

Chemoresistant Colorectal Cancer Cells, the Cancer Stem Cell Phenotype, and Increased Sensitivity to Insulin-like Growth Factor-I Receptor Inhibition

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Abstract

5-Fluorouracil (5FU) and oxaliplatin are standard therapy for metastatic colorectal cancer (CRC), but the development of chemoresistance is inevitable. Because cancer stem cells (CSC) are hypothesized to be chemoresistant, we investigated CSC properties in newly developed chemoresistant CRC cell lines and sought to identify targets for therapy. The human CRC cell line HT29 was exposed to increasing doses of 5FU (HT29/5FU-R) or oxaliplatin (HT29/OxR) to achieve resistance at clinically relevant doses. Western blotting and flow cytometry were done to determine molecular alterations. The insulin-like growth factor-I receptor (IGF-IR) monoclonal antibody (mAb) AVE-1642 was used to inhibit signaling *in vitro* and *in vivo* using murine xenograft models. HT29/5FU-R and HT29/OxR showed 16- to 30-fold enrichment of CD133⁺ cells and 2-fold enrichment of CD44⁺ cells (putative CRC CSC markers). Resistant cells were enriched 5- to 22-fold for double-positive (CD133⁺/CD44⁺) cells. Consistent with the CSC phenotype, resistant cells exhibited a decrease in cellular proliferation *in vitro* (47–59%; $P < 0.05$). Phosphorylated and total IGF-IR levels were increased in resistant cell lines. HT29/5FU-R and HT29/OxR cells were ~5-fold more responsive to IGF-IR inhibition relative to parental cells ($P < 0.01$) *in vitro*. Tumors derived from HT29/OxR cells showed significantly greater growth inhibition in response to an IGF-IR mAb than did parental cells ($P < 0.05$). Chemoresistant CRC cells are enriched for CSC markers and the CSC phenotype. Chemotherapy-induced IGF-IR activation provided for enhanced sensitivity to IGF-IR-targeted therapy. Identification of CSC targets presents a novel therapeutic approach in this disease. [Cancer Res 2009;69(5):1951–7]

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer death in the United States with about 50,000 deaths estimated for 2008 alone (1). Almost 25% of patients who present with CRC present with metastatic disease, and thousands of patients receive treatment for metastatic CRC each year (2). Significant improvements in patient survival rates have been achieved in recent years, largely due to the availability of targeted molecular therapies in addition to the

standard chemotherapeutic regimens. The median overall survival duration for patients with metastatic CRC is currently about 20 months; however, most patients still die of their disease (3). Although the response rate to current systemic therapies is ~50%, resistance develops in nearly all patients. Therefore, it is essential to understand mechanisms of resistance as a first step in developing approaches to preventing or reversing chemoresistance in patients who receive systemic therapy for metastatic CRC.

5-Fluorouracil (5FU) and oxaliplatin are the mainstays of chemotherapeutic regimens for metastatic CRC. 5FU inhibits activity of the enzyme thymidylate synthase during DNA replication (4). In contrast, oxaliplatin covalently binds DNA, forming platinum-DNA adducts that cause prolonged G₂ arrest and inhibition of growth, which leads to apoptotic cell death (5). Although resistance mechanisms have been extensively studied for both of these agents, therapies to target resistance pathways have yet to be identified. There is an emerging body of evidence that tumor cells that are resistant to chemotherapy represent a subpopulation of cells from the original tumor that are molecularly and phenotypically distinct. These cells are referred to by several names, including tumor-initiating cells, tumor-promoting cells, or, more commonly, cancer stem cells (CSC; ref. 6).

Experimental evidence for the existence of CSCs in CRC was recently shown using human surgical specimens (7–9). Cells characterized by the expression or the absence of the transmembrane surface marker CD133 were isolated from fresh CRC tumors and injected into nonobese diabetic-severe combined immunodeficient mice. Tumors resulting from CD133⁺ cells resembled the primary tumor from which they were derived and were more tumorigenic than were CD133⁻ cells. Several other markers have been identified as putative CSC markers, including CD44, epithelial surface antigen, and CD166 (9). There is no consensus as to the exact criteria that define a CSC, as markers may vary according to tumor type. However, several functional studies have identified characteristics of these cells, including inherent chemoresistance, the ability to efflux Hoechst dye, the tendency to form spherical colonies *in vitro*, and the ability, in limited numbers, to form tumors in immunodeficient mice [reviewed in Tang and colleagues (10)]. Several other properties have been identified in organ-specific CSCs, and new studies are continuously being reported further characterizing the CSC phenotype.

Perhaps the most clinically relevant variable of CSCs is their resistance to standard chemotherapeutic agents, and thus, there is a great deal of interest in attempting to identify new targets for specifically eradicating these cells. Recent attention has been given to notch signaling (11), the sonic hedgehog pathway (12, 13), and growth factor receptors.

Given the clinical significance of chemoresistance and the ineffectiveness of chemotherapy in eliminating CSCs, we chose

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to evaluate the relationship between chemoresistance and the CSC phenotype. We developed two chemoresistant cell lines from a chemosensitive parental human CRC cell line and investigated their molecular and phenotypic alterations *in vitro* and *in vivo*. After we detected activation of the insulin-like growth factor-I receptor (IGF-IR) pathway in the chemoresistant cell lines, we targeted this pathway in several xenograft studies.

Materials and Methods

Cell lines and culture conditions. The human CRC cell line HT29 was obtained from the American Type Culture Collection. The oxaliplatin-resistant cell line HT29/OxR was developed in our laboratory as previously described (14). Cells stably resistant to 5FU were developed by exposing parental HT29 cells to an initial dose of 0.1 $\mu\text{g}/\text{mL}$ and culturing surviving cells to a confluence of 80% for three passages (~6 wk). The cells that survived initial 5FU treatment were then exposed to 0.5 $\mu\text{g}/\text{mL}$ 5FU for three passages (~8 wk) and then 1.0 $\mu\text{g}/\text{mL}$ for three passages (~8 wk). Finally, the 5FU concentration was increased to the clinically relevant plasma concentration of 2 $\mu\text{g}/\text{mL}$ for 3 wk (10 wk). The surviving resistant cells were named HT29/5FU-R.

All cells were cultured in MEM supplemented with 10% fetal bovine serum (FBS), vitamins, nonessential amino acids, penicillin-streptomycin, sodium pyruvate, and L-glutamine (Life Technologies). Oxaliplatin- and 5FU-resistant cells were continuously cultured in 2 $\mu\text{mol}/\text{L}$ and 2 $\mu\text{g}/\text{mL}$ of the respective drugs, unless otherwise indicated. *In vitro* experiments were carried out at 70% cell confluence and confirmed in at least three independent experiments.

Drugs and antibodies. Oxaliplatin and 5FU were purchased from the M. D. Anderson Cancer Center pharmacy. The monoclonal antibody (mAb) AVE-1642 (provided by Sanofi-Aventis) was used to inhibit IGF-IR signaling *in vitro* and *in vivo* and has been previously described (15). Antibodies used for flow cytometry, immunohistochemical analysis, immunofluorescence, or Western blotting were as follows: rabbit anti-CD133, rabbit anti-phosphorylated IGF-IR (Cell Signaling Technology), mouse anti-vinculin, rabbit anti- β -actin (Sigma-Aldrich), mouse anti-CD44 (Abcam), phycoerythrin (PE)-conjugated anti-CD133, PE-conjugated mouse-IgG1 (Miltenyi Biotec),

FITC-conjugated anti-CD44, FITC-conjugated mouse-IgG2b, rabbit anti-IGF-IR (Santa Cruz Biotechnology), and mouse anti-Ki67 (Dako).

Western blotting. For all Western blot analyses, protein was harvested from cells plated to 70% to 80% confluence. Whole-cell lysates were isolated using radioimmunoprecipitation assay B protein lysis buffer as previously described (16). Secreted proteins were obtained from conditioned medium after cells were plated in 1% FBS medium for 48 h; medium was harvested and concentrated using Amicon Ultra Centrifugal Filter Devices (Millipore Corp.). Quantification of these proteins was completed using a modified Bradford assay (Bio-Rad Laboratories). Protein samples for Western blotting were prepared by boiling after the addition of denaturing sample buffer. Proteins were separated using SDS-PAGE on an 8% or 15% gel and transferred to a polyvinylidene difluoride membrane (Millipore) by electroblotting. Antibodies were diluted in TBS and 0.1% (v/v) Tween with 5% nonfat dry milk after 1 h of protein blocking in the absence of antibody. Membranes were incubated at 4°C overnight with primary antibody. Membranes were subsequently washed and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences) for 1 h at room temperature. Membranes were again washed, and protein bands were visualized using a commercially available enhanced chemiluminescence kit (Amersham Biosciences). When appropriate, membranes were incubated in stripping solution for 30 min at 65°C, washed, and reprobed with a second primary antibody for verification of loading control.

Flow cytometry and cell cycle analysis. Cells were prepared for analysis of cell surface marker expression by plating to 70% confluence the day before analysis. Cells were then detached from plates by incubation with enzyme-free cell dissociation buffer (Invitrogen). Cells were washed in PBS and resuspended in 1% bovine serum albumin plus fluorophore-conjugated primary antibodies for 30 min at room temperature. Samples were then washed and analyzed using a Cell Lab Quanta flow cytometer coupled to a computer with data acquisition and analysis software (Beckman Coulter).

Proliferation and chemosensitivity assay. Rates of proliferation and sensitivity to drugs were assessed using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (17). Briefly, 2,500 cells of each cell line were plated per well in 96-well plates in 200 μL medium with or without drug (5FU, oxaliplatin, and

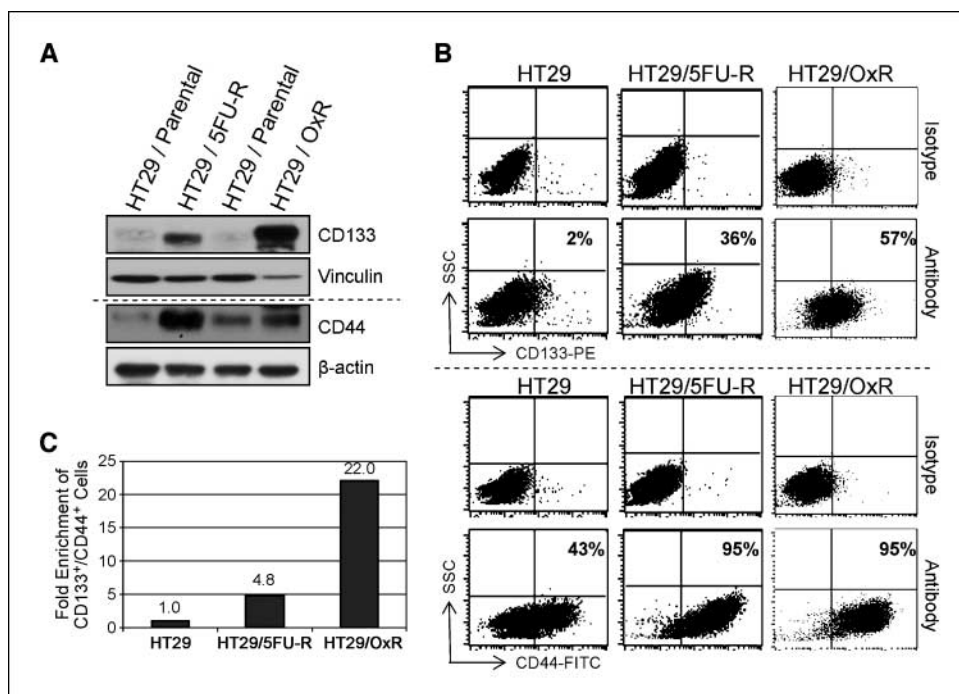
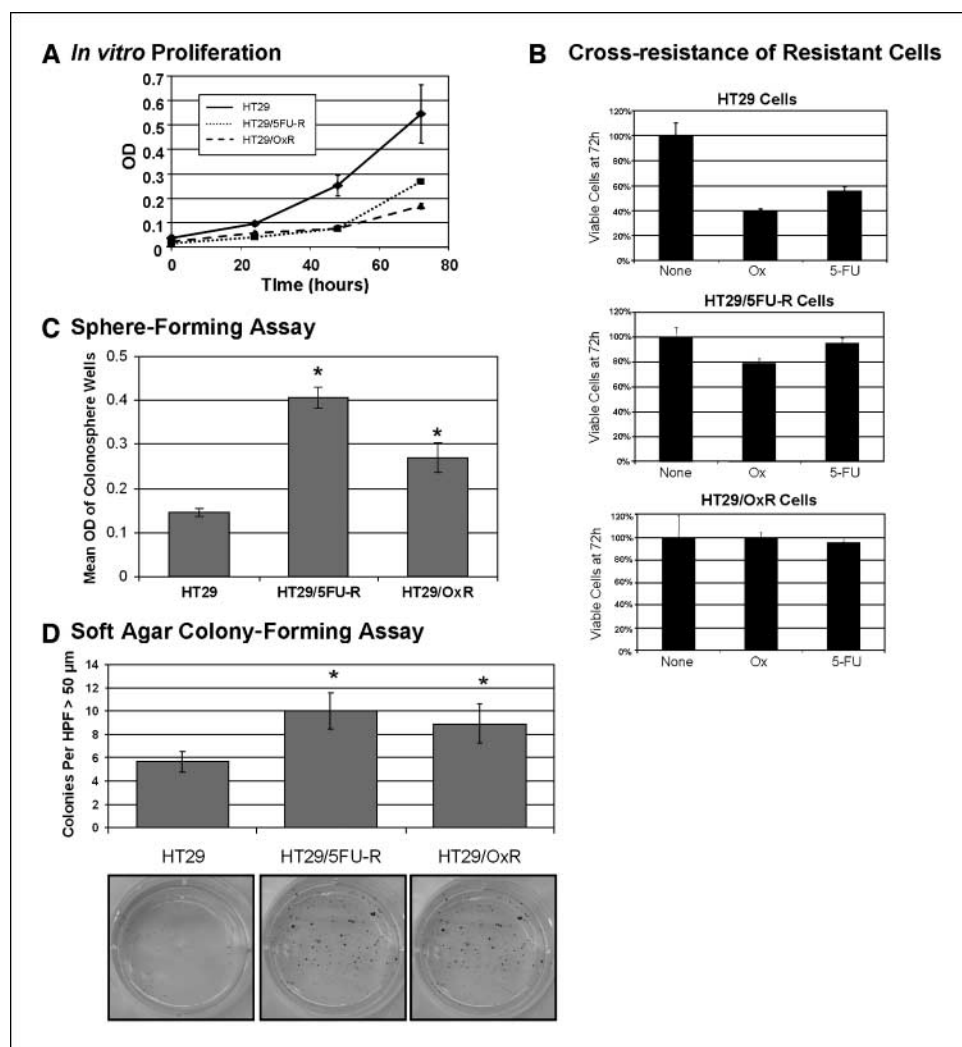


Figure 1. Chemoresistant cell lines are enriched for tumor stem cell markers. *A*, Western blotting showed that expression of the CRC stem cell markers CD133 and CD44 was higher in the chemoresistant cell lines HT29/5FU-R and HT29/OxR than in the parental HT29 human CRC cell line. Vinculin and β -actin levels are provided as loading controls. *B*, flow cytometric analysis showed that both chemoresistant cell lines were enriched for cells that expressed CD133 and CD44 when compared with the parental cell line. Thirty-six percent of HT29/5FU-R cells and 57% of HT29/OxR cells expressed CD133 relative to 2% of HT29 cells. Similarly, 95% of chemoresistant cells expressed CD44 relative to only 43% of parental cells. Cytometric analysis plots using isotype control antibodies are provided as staining controls. *C*, when cells were labeled with both markers, flow cytometric analysis showed ~5-fold enrichment of double-positive cells in the HT29/5FU-R cell line and ~22-fold enrichment in the oxaliplatin-resistant cell line compared with the parental HT29 cell line. SSC, side scatter.

Figure 2. Chemoresistant cell lines are enriched for tumor stem cell phenotype. *A*, chemoresistant cells proliferate at a significantly slower rate than do parental cells by MTT analysis. *OD*, absorbance. *B*, parental cells show sensitivity to both 5FU and oxaliplatin (*Ox*) after exposure to drug for 72 h, with only 40% and 58% of cells remaining, respectively, relative to untreated cells. In contrast, HT29/5FU-R cells are resistant to 5FU as expected; however, they also show an increase in resistance to oxaliplatin relative to parental cells. Similarly, oxaliplatin-resistant cells are resistant to 5FU. *C*, cells were plated in an ultralow-attachment 96-well plate in the absence of serum, and 14 d later, the number of viable sphere-forming cells was assayed by MTT. Chemoresistant cells showed a 2- to 3-fold increase in colonosphere-forming cells relative to parental cells. *D*, in a soft agar assay, 5FU- and oxaliplatin-resistant cells formed significantly more colonies larger than 50 μm in diameter under anchorage-independent growth conditions when compared with HT29 cells. These findings are consistent with the described CSC phenotype. *Bars*, SE. *, $P < 0.05$. *HPF*, high-power field.



AVE-1642). At each time point (0, 24, 48, and 72 h), 40 μL MTT solution was added to each well and the plate was incubated for 1 h at 37°C. Medium was then aspirated from each well, and 100 μL DMSO was added. Colorimetric analysis was performed at a wavelength of 570 nm using a standard microplate reader. Doubling times were calculated by plotting growth curves on Excel v2003.SP2 software (Microsoft Corp.) and using the built-in exponential regression algorithm.

Colonosphere assay. The ability of cell lines to form spheres in suspension was evaluated as described by Liu and colleagues (13), with modifications. DMEM with B27 supplement (Life Technologies), 20 $\mu\text{g}/\text{mL}$ epidermal growth factor (Invitrogen), 20 $\mu\text{g}/\text{mL}$ fibroblast growth factor (Invitrogen), and penicillin-streptomycin served as the stem cell medium (SCM) for these experiments. An equal number of cells from parental HT29 cells and each chemoresistant cell line were plated at a concentration of 200 cells/100 μL SCM in each of 32 wells of a 96-well ultralow-attachment plate (Corning Life Sciences). Cells were supplemented with 100 μL SCM after 5 d of incubation and analyzed on day 10, when the formation of colonospheres was evaluated by light microscopy. To quantitate the difference in numbers of spheres between cell lines, MTT solution (40 μL) was added to each well, and colorimetric assessment was conducted as above. The average absorbance measurements for each cell line from 32 wells were used as an index of sphere number.

Anchorage-independent growth assay. Soft agar assays were used to determine the ability of parental and chemoresistant cell lines to grow

under anchorage-independent conditions. Each well of a six-well plate was coated with 1 mL of 10% FBS medium with 1% agarose. After 20 min of incubation at 37°C, cell suspensions of an equal number (500) of parental and chemoresistant cells were added in 1 mL of medium with 0.5% agarose. Cells were incubated for 14 d under standard conditions (37°C, 5% CO_2) and with the addition of 300 μL of medium every 3 d to hydrate the exposed agarose. At the end of the incubation period, wells were examined under a light microscope at $\times 20$ magnification and the number of colonies larger than 50 μm was counted per well.

S.c. xenograft model and IGF-IR inhibition. Male athymic nude mice, 6 to 8 wk old, were obtained from the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD) and acclimated for 2 wk. All animal studies were conducted under approved guidelines of the Animal Care and Use Committee of M. D. Anderson Cancer Center. Equal numbers of cells (10^6) from parental and chemoresistant cell lines were suspended in 100 μL PBS and injected s.c. into the right rear flank of each mouse (20 mice per group). When tumors reached $\sim 100 \text{ mm}^3$, i.p. treatment with 1 mg/mouse AVE-1642 was initiated at a dosing frequency of twice per week for 10 mice in each group. Tumor growth was observed and recorded over 10 wk. When tumors in the control group exceeded 1.5 cm in longest diameter, mice were killed by CO_2 asphyxiation according to protocol, and tumors were excised. Tumors were weighed and measured, and a portion of each was placed in 10% formalin (for paraffin embedding), placed in OCT compound, and snap frozen in liquid nitrogen. Tumor volume was calculated as $(\text{length})/2 \times (\text{width})^2$.

Immunohistochemistry and immunofluorescent analysis. Tumors preserved in formalin were placed in paraffin blocks and sectioned onto positively charged microscope slides. They were deparaffinized in xylene, hydrated in graded alcohol, and pretreated for antigen retrieval in citrate buffer for 20 min in a 98°C steamer. Tumor sections embedded in OCT compound were sectioned onto positively charged microscope slides and serially immersed in acetone, acetone/chloroform mixture (1:1), and then acetone. Slides were then stained for H&E to assess morphology or anti-Ki67 antibodies to visualize proliferative nuclei. All immunohistochemical sections were counterstained with Gill No. 3 hematoxylin (Sigma-Aldrich). The DeadEnd Fluorometric TUNEL System (Promega) was used to identify and quantitate apoptotic cells in *in vivo* sections. Immunofluorescent slides were examined using a Nikon Microphot FXA fluorescent microscope, and representative images were obtained. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) images were processed using NIH ImageJ v1.34 software, converting TUNEL fluorescent images to binary images to quantitate staining. Immunohistochemical staining and fluorescence were quantitated using commercially available image analysis software (Photoshop v7.0, Adobe Systems, Inc.).

Results

Effect of chemoresistance on expression of CSC markers. The expression profiles of parental HT29 human CRC cells and

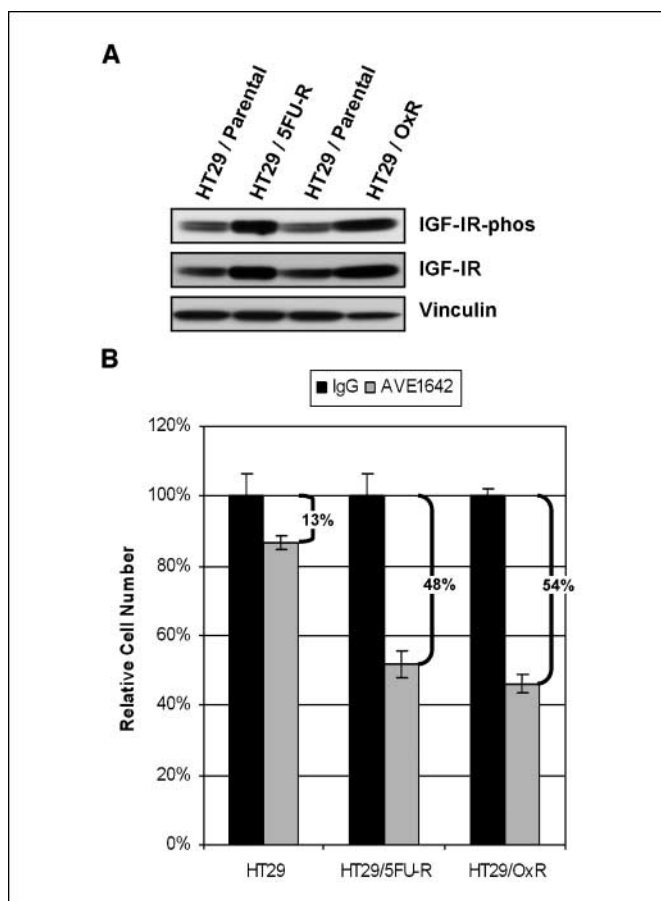


Figure 3. Effect of IGF-IR inhibition on chemoresistant cells. *A*, analysis of whole-cell lysates from parental and resistant cells showed an increase in both phosphorylation and total levels of IGF-IR in chemoresistant cells relative to parental cells. *B*, cells were treated with control or IGF-IR-targeting antibodies, and the cell number relative to that of the control treatment was analyzed. There was a significantly greater decrease in cell number in the chemoresistant cells treated with AVE-1642 than in the parental cells (48–54% versus 13%; $P < 0.05$).

cells resistant to 5FU or oxaliplatin (HT29/5FU-R or HT29/OxR, respectively) were evaluated by Western blot and flow cytometric analyses. HT29/5FU-R and HT29/OxR cells expressed significantly more CD133 and CD44, putative CSC markers (7, 8), than did parental HT29 cells (Fig. 1A). The number of cells that expressed CD133 and CD44 was also significantly greater in the chemoresistant cell lines than in parental cells (Fig. 1B). Whereas only 2% of parental cells expressed CD133 and 43% expressed CD44, more than 36% of HT29/5FU-R and 57% of HT29/OxR cells expressed CD133, and 95% of both types of chemoresistant cells expressed CD44. In fact, 5FU-resistant cells were ~5-fold and oxaliplatin-resistant cells ~22-fold enriched for cells positive for both CD133 and CD44 (Fig. 1C).

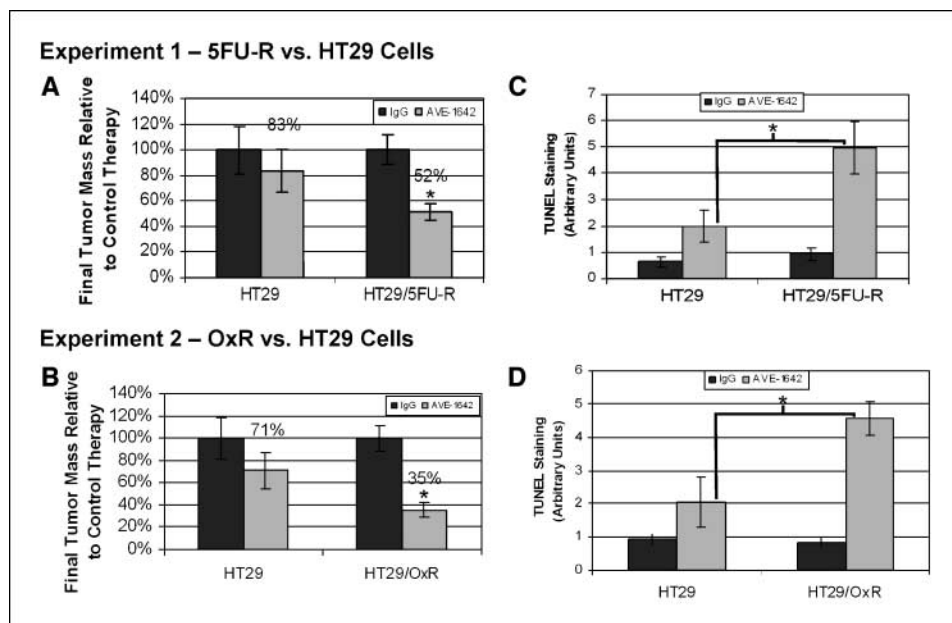
Effect of chemoresistance on the cellular phenotype. *In vitro* proliferation was assessed by plating equal numbers of cells of each cell line and by using MTT assay as an index of cell number. The proliferation rates of both 5FU- and oxaliplatin-resistant cells were significantly lower than that of the parental cells ($P < 0.05$; Fig. 2A). Specifically, the doubling time of parental HT29 cells was 17 hours, whereas the doubling times of HT29/5FU-R and HT29/OxR cells were 32 and 42 hours ($P < 0.05$ versus parental cells), respectively. MTT assay was also used to evaluate sensitivity to chemotherapeutic agents. Parental and 5FU- and oxaliplatin-resistant cells were exposed to clinically relevant doses of 5FU and oxaliplatin, and the remaining number of cells was evaluated after 48 hours. Parental cells were sensitive to both oxaliplatin and 5FU, with only 40% and 58% of viable cells remaining after exposure to drug, respectively (Fig. 2B). 5FU-resistant cells were resistant to 5FU as expected, but these cells were also resistant to oxaliplatin, with 80% of viable cells remaining after 72 hours of exposure. Similarly, oxaliplatin-resistant cells were resistant to oxaliplatin as expected but also showed cross-resistance to 5FU.

CSCs have the described ability to form colonies, or spheres, in the absence of serum and without attachment to culture plates (13). We evaluated the ability of parental and chemoresistant cell lines to grow colon cancer cell spheres, or colonospheres, under serum-free conditions. Differences between cell lines were quantitated by plating a limited number of cells in each well of a low-attachment 96-well plate and evaluating the ability of cells to form colonospheres. HT29/5FU-R and HT29/OxR cells had an increased number of spheres relative to parental cells ($P < 0.05$; Fig. 2C). Chemoresistant cells also showed an increased ability to form colonies under anchorage-independent conditions in a standard soft agar assay after 14 days in culture (Fig. 2D).

Effect of chemoresistance on IGF-IR signaling. Constitutive signaling in parental and chemoresistant cell lines, with a focus on targets for which agents that inhibit target function are readily available, was evaluated by Western blotting. We studied the activation status of several growth factor receptors but focused our efforts on IGF-IR, for which we observed the most marked alterations. Levels of phosphorylated IGF-IR were higher in both HT29/5FU-R and HT29/OxR cells than in parental cells (Fig. 3A). Levels of total IGF-IR were also increased in the chemoresistant cell lines.

The IGF-IR mAb AVE-1642 was used to determine the dependence of the cells on IGF-IR signaling for survival. MTT assay showed that AVE-1642 treatment of chemoresistant cells led to only a minor decrease (13%) in cell number in the parental cells but a significantly greater reduction in cell number in the chemoresistant cells relative to the parental cells (48% for HT29/5FU-R and 54% for HT29/OxR; $P < 0.05$ in each case; Fig. 3B).

Figure 4. Effect of IGF-IR inhibition on *in vivo* tumor growth, proliferation, and apoptosis. Mice were s.c. injected with 1×10^6 HT29 or HT29/5FU-R cells in one experiment or HT29 or HT29/OxR cells in a second experiment and treated with control IgG or AVE1642 twice weekly. The final tumor masses were measured and compared between mice bearing tumors from each cell line. Relative to control-treated mice, HT29/5FU-R-derived (A) and HT29/OxR-derived tumors (B) showed significantly greater growth inhibition than did HT29-derived tumors. TUNEL staining revealed significantly greater apoptosis in response to AVE1642 in tumors derived from HT29/5FU-R-derived (C) and HT29/OxR-derived tumors (D) than in tumors derived from parental cells. Bars, SE. *, $P < 0.05$.



We further evaluated whether treatment with AVE-1642 would restore chemosensitivity in our chemoresistant cell lines. Using the MTT assay, there were no differences between cells treated with AVE-1642 and those treated with both AVE-1642 and either 5FU or oxaliplatin after 48 hours of exposure (data not shown). This chemosensitivity assay was performed with both HT29/5FU-R and HT29/OxR cells, yielding similar results.

Effect of IGF-IR inhibition on *in vivo* tumor growth. Parental and chemoresistant cells were injected s.c. in the right flanks of nude mice, and tumor growth was assessed during biweekly treatment with AVE-1642 or with nonspecific, isotype-matched control mAb. After ~4 weeks (at which time maximum tumor size approached 1.5 cm³), tumors were harvested and analyzed. Tumors derived from parental and chemoresistant cells that were treated with AVE1642 were significantly smaller than those treated with control antibody (Fig. 4A and B). However, HT29/5FU-R tumors and HT29/OxR tumors showed significantly greater tumor growth inhibition with AVE-1642 treatment than did parental cells. 5FU-resistant cells showed 48% growth inhibition and oxaliplatin-resistant cells 65% growth inhibition relative to parental cells (17%; $P < 0.05$).

Evaluation of a proliferative marker (Ki67) by staining of tumor sections showed that the mAb to IGF-IR led to a decrease in the number of proliferating cells in all tumors compared with those treated with control antibody. However, similar to tumor growth inhibition, blockade of IGF-IR led to a greater effect in chemoresistant tumors than in tumors from parental cells, although this difference did not reach statistical significance. TUNEL staining was used to evaluate apoptosis in xenografts. Quantification of TUNEL staining showed that AVE-1642 treatment caused significantly more apoptosis in tumors derived from HT29/5FU-R and HT29/OxR cells than in tumors derived from HT29 cells ($P < 0.05$; Fig. 4C and D). Specifically, HT29 tumors showed a 2.7-fold increase in apoptotic nuclei with IGF-IR inhibition compared with a 5.3-fold increase in HT29/5FU-R-derived and a 5.5-fold increase in HT29/OxR-derived tumors ($P < 0.05$ in each case). Representative images from all tumor sections analyzed, in addition to photographs of s.c. tumors, can be seen in Fig. 5.

Discussion

CRC is the second leading cause of cancer death in the United States. Despite recent therapeutic regimens that have significantly increased survival in metastatic disease, invariably, nearly all tumors become chemoresistant. Understanding the mechanisms of resistance in CRC is essential to optimizing current therapeutic strategies.

To address the issue of identifying potential targets in chemoresistant cell lines, we created two chemoresistant cell lines from the parental human CRC cell line HT29. HT29/5FU-R and HT29/OxR cells were developed to be resistant to 5FU and oxaliplatin, respectively, at the clinically relevant plasma concentrations of patients receiving these drugs. The oxaliplatin-resistant cell line was characterized in a previous study from our laboratory whereby oxaliplatin-resistant cells were shown to undergo epithelial-to-mesenchymal transition (EMT; ref. 14). EMT-consistent changes were likewise observed in the HT29/5FU-R-resistant cell line (data not shown). In attempting to determine the mechanisms that imparted chemoresistance on our cell lines, we investigated these cells for CRC stem cell markers because CSCs are characterized by being chemoresistant.

Many investigators have used fluorescence-activated cell sorting to identify and isolate CSCs and determined that these cells are chemoresistant. We took the opposite approach by developing chemoresistant cells and then determining whether these cells acquired CSC characteristics. We determined that both 5FU- and oxaliplatin-resistant cells were significantly enriched for the CSC markers CD133 and CD44. Chemoresistant cells were also more quiescent *in vitro*, showing a decrease in cellular proliferation relative to parental cells. However, HT29/5FU-R and HT29/OxR cells showed an increased ability to form colonies in soft agar under anchorage-independent conditions and an increased ability to form spheres in specialized serum-free medium, all properties consistent with the CSC phenotype (13, 18–20). Oxaliplatin-resistant cells showed an increase in resistance to 5FU, and likewise, 5FU-resistant cells were cross-resistant to oxaliplatin. This finding suggests that acquired resistance to one chemotherapeutic

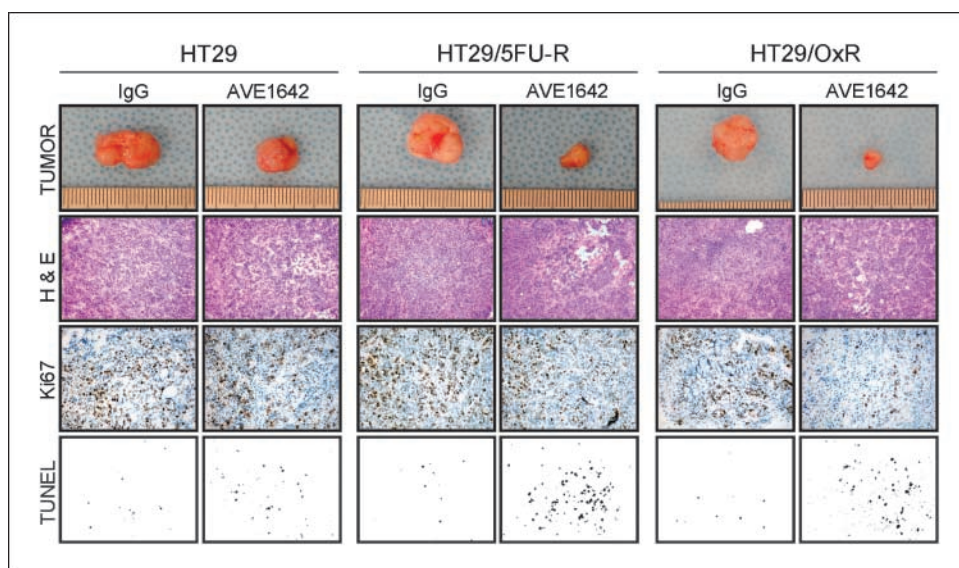


Figure 5. Effect of IGF-IR inhibition on *in vivo* tumor characteristics. Immunohistochemical analysis of tumors was conducted, and multiple tumor fields were evaluated per group. Representative images for all groups from both experiments are presented. H&E staining revealed similar s.c. tumor morphology among all groups of tumors. Ki67 staining showed decreased proliferative cells in tumors treated with AVE1642; however, differences in reduction of proliferative cells between parental and resistant cell-derived tumor sections were not significant. TUNEL staining revealed significantly greater apoptosis in response to AVE1642 in tumors derived from chemoresistant cells than in tumors derived from parental cells ($P < 0.05$).

agent activates general resistance pathways that impart resistance to multiple agents. Chemoresistant cells show enrichment of CSC markers and properties consistent with the CSC phenotype; however, definitive evidence that these cells are true CSCs has yet to be obtained, and alternate explanations for our findings do exist. For example, it is certainly plausible that the process of developing chemoresistant cell lines may include increased expression of CSC markers rather than enrich for those cells already expressing these markers. Testing this hypothesis would be difficult, however, as little is known about the pathways involved and the expression pattern of CD133 in epithelial malignancies. In addition, studies in our laboratory have shown that CSC marker expression is elevated in lysates obtained from cell line-derived CRC spheres relative to adherent tumor cells (data not shown). Given this finding and the data presented in this study, we have shown two mechanisms by which selection of cells using either sphere formation or chemoresistance enriches for CSC marker-expressing cells. To definitively explain our findings, further studies to elucidate the mechanism of resistance and increased marker expression are needed.

We also investigated the activation status of growth factor receptors in response to chemoresistance in a focused attempt to identify potential mediators in the chemoresistant cells that would allow specific targeting of these cells with available agents. One such pathway was the IGF-IR signaling pathway, known to be involved in CRC progression and growth (21, 22). In this study, constitutive IGF-IR levels were greater in chemoresistant cell lines relative to parental cell lines, more notably in the oxaliplatin-resistant line. The increase in IGF-IR phosphorylation is associated with the increase of total levels of the receptor. The attribution of the increase in IGF-IR phosphorylation being due to an increase in total levels of IGF-IR is supported by the fact that there were no demonstrable changes in expression of the ligands for IGF-IR (IGF-I and IGF-II; data not shown). The precise mechanism of the increase in IGF-IR expression in these cells remains unknown. Inhibition of Src and Akt signaling failed to block the increase in IGF-IR expression, ruling out these pathways as the mechanism for IGF-IR induction in chemoresistant cell lines.

Inhibition of IGF-IR *in vitro* led to a decrease in cell growth as determined by MTT assay, and these effects were greater in the chemoresistant cell lines. *In vivo*, after treatment with AVE-1642, inhibition of growth of tumors derived from 5FU- and oxaliplatin-resistant cells was significantly higher than the growth inhibition noted in tumors derived from parental cells. Our laboratory previously showed that IGF-IR inhibition in an orthotopic model of metastatic colon cancer in the murine liver leads to decreased tumor growth by induction of tumor cell apoptosis (23). In this study, we showed that IGF-IR inhibition caused a modest decrease in the growth of parental tumors (17% inhibition), whereas the effect on growth of chemoresistant cell-derived tumors was significantly greater (48% for HT29/5FU-R and 65% for HT29/OxR; $P < 0.05$), which was largely due to an increase in apoptosis. Inhibition of IGF-IR *in vitro* did not lead to increased sensitivity to 5-FU or oxaliplatin, and thus, *in vivo* studies using both AVE-1642 and 5FU or oxaliplatin were not performed.

In addition to IGF-IR, we studied several other growth factor receptors in the chemoresistant cell lines, including epidermal growth factor receptor, cMET, and RON (data not shown). However, we focused our studies in IGF-IR because preliminary studies *in vitro* using inhibitors of each of the above-mentioned pathways did not show significant effects on chemoresistant cell proliferation.

5FU and oxaliplatin act by distinct mechanisms to cause tumor cell cytotoxicity, and it is unlikely that acquired chemoresistance to these agents would yield common molecular alterations. However, our data show that when cells are chronically exposed to either agent, the resulting cells acquire similar molecular alterations that are characteristic of the CSC phenotype. Interestingly, these resistant cells expressed increased levels of IGF-IR (and, in turn, phosphorylated IGF-IR), making these cells more sensitive to blockade of this pathway. Recent data have suggested that the targeting of growth factor receptors, such as that encoded by the *MET* proto-oncogene, may be an effective method for targeting chemoresistant CSCs [reviewed in Boccaccio and Comoglio (24)]. Similar to the activity of MET kinase, our data suggest that IGF-IR inhibition may also target these cells. Our data lead us to further hypothesize that inhibition of IGF-IR signaling in the chemoresistant setting may be more effective than inhibition of this

pathway in the frontline setting. This hypothesis requires further study in preclinical and clinical trials. IGF-IR inhibition may prove to be the therapeutic strategy that is effective in patients with otherwise untreatable disease.

Disclosure of Potential Conflicts of Interest

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