft tumors. **METHODS:** Colonosphere cultures were established from human colorectal tumor specimens obtained from patients who underwent colon or liver resection for primary or metastatic adenocarcinoma. Stem cell and differentiation markers were analyzed by immunoblotting and fluorescence-activated cell sorting. Clone- and tumor-initiating capacities were assessed by single-cell cloning and in immune-deficient mice. Sensitivity to irinotecan was assessed in vitro and in tumor-bearing mice. The relationship between drug resistance and tumor-initiating capacity was tested by fluorescence-activated cell sorting of colonosphere cells, based on expression of ABCB1 and aldehyde dehydrogenase (ALDH) activity. **RESULTS:** Colonosphere cultures had a high capacity to initiate tumors in mice and were resistant to irinotecan. Inhibition of the drug-efflux pump ABCB1 by PSC-833 allowed irinotecan to eradicate tumor-initiating cells. However, ABCB1 was expressed only by a subpopulation of differentiated tumor cells that did not form clones or tumors. Conversely, tumor-initiating cells were ABCB1-negative and were identified by high ALDH activity. Tumorigenic $ALDH^{high}/ABCB1^{negative}$ cells generated nontumorigenic $ALDH^{low}/ABCB1^{positive}$ daughter cells in vitro and in tumor xenografts. PSC-833 increased the antitumor efficacy of irinotecan in mice. **CONCLUSIONS: The resistance of colorectal tumors to irinotecan requires the cooperative action of tumor-initiating $ALDH^{high}/ABCB1^{negative}$ cells and their differentiated, drug-expelling, $ALDH^{low}/ABCB1^{positive}$ daughter cells.**

Keywords: Tumor Progression; Cancer Stem Cell; Drug Resistance; Metastasis; Colon Cancer.

Intestinal stem cells drive normal tissue turnover by generating daughter stem cells and a rapidly proliferating pool of progenitor cells that give rise to differentiated cell types with specialized functions.[1] This functional hierarchy is preserved in colon tumors in which a small tumor-initiating cell compartment drives the formation of proliferating and differentiated cell types with reduced tumorigenic potential.[2–5] Normal tissue stem cells recently have been identified as the cells of origin of several tumor types, including those of intestinal origin.[6–10] Preservation of the resistance mechanisms that characterize normal tissue stem cells during oncogenic transformation would provide the resulting tumor-initiating cells with an innate resistance to genotoxic agents. Therefore, tumor-initiating cells not only may drive tumor progression, but also may be responsible for resistance to chemotherapy and for subsequent tumor recurrence.[11–13] Indeed, tumor-initiating cells from a diverse range of tumors were found to display intrinsic chemoresistance.[13–18]

Chemotherapy of colorectal tumors is based primarily on 5-fluorouracil, oxaliplatin, and irinotecan.[19] However, colorectal tumors frequently fail to respond to chemotherapy, leaving the majority of tumor cells to survive treatment. Response rates are limited to 40%–50%.[19] It is presently unknown how these clinical observations fit a model in which chemoresistance is mediated by the small fraction of tumor cells with tumor-initiating capacity. Furthermore, drug-efflux capacity does not appear to mark intestinal or colorectal tumor-initiating cells.[20,21] Nevertheless, chemotherapy does enrich the population of clonogenic cancer cells in colorectal xenograft models, suggesting that these cells do display some form of selective drug resistance.[4,22]

Here, we studied the relationship between tumor-initiating potential and irinotecan resistance by using a series of highly clonogenic and tumorigenic colonosphere cultures isolated from freshly resected primary colorectal tumors and liver metastases.

Materials and Methods

Collection of Tumor Specimens

Human colorectal tumor specimens were obtained from 28 patients undergoing a colon or liver resection for primary or metastatic adenocarcinoma, in accordance with the ethical committee on human experimentation. Informed consent was obtained from all patients. Diagnosis of tumor type and grade was based on histologic examination.

Abbreviations used in this paper: ALDH1, aldehyde dehydrogenase 1; FACS, fluorescence-activated cell sorting.

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Isolation and Expansion of Colorectal Tumor-Initiating Cell Cultures

A detailed procedure describing the isolation and expansion of colorectal tumor-initiating cell cultures is given in the Supplementary Materials and Methods section.

Colonosphere-Forming Efficiency

Single-cell suspensions derived from colonospheres and differentiated tumor cell cultures were counted, suspended in Matrigel (100 cells in 100 μL) (BD Biosciences, Franklin Lakes, NJ), and allowed to set in 48-well plates. After setting, stem cell medium with fresh growth factors was added. Clone formation was analyzed after 3 weeks of culture. Colonies were counted using a Leica DM IRBE microscope (Leica, Solms, Germany) and the colonosphere-forming efficiency was calculated as the percentage of seeded cells that formed colonospheres.

Irinotecan Sensitivity Assay

Both colonosphere cultures and their differentiated derivatives were cultured in stem cell medium in the presence of irinotecan (Campo; Pfizer, Capelle a/d IJssel, The Netherlands) at the indicated concentrations for 5 days. In addition, colonosphere cultures were cultured in the presence of the ABCB1 inhibitor PSC833 (2 μmol/L; Novartis, Basel Switzerland), the ABCG2 inhibitor Ko143 (200 nmol/L; kindly provided by Dr Alfred Schinkel, Netherlands Cancer Institute, The Netherlands), and the ABCC1 inhibitor MK571 (30 μmol/L; Sigma, Zwijndrecht, The Netherlands) either alone, or in combination with 50 μg/mL irinotecan for 3 consecutive days. Mitochondrial activity was evaluated using CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI). All absorbance values are expressed as percentages of vehicle-treated control wells.

Immunofluorescence and Immunohistochemistry

Immunofluorescence was performed according to standard procedures, as specified in the Supplementary Materials and Methods section.

Statistical Analysis

The Student *t* test (unpaired, 2-tailed) was performed to analyze statistically significant differences between groups. Outcome variables are depicted as point estimates, with a 95% confidence interval. In vivo tumor formation and drug treatment experiments were analyzed by 1-way analysis of variance followed by a Bonferroni multiple comparison test using GraphPad Prism software (GraphPad, San Diego, CA). Differences with a *P* value of less than .05 were considered statistically significant.

Results

A Novel Series of Undifferentiated Colonosphere Cultures From Primary Colorectal Tumors and Liver Metastases

To study the relationship between tumor-initiating capacity and irinotecan resistance, we established a series of colonosphere cultures isolated from freshly resected primary colorectal tumors and liver metastases. Immediately after surgery, biopsies were obtained from the resection specimens. Single-cell cultures were prepared from the biopsies and these were cultured in serum-free stem cell medium in low-adherence flasks. Colonospheres formed in 8 of 20 of these cultures, 4 originating from primary colorectal cancer tumors and 4 originating from liver metastases (Supplementary Table 1). Single-cell suspensions of all 8 colonosphere cultures displayed high colonosphere-forming efficiency ranging from 20%–62% (Supplementary Table 1). Furthermore, these cells were highly tumorigenic when injected into immune-deficient mice (Supplementary Table 1). Flow cytometry and Western blot analysis for the expression of published markers for tumor-initiating cells[2,3,5,22–24] showed that all colonospheres were positive for CD44 (50%–100% of the cells) and aldehyde dehydrogenase 1 (ALDH1), whereas expression of CD133 and CD166 was more variable, ranging from 0%–100% CD133-positive cells, and from 15%–100% CD166-positive cells (Supplementary Table 1). Colonosphere-initiated xenografts displayed extensive differentiation and were histologically similar to the tumors from which they were derived (Supplementary Figure 1).

When exposed to serum-containing medium the colonospheres attached to the culture dish and grew out to form adherent cultures with heterogeneous morphology (Figure 1*A*). A subpopulation of cells was positive in the periodic acid–Schiff stain for mucin-producing goblet cells (Figure 1*A*) and all adherent cultures expressed the differentiation marker cytokeratin 20 (Figure 1*B*). Distinct subpopulations of the adherent tumor cell populations expressed cytokeratin 20 and the enterocyte brush-border marker phospho-ezrin (Figure 1*C*). An unbiased proteomics screen of the tumorigenic and nontumorigenic differentiated tumor cell populations revealed that ALDH1 was strongly expressed in all tumor-initiating cell-enriched cultures, but not in the differentiated cultures. Western blot analysis confirmed the selective expression of ALDH1 in the colonosphere cultures (Figure 2*A*). Colonosphere-derived single-cell populations displayed heterogeneous ALDH activity as measured by Aldefluor (STEMCELL Technologies, Grenoble, France) (Supplementary Figure 2), and cells with high ALDH activity displayed a significantly higher clone-forming potential when compared with cells with low ALDH activity (56% vs 14%; difference, 42%; 95% confidence interval, 34%–49%; $P < .0001$) (Figure 2*B*). Co-staining with Aldefluor and CD44 showed that the vast majority (>96%) of Aldefluor-positive colonosphere cells also were positive for CD44 (Supplementary Figure 2). Importantly, serial transplantation of $ALDH^{high}$ cells into immune-deficient mice revealed their capacity to self-renew and to form tumors (Figure 2*C*, Supplementary Figure 3). Upon in vitro differentiation, ALDH1 expression decreased, and this was accompanied by a drastic reduction in clone- and tumor-forming potential (Supplementary Table 1, Figure 2*D–E*) (for statistics see Supplementary