Chronic Oxaliplatin Resistance Induces Epithelial-to-Mesenchymal Transition in Colorectal Cancer Cell Lines

Anthony D. Yang,1 Fan Fan,2 E. Ramsay Camp,1 George van Buren,1 Wenbiao Liu,2 Ray Somcio,2 Michael J. Gray,2 Haiyun Cheng,2 Paulo M. Hoff,3 and Lee M. Ellis1,2

Abstract

**Purpose:** Epithelial-to-mesenchymal transition (EMT) is a process whereby cells acquire molecular alterations that facilitate cell motility and invasion. In preliminary studies, we observed that oxaliplatin-resistant (OxR) colorectal cancer (CRC) cells underwent morphologic changes suggestive of a migratory phenotype, leading us to hypothesize that OxR CRC cells undergo EMT.

**Experimental Design:** The human CRC cell lines KM12L4 and HT29 were exposed to increasing doses of oxaliplatin to establish stable cell lines resistant to oxaliplatin. Migration and invasion were assessed by modified Boyden chamber assays. Morphologic and molecular changes characteristic of EMT were determined by immunofluorescence staining and Western blot analyses.

**Results:** The OxR cells showed phenotypic changes consistent with EMT: spindle-cell shape, loss of polarity, intercellular separation, and pseudopodia formation. KM12L4 and HT29 OxR cells exhibited an 8- to 15-fold increase in migrating and invading cells, respectively (P < 0.005 for both). Immunofluorescence staining of OxR cells revealed translocation of E-cadherin and β-catenin from their usual membrane-bound complex to the cytoplasm and nucleus, respectively. The OxR cells also had decreased expression of the epithelial adhesion molecules E-cadherin and plakoglobin and an increase in the mesenchymal marker vimentin. The KM12L4 OxR cells exhibited increased nuclear expression of Snail, an EMT-regulatory transcription factor, whereas the HT29 OxR cells exhibited an increase in nuclear expression of the EMT-associated transcription factor nuclear factor κB.

**Conclusion:** We hypothesize that induction of EMT may contribute to the decreased efficacy of therapy in chemoresistant CRC, as the tumor cells switch from a proliferative to invasive phenotype. Further understanding of the mechanisms of chemoresistance in CRC will enable improvements in chemotherapy for metastatic disease.

Oxaliplatin is a third-generation platinum compound and is the first platinum-based compound to show efficacy in the treatment of colorectal cancer (CRC; ref. 1). Its use in combination with 5-fluorouracil and leucovorin (FOLFOX) for metastatic CRC has led to response rates >50% and median survival approaching 2 years (2, 3). FOLFOX has also been found to be very effective in the adjuvant setting, leading to an increase in the number of patients who are cured after surgical resection when compared with the use of 5-fluorouracil and leucovorin alone (4). Despite these impressive accomplishments, virtually all metastatic CRC eventually become resistant to oxaliplatin, with a median time to progression of 8 months (5). Hypotheses on the mechanisms of oxaliplatin resistance include defects in oxaliplatin uptake, impaired DNA adduct formation, and increased expression of a copper efflux transporter (6–9).

Epithelial-to-mesenchymal transition (EMT) is a process initially observed in embryonic development in which cells lose epithelial characteristics and gain mesenchymal properties to increase motility and invasion (10). Previous research suggests that EMT is also important in tumor progression and metastasis (10, 11) and is induced by growth factors implicated in these processes such as hepatocyte growth factor, transforming growth factor β, and epidermal growth factor (12).

In preliminary studies, our laboratory observed that oxaliplatin-resistant (OxR) CRC cells exhibit an altered phenotype whereby cells disperse, develop pseudopodia, and assume a spindle shape, properties associated with the EMT phenotype. Based on the above observations, we hypothesized...
that oxaliplatin resistance leads to an EMT phenotype, including its characteristic molecular alterations. To test this hypothesis, we assessed OxR cells derived from two human CRC cell lines for gross morphologic, immunohistochemical, and molecular changes consistent with EMT.

Materials and Methods

Cell lines and culture conditions. The human CRC cell line KM12L4 was obtained from I.J. Fidler, D.V.M., Ph.D. (The University of Texas M.D. Anderson Cancer Center, Houston, TX). The human CRC cell line HT29 was obtained from the American Type Culture Collection (Manassas, VA). Cell lines were cultured in MEM supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin, vitamins, sodium pyruvate, l-glutamine, nonessential amino acids (Life Technologies, Grand Island, NY), and HEPES buffer (MP Biomedicals, Irvine, CA) at 37°C in 5% CO2 and 95% air. Cells were confirmed to be free of Mycoplasma using a Mycoplasma Detection Kit (American Type Culture Collection). In vitro experiments were done at 50% to 70% cell confluence. Results from all studies were confirmed in at least three independent experiments.

Drugs and antibodies. Oxaliplatin (Sanofi-Synthelabo, New York, NY) was purchased from the pharmacy at M.D. Anderson Cancer Center. Antibodies used for immunofluorescence staining and Western blot analyses were as follows: mouse anti-E-cadherin (Zymed Laboratories, Carlsbad, CA), mouse anti-plakoglobin, mouse anti-vimentin, mouse anti-smooth muscle actin, mouse anti-fibronectin (Chemicon International, Temecula, CA), mouse anti-N-cadherin, rabbit anti-i

Fig. 1. Acquisition of oxaliplatin resistance induces morphologic changes consistent with EMT in CRC cells. KM12L4 (A) and HT29 (B) parental and OxR cells were assessed for morphologic changes consistent with EMT. Spindle-shaped cells with loss of polarity (red arrows), increased intercellular separation (green arrows), and pseudopodia (white arrows) were noted in the OxR cells but not in parental cells from both cell lines.
Western blot analysis. For protein extraction, KM12L4 and HT29 parental and OXR cells were plated and grown to 70% to 80% confluence. Whole-cell protein was isolated using radioimmunoprecipitation assay B protein lysis buffer as previously described (14). Nuclear protein was extracted with a commercially available kit (Active Motif). The isolated protein was quantified by a commercially available Bradford assay (Bio-Rad Laboratories, Hercules, CA). Western blot protein samples were prepared by boiling the isolated protein with denaturing sample buffer. The protein was then separated by SDS-PAGE on a 10% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore Corp., Billerica, MA). The membranes were blocked with 5% nonfat dry milk in TBS and 0.1% Tween 20 for 1 hour and probed with the appropriate primary antibody overnight at 4°C. The next morning, the membranes were washed and incubated with the appropriate horseradish peroxidase–conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ) for 1 hour at room temperature. The membranes were then washed and protein bands visualized by using a commercially available enhanced chemiluminescence kit (Amersham Biosciences). To verify the accuracy of whole-cell lysate and nuclear extract protein loading, membranes were incubated in stripping solution for 30 minutes at 65°C, washed, and reprobed with β-actin, vinculin, or lamin-B1 antibody as a loading control.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. KM12L4 and HT29 parental and OXR cells were plated in 96-well plates at 4,000 per well with MEM plus 10% FBS and incubated for 24, 48, or 72 hours. At the end of the incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) was added to achieve a final concentration of 0.5 mg/mL and the cells were incubated for another 60 minutes. Medium and MTT were then removed, DMSO was added for 1 minute to induce cell lysis, and absorption was read at 570 nm.

Flow cytometry and cell cycle analysis. KM12L4 and HT29 parental and OXR cells were grown to 70% to 80% confluence in MEM plus 10% FBS (parental cell lines) or MEM plus 10% FBS plus 2 μmol/L oxaliplatin (OXR cell lines) over 48 hours. After trypsinization, cells were washed in PBS and fixed in 70% ethanol at 4°C for 2 hours. DNA staining was done with 10 mg propidium iodide/mL PBS and 0.1% RNase (Roche Diagnostics)/PBS for at least 30 minutes before flow cytometry in a Coulter EPICS XL flow cytometer (Beckman Coulter, Inc., Fullerton, CA). Cell cycle profiles were generated from flow cytometry analysis with MultiCycle software (Phoenix Flow Systems, San Diego, CA).

Statistical analysis. Student’s t test was used in all statistical analyses of MTT, invasion, and migration assay results using InStat Statistical Software version 2.03 (GraphPad Software, San Diego, CA). Statistical significance was defined as two-tailed P ≤ 0.05.

Results

Acquisition of oxaliplatin resistance induces morphologic changes consistent with EMT in CRC cells. We first noted that cells from the human KM12L4 and HT29 CRC cell lines that had acquired resistance to oxaliplatin at the clinically relevant concentration of 2 μmol/L had a markedly different light-microscopic appearance from cells of the parental cell lines. The phenotypic changes observed in OXR cells included loss of cell polarity causing a spindle-cell morphology, increased intercellular separation signifying loss of intercellular adhesion, and increased formation of pseudopodia (Fig. 1). These changes are typical of cells with a mesenchymal phenotype.

OXR CRC cells have increased migratory and invasive capacity. Boyden chamber assays were done to compare the migratory and invasive capabilities of KM12L4 OXR and parental cells. At 48 hours, the OXR cells showed an ~7.5-fold increase in the number of cells migrating through the collagen insert. Also at 48 hours, the OXR cells exhibited an ~15-fold increase in the number of cells invading through the Matrigel-coated collagen insert. HPE, high-power field; Ox, oxaliplatin.
48 hours, the OxR cells showed an ~7.5-fold increase in the number of cells migrating through the collagen membrane (P < 0.001; Fig. 2A). The capacity of OxR cells to invade through a Matrigel-coated membrane was even greater, with an ~15-fold increase in the number of invading OxR cells compared with parental cells (P < 0.005; Fig. 2B). The presence or absence of 2 μmol/L oxaliplatin in the cell culture medium did not significantly affect the migratory or invasive capacity of the OxR cells.

OxR CRC cells exhibit changes in the localization of cellular EMT markers. Immunofluorescence staining for E-cadherin and β-catenin was done on KM12L4 (A) and HT29 (B) parental and OxR cells. OxR cells from both cell lines showed changes in localization of E-cadherin and β-catenin from their usual cell membrane-associated site. OxR cells exhibited E-cadherin in a disorganized cytoplasmic location and β-catenin was noted to translocate to the nucleus.

OxR CRC cells exhibit molecular changes consistent with EMT. To determine if the acquisition of oxaliplatin resistance induced the specific molecular changes consistent with EMT, Western blotting was done on cell lysates and nuclear extracts from the KM12L4 OxR and parental cells (Fig. 4A). Expression of the epithelial adhesion molecules E-cadherin and plakoglobin was decreased in the OxR cells compared with the parental cells. A concurrent marked increase in the expression of the mesenchymal marker vimentin was also observed. There was no change in expression of the mesenchymal markers N-cadherin, α-smooth muscle actin, and fibronectin (data not shown). Furthermore, increased nuclear expression of the EMT-related transcription factor Snail was observed in OxR KM12L4 cells compared with the parental cells (15, 17). No significant changes were observed in nuclear levels of the EMT-related transcription factors Slug and Twist (also known as Snail-2 and Snail-3, respectively). Similar results were noted when validation studies were done in HT29 OxR and parental cells for the epithelial markers E-cadherin and plakoglobin. In contrast to the KM12L4 OxR cells, no change in expression of...
mesenchymal markers (data not shown) or nuclear expression of Snail, Slug, and Twist was observed in the HT29 OxR cells when compared with the parental cells (Fig. 4B). However, in the HT29 OxR cells, nuclear expression of the transcription factor nuclear factor \( \kappa \)B was found to be increased (Fig. 4B). The expression level of \( \beta \)-catenin was not changed in either the KM12L4 or HT29 OxR cell line when compared with the parental cell lines in whole-cell protein extracts, cytoplasmic protein extracts, or nuclear protein extracts (data not shown).

**OxR CRC cells exhibit reduced proliferation rates.** The KM12L4 (Fig. 5A) and HT29 (Fig. 5B) parental and OxR cell numbers were compared at different time points after plating by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. At 48 and 72 hours, cell counts increased in all groups but both the KM12L4 and HT29 OxR cell lines exhibited reduced cell counts compared with the parental cell lines (\( P < 0.005 \) for both comparisons). Cell cycle analysis showed an increase in the percentage of cells in G2 and no change in the percentage of cells undergoing apoptosis (sub-G0). This suggests that the finding of decreased cell number in the MTT assay can be attributed to decreased proliferation of the OxR cells.

**Discussion**

The last decade has witnessed major innovations in the treatment of metastatic CRC. The median survival for affected patients has increased steadily and is now crossing the 2-year barrier (18). The advent of oxaliplatin played a major role in this disease and it is now part of the most commonly used regimens both in the metastatic as well as in the adjuvant setting. However, it is far from being the perfect treatment. Despite the oxaliplatin activity, the average responding patient can expect to develop resistance – 6 to 8 months after the treatment was initiated. Given enough time, the development of resistance is uniform, and rare patients with metastatic disease, if any, are cured without surgery. Cellular levels of glutathione and ERCC-1 seem to be associated with inherent resistance to oxaliplatin and may play a role in the acquired resistance as well (7). Decreased cellular uptake and adduct formation have been shown but the mechanisms of development of resistance during treatment are still poorly understood (8).

We showed in this series of experiments that acquisition of oxaliplatin resistance by CRC cells leads to morphologic and molecular alterations consistent with a change to a mesenchymal-like phenotype. Furthermore, we showed that OxR CRC cell lines had changes in the cellular localization of two major factors associated with EMT, E-cadherin and \( \beta \)-catenin. Although expression of E-cadherin was decreased in both OxR cell lines, we did not observe changes in \( \beta \)-catenin protein expression levels in whole-cell, cytoplasmic, or nuclear extracts. This lack of change in \( \beta \)-catenin expression could be due to high constitutive expression of \( \beta \)-catenin in these cell lines secondary to APC mutation or it may reflect the
fact that this may be a relocation phenomenon that is not accompanied by changes in β-catenin protein levels. Of note, although we discovered molecular evidence that the EMT changes in the KM12L4-OxR cell line were associated with an increase in the nuclear expression of the transcription factor Snail, we were not able to detect changes in Snail, Slug, or Twist in the HT29 OxR cell line. However, nuclear expression of the transcription factor nuclear factor κB, which has been shown to be associated with EMT in a mammary carcinoma model (19), was increased in the HT29 OxR cell line only. Given that we were unable to observe an increase in any mesenchymal markers in the HT29 OxR cell line, we postulate that the HT29 OxR cells may be undergoing an incomplete change to a mesenchymal-like phenotype rather than the full EMT observed in the KM12-OxR cells. This could be explained by the different signaling pathways (Snail versus nuclear factor κB) inducing the phenotypic change in the two OxR cell lines. Finally, we also showed that the OxR cells undergoing EMT had increased migratory and invasive capabilities. This finding of increased aggressiveness was consistent in two OxR CRC cell lines.

Recent research has implicated EMT in cancer progression by noting that epithelial-derived tumor cells can switch their phenotype to a more primitive mesenchymal phenotype that facilitates motility and invasion (10). Several studies have examined the possible role of EMT in CRC progression (20, 21). Although two studies have shown that loss of E-cadherin-mediated adhesion decreases chemoresistance (22, 23), to our knowledge, no studies have described EMT with loss of E-cadherin-mediated adhesion occurring in cells that have acquired chemoresistance. We believe that our finding that OxR CRC cells undergo epithelial to mesenchymal or mesenchymal-like transition reflects an important process by which cancer cells may potentially acquire chemoresistance. We further hypothesize that OxR cells may switch their “molecular machinery” from a proliferative, epithelial phenotype to a more invasive and migratory mode. Because proliferation is required for oxaliplatin-induced chemosensitivity, the decrease in proliferation of OxR cells may be one means whereby resistant cells can escape the effects of chemotherapy.

In conclusion, this is, to our knowledge, the first description of chemoresistance-induced EMT, and we postulate that EMT induced by acquisition of oxaliplatin resistance could be a possible survival mechanism for chemoresistant CRC cells. If our hypothesis is true, blocking or reversing EMT changes may cause chemoresistant cells to revert to chemosensitive cells. Furthermore, it is yet to be determined if these findings in OxR cells are applicable in cells that develop resistance to other chemotherapeutic drugs. We believe that induction of EMT in chemoresistant CRC represents a new potentially exciting area of research into the mechanism of CRC progression and may eventually be of benefit to patients with advanced, chemoresistant CRC patients who currently do not have many effective treatment options.

**Acknowledgments**

We thank Melissa G. Burkett from the Department of Scientific Publications and Rita Hernandez from the Department of Surgical Oncology for editorial assistance.
References

Chronic Oxaliplatin Resistance Induces Epithelial-to-Mesenchymal Transition in Colorectal Cancer Cell Lines
