Dual silencing of insulin-like growth factor-I receptor and epidermal growth factor receptor in colorectal cancer cells is associated with decreased proliferation and enhanced apoptosis

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Abstract

Overexpression and activation of tyrosine kinase receptors are common features of colorectal cancer. Using the human colorectal cancer cell lines DLD-1 and Caco-2, we evaluated the role of the insulin-like growth factor-I (IGF-I) receptor (IGF-IR) and epidermal growth factor receptor (EGFR) in cellular functions of these cells. We used the small interfering RNA (siRNA) technology to specifically down-regulate IGF-IR and EGFR expression. Knockdown of IGF-IR and EGFR resulted in inhibition of cell proliferation of DLD-1 and Caco-2 cells. An increased rate of apoptosis was associated with siRNA-mediated silencing of IGF-IR and EGFR as assessed by activation of caspase-3/caspase-7. The combined knockdown of both EGFR and IGF-IR decreased cell proliferation and induced cell apoptosis more effectively than did silencing of either receptor alone. Comparable effects on cell proliferation and apoptosis were observed after single and combinational treatment of cells by the IGF-IR tyrosine kinase inhibitor NVP-AEW541 and/or the EGFR tyrosine kinase inhibitor erlotinib. Combined IGF-IR and EGFR silencing by either siRNAs or tyrosine kinase inhibitors diminished the phosphorylation of downstream signaling pathways AKT and extracellular signal–regulated kinase (ERK)-1/2 more effectively than did the single receptor knockdown. Single IGF-IR knockdown inhibited IGF-I-dependent phosphorylation of AKT but had no effect on IGF-I– or EGF-dependent phosphorylation of ERK1/2, indicating a role of EGFR in ligand-dependent ERK1/2 phosphorylation. The present data show that inhibition of the IGF-IR transduction cascade augments the antiproliferative and proapoptotic effects of EGFR inhibition in colorectal cancer cells. A clinical application of combination therapy targeting both EGFR and IGF-IR could be a promising therapeutic strategy. [Mol Cancer Ther 2009;8(4):821–33]

Introduction

Colorectal cancer is one of the most common malignancies in the Western world (1, 2). Long-term survival of colorectal cancer is related to the stage of the disease. If detected early, surgery is the main modality of treatment. At least 40% of patients with colorectal cancer develop metastases during their illness (3). At this stage of disease, the prognosis becomes poor. Although various combinations of surgery, radiotherapy, and chemotherapy are used, innovative approaches are needed to improve the treatment of advanced colorectal cancer.

Some of the characteristic properties of the malignant phenotype of colorectal cancer are mediated by overexpression and/or activation of receptor tyrosine kinases, pointing to these molecules as attractive anticancer treatment targets. One of these approaches is the inhibition of epidermal growth factor (EGF) receptor (EGFR) either by EGFR tyrosine kinase inhibition or antibody-induced receptor blockade. Inhibition of EGFR signaling significantly inhibits tumor growth in numerous preclinical models including colon cancer models (4). These effects have been associated with reduced tumor cell proliferation and increased tumor cell apoptosis (4). In the clinic, EGFR inhibitors have shown beneficial effects in the treatment of different cancer entities including colon cancer (5).

Resistance to EGFR inhibitors was associated with signaling via the type I insulin-like growth factor (IGF) receptor (IGF-IR), which belongs also to the family of receptor tyrosine kinases. Several reports indicate that IGF-IR is overexpressed in the majority (>90%) of colorectal carcinomas, most likely contributing to the aggressive growth characteristics of these tumors and poor prognosis (6–12). In colorectal cancer, IGF-IR signaling plays a pivotal role in cellular transformation, tumorigenesis, and tumor vascularization through the mitogen-activated protein kinase
(MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways (7, 13–15). In addition, the ligand-binding activation of IGF-IR through the phosphatidylinositol 3-kinase signaling pathway is known to protect cells from a variety of proapoptotic damage (16–18). The promoter region of the IGF-IR gene is a target for oncoproteins such as KRAS and c-Myc, which are associated with colorectal cancer. Mutations of these oncogenes lead to increased expression of IGF-IR and signaling (19). Similarly, loss of the p53 tumor suppressor gene (the most frequently mutated tumor suppressor gene in colorectal cancer) has also been shown to increase IGF-IR expression (20–22). The ability of IGF-IR to promote invasive tumor growth and metastatic spread of colorectal cancer has been explained by different mechanisms (23): (a) increased production of the angiogenic vascular endothelial growth factor by activation of IGF-IR, and (b) an increase in cellular motility and cellular invasiveness of colon cancer cells by activation of IGF-IR (24). Furthermore, interactions of IGF-IR with other receptor tyrosine kinases to potentiate the biological effects of each other have been reported. In colorectal cancer, interactions of IGF-IR with EGFR might be of special importance. Of interest, IGF-IR is capable of trans-activating EGFR tyrosine kinase and abrogating the antiproliferative effects of EGFR antibody treatment (25–27). Therefore, in the current work we analyzed the effect of dual silencing of IGF-IR and EGFR on cellular function such as proliferation and apoptosis in colorectal cancer cells.

Materials and methods

Materials

Chemicals were reagent grade and commercially obtained as mentioned: sodium $^{125}$I iodide (carrier-free, specific activity 16.85 mCi/mg), Hybond-N membranes, X-ray Hyperfilm, Rainbow color protein molecular weight markers, and Nick column (Amersham Biosciences); CL-Xposure film (Clear blue X-ray); tyrosine, bovine serum albumin, and L-glutamine (PAA); CHAPS (Roche Pharma); Tween 20 (Serva); recombinant human IGF-I (Gropep); IGF-IR tyrosine kinase inhibitor NVP-AEW541 (Novartis); EGFR tyrosine kinase inhibitor erlotinib (Roche Pharma AG); recombinant platelet-derived growth factor (PDGF)-BB, protease inhibitors, sodium orthovanadate, $\alpha$-glycerophosphate, and $\beta$-glycerophosphate (Sigma); and hybridization solution Quick hyb (Stratagene Europe).

Antibodies

The following antibodies and sera were purchased from commercial sources as indicated: rabbit polyclonal antisera against the IGF-IR $\beta$ subunit (IGF-IR$\beta$), insulin receptor, and rabbit polyclonal antibody against EGFR (Santa Cruz Biotechnology); mouse monoclonal antibodies directed against phospho-extracellular signal–regulated kinase (ERK)-1/2 (Thr202/Tyr204), ERK1/2, phospho-Akt, and Akt (Cell Signaling); mouse monoclonal antibody raised against $\alpha$-tubulin (Sigma); and secondary peroxidase-conjugated antibodies (DAKO).

Cell Lines and Cell Cultures

The human colon cancer cell lines Caco-2 and DLD-1 were obtained from American Type Culture Collection. Caco-2 cells were maintained in MEM supplemented with 20% fetal bovine serum and DLD-1 cells in RPMI 1640 supplemented with 10% fetal bovine serum and 1.2% penicillin/streptomycin (PAN-Systems) at 37°C and 5% CO$_2$ in humidified air. Medium was changed thrice a week and cells were passaged using trypsin/EDTA.

Treatment of DLD-1 and Caco-2 Cells

Before addition of stimuli, cells were allowed to grow until 70% confluency and then washed with PBS. All cultures were maintained under serum-reduced conditions by addition of the specified media supplemented with 0.3% fetal bovine serum (serum-reduced medium) for 1 h and incubated then with or without growth factors for 36 h at 37°C. All cultures received the same volume of serum-reduced medium. After treatment, conditioned media were collected and frozen at −20°C until further analysis. The cells were washed with cold PBS and immediately processed for RNA isolation or protein extraction, respectively. For treatment with pharmacologic inhibitors, cells were serum starved overnight and incubated with NVP-AEW451 (5 and 10 μmol/L) and/or erlotinib (0.01, 0.1, 1.0, and 10 μg/mL) in serum-reduced medium for 24 to 72 h. Stimulation with IGF-I (1 nmol/L) and EGF (100 ng/mL) was done after 24-h treatment in the presence of the inhibitor.

Protein Extraction and Western Blot Analysis

Cell lysates were prepared using lysis buffer containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, 0.25% sodium deoxycholate, 1 μg/mL leupeptin, 1 μg/mL aprotinin, 1 μg/mL pepstatin A, 1 μg/mL phenylmethylsulfonyl fluoride, 1 mmol/L sodium fluoride, and 1 mmol/L sodium orthovanadate. Protein concentration was determined using the Bradford assay (Nanoquant, Carl Roth). Fifty micrograms of total cell lysates were boiled and denatured in sample buffer at 4°C overnight. After washing the membrane thrice, proteins were visualized by enhanced chemiluminescence according to the manufacturer’s instructions (ECL Plus, GE Healthcare).

$^{125}$I-IGF-I Western Ligand Blot

Western ligand blotting of conditioned media from colorectal cancer cell lines using $^{125}$I-IGF-I as tracer was done according to ref. 28 with slight modifications as described (29). In brief, 150 μg of conditioned media were separated on a 10% SDS-PAGE under nonreducing conditions and subsequently transferred to nitrocellulose sheets. To block nonspecific binding, nitrocellulose sheets were soaked overnight at 4°C in buffered saline (0.9% NaCl, 50 mmol/L...
Tris-HCl (pH 7.4), 0.5% fish gelatin]. Membranes were incubated with labeled IGF-I (5–10 × 10^6 cpm of 125I-IGF-I) at 4°C for 24 h in 30 mL incubation buffer [50 mmol/L Tris-HCl (pH 7.4), 0.9% NaCl, 0.5% fish gelatin, and 0.2% NP40]. Following washing, membranes were air-dried and exposed for 24 h at −80°C to X-ray films.

Reverse Transcription and Quantitative Reverse Transcription-PCR

Total RNA from cells was extracted using TriReagent (Sigma). RNA integrity and quantity were assessed by agarose gel electrophoresis and spectrophotometry, respectively. Subsequently, 1 μg of total RNA was reverse transcribed using Oligo dT primers and the Superscript Plus Kit (both from Invitrogen) according to the manufacturer’s instructions. Quantification of IGF-IR and EGFR expression and expression of the two housekeeping genes porphobilinogen deaminase (PBGD) and TATA box binding protein (TBP) was done using the 7900HT sequence detection system (Applied Biosystems) with the SYBR-Green chemistry kit (Qiagen). The 5-μL reaction from the kit was supplemented with 2.5 μL cDNA (diluted 1:20) and 0.25 μmol/L gene-specific primers for IGF-IR, EGFR, PBGD, and TBP. All primers (Operon) were designed using the primer3 online primer design program.4 Primers used for quantitative reverse transcription-PCR (RT-PCR) were as follows: hIGFIR-For-Q2, 5′-CGCACCAATGCTTCAGTTCCCTC-3′; hIGFIR-Rev-Q2, 5′-CTCCCCCACCACACACCTCAGTCT-3′; hIGFIR-Fw-Q3, 5′-GAGAACCCCAAGACTGAGGTGTGT-3′; hIGFIR-Rev-Q3, 5′-GTTCGGTGATGTTGTAGGTGTCTG-3′; hIGFIR-For-Q4, 5′-AAGGGGACATAAACACCAGGAACA-3′; hIGFIR-Rev-Q4, 5′-CCTGCCCATCATACTCTGTGACAT-3′; hEGFR-Fw-Q, 5′-CAATAACTGTGAGGTGGTCCTTGG-3′; hEGFR-Rev-Q, 5′-CTCCTTCAGTCCGGTTTTATTTGC-3′; hEGFR-Fw-Q2, 5′-AGGACGAGTAACAAGCTCAC-3′; hEGFR-Rev-Q2, 5′-CAATGAGGACATAACCAGCCAC-3′; PBGD-For-Q, 5′-GCAATGCGGCTGCAACGGCGGAAG-3′; PBGD-Rev-Q, 5′-CCTGTGGTGGACATAGCAATGATT-3′; TBP-For-Q, 5′-AGCCTGCCACCTTACGCTCAG-3′; TBP-Rev-Q, 5′-TGCTGCCTTTGTTGCTCTTCCA-3′.

A standard curve for quantitative PCR was generated with the same reaction setup using one control sample as a standard (1:4 to 1:80). Fluorescence signals were monitored by the 7900HT sequence detection system and terminated when all reactions reached an amplification plateau while a template-free control stayed at a basal level. Data analysis was done with the detection system software SDS 2.1 (Applied Biosystems). To verify that only specific PCR products evoked fluorescence signals, PCR products were run on 2% agarose gels and analyzed using the E.A.S.Y. Win 32 software (Herolab). IGF-IR and EGFR mRNA expression was normalized to both PBGD and TBP mRNA expression, respectively, to compensate for different sample capacities. All quantification assays were done in triplicates.

4 http://www-genome.wi.mit.edu/genome_software/other/primer3.html

Figure 1. Effect of IGF-I, PDGF-BB, and EGF on IGF-IRβ and EGFR protein expression in DLD-1 (A and B) and Caco-2 cells (C and D). Colorectal cancer cells were grown in serum-reduced conditions in the presence or absence of IGF-I (100 nmol/L), PDGF-BB (10 ng/mL), and EGF (100 ng/mL) for 36 h. Proteins (25 μg) from whole-cell lysates were size-fractionated by SDS-PAGE under reducing conditions, transferred onto membranes, and incubated with antibodies as indicated. A and C, representative Western blots of IGF-IRβ and EGFR proteins in DLD-1 cells (A) and Caco-2 cells (C). B and D, densitometric analysis of IGF-IRβ and EGFR proteins in DLD-1 cells (B) and Caco-2 cells (D). Antigen-antibody interaction signals corresponding to the 97-kDa IGF-IRβ protein and the 170-kDa EGFR protein were densitometrically quantified and expressed as percent increase or decrease compared with untreated controls. Columns, means (n = 3); bars, SD. **, P < 0.01, statistically significant differences in receptor protein expression relative to untreated controls (Student’s t test).
Transfection

Colorectal cancer cells were plated in 12-well plates at a density of 6.5 × 10^4 per well before transfection with small interfering RNA (siRNA) oligonucleotides. After a 24-h incubation, transfection of the cells was accomplished using Oligofectamine reagent and OPTIMEM I medium (both from Invitrogen) according to the supplier’s instructions with different IGF-IR or EGFR gene-specific stealth siRNA duplex oligonucleotides (Invitrogen) at a final concentration of 80 nmol/L, respectively. Target sequences of the gene-specific stealth siRNA were as indicated: Luc, 5′-CGTACGCGGAATACTTCGATT-3′; IGF-IR-53, 5′-TTAATGAGCAAATTGCCCTTGAAGA-3′; IGF-IR-54, 5′-CCTGTGAAAGTGAC-GTCCTGCATTT-3′; EGFR-528, 5′-CACAGTGGAGCGAATTCCTTTGGAA-3′; EGFR-1246, 5′-CGCAAAGTGTG-TAACGGAATAGGTA-3′; EGFR-2438, 5′-GGATCCCAGAAGGTGAGAAAGTTAA-3′.

As control, cells were transfected with siRNA duplex oligonucleotides (Eurogentec) against the firefly (Photinus pyralis) luciferase gene (Luc). Forty-eight hours and 72 h after transfection, cells were collected and used in the following experiments.

Determination of Cell Proliferation

After transfection with siRNA oligonucleotides or treatment with the accordant inhibitors, cells (5 × 10^3 per well) were plated in 100 μL cell culture medium in a 96-well plate as described above. After different periods of incubation (3, 24, 48, and 72 h), cell proliferation was determined using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (MTS; Promega) according to the manufacturer’s instructions. For detection, a microplate reader (E800x, BioTek Instruments, Inc.) was used to measure the absorbance at a wavelength of 495 nm and a reference wavelength of 620 nm. All experiments were done in triplicates.

Determination of Cell Viability and Apoptosis

Cell viability was evaluated using the MultiTox-Fluor Multiplex Cytotoxicity Assay (Promega) according to the manufacturer’s instructions. Briefly, after transfection with siRNA or treatment with the accordant inhibitors, cells were plated into 96-well plates (5 × 10^3 per well). After 72 h, the solution containing substrate for live-cell and dead-cell protease activities was added, incubated for 2 h, and fluorescence was measured in a microplate reader (Flx800, Biotek) at 400Ex/505Em for living cells and at 485Ex/520Em for dead cells.

Figure 2. siRNA-mediated knockdown of EGFR in DLD-1 cells. DLD-1 cells were transiently transfected with different EGFR gene-specific siRNAs [EGFR-528, EGFR-1246, and EGFR-2438] or luciferase (Luc) gene-specific siRNAs. A, total RNA (1 μg) from transfected DLD-1 cells was reverse transcribed to cDNA and used to determine EGFR mRNA expression by means of quantitative RT-PCR. The relative expression of EGFR mRNA was expressed as percent decrease compared with the Luc siRNA–transfected DLD-1 cells after normalization against the expression of housekeeping genes PBGD and TBP. Columns, mean; bars, SD. **, P < 0.01, statistically significant differences relative to Luc siRNA–transfected DLD-1 cells (Student’s t test). B, cell lysates were size-fractionated by SDS-PAGE, blotted, and analyzed by Western blotting with antibodies specific for EGFR. The membrane was stripped and reprobed with an α-tubulin–specific antibody to check for equal loading of total protein. C, protein bands were densitometrically quantified. The relative densities of bands were indicated as percent decrease compared with Luc siRNA–transfected DLD-1 cells (Student’s t test). D, transfected DLD-1 cells were cultured in serum-reduced media for 24 h. Equal amounts of proteins extracted from culture supernatants were size-fractionated by SDS-PAGE under nonreducing conditions followed by transfer to membranes and ligand blotting with 125I-IGF-I. IGFBP species at 32 and 24 kDa representing IGFBP-2 and IGFBP-4 are indicated.

Induction of apoptosis was determined by measuring active caspase-3/caspase-7. Cells were transfected with siRNA or treated with the accordant inhibitors as described above and plated into 96-well plates (8 × 10³ per well). After 72 h, active caspase-3/caspase-7 was measured using the caspase-Glo 3/7 assay system (Promega) according to the manufacturer’s instructions.

**Statistical Analysis**

All experiments were replicated three to four times. Autoradiographs of Western ligand and Western blots were scanned (Bio-Rad). After background subtraction, densitometry of individual bands was analyzed by ImageJ software (version 1.34s, NIH) according to the instructions of the manual. The relative densities of the bands were expressed as a percentage of control. The proliferation assays, cell viability experiments, and apoptosis studies were done in triplicates. Means ± SD were indicated relative to the control. The Student’s t test for paired values was used, with \( P < 0.05 \) considered significant.

**Results**

**Expression of IGF-IR and EGFR in DLD-1 and Caco-2 Cells**

Protein levels of IGF-IR and EGFR were evaluated by Western blotting in the colorectal cancer cell lines DLD-1 and Caco-2 (Fig. 1). An IGF-IRβ-specific protein band was detectable at 97 kDa in DLD-1 and Caco-2 cells, respectively. The expression level of IGF-IR was ∼3- to 4-fold higher in Caco-2 cells as compared with DLD-1 cells after densitometric analyses of three independent experiments. In DLD-1 cells, IGF-I treatment showed different effects on IGF-IR expression from PDGF-BB and EGF. As shown by Western blotting, addition of IGF-I reduced the protein levels of IGF-IR (Fig. 1). In contrast, treatment of cells with recombinant PDGF-BB and EGF slightly increased the levels of IGF-IR. In DLD-1 cells, IGF-I, PDGF-BB, and EGF stimulated the protein levels of EGFR. Maximal increase of EGFR was observed when DLD-1 cells were treated with PDGF-BB (Fig. 1A and B). In Caco-2 cells, levels of IGF-IR were not significantly altered by incubation of cells with IGF-I, PDGF-BB, or EGF (Fig. 1C and D) whereas abundance of EGFR-specific protein bands was stimulated by addition of IGF-I and PDGF-BB.

**Knockdown of EGFR and IGF-IR Expression in DLD-1 and Caco-2 Cells**

For down-regulation of EGFR expression in DLD-1 and Caco-2 cells, three different EGFR-specific siRNAs (EGFR-528, EGFR-1246, and EGFR-2438) were used for transfection studies. As a control, cells were transfected with siRNA against the luciferase gene (Luc). To quantitatively determine
Figure 4. Dual knockdown of IGF-IR and EGFR in DLD-1 cells. DLD-1 cells were transfected with luciferase (Luc) gene-specific siRNAs, IGF-IR gene-specific siRNAs (IGF-IR-53), EGFR gene-specific siRNAs (EGFR-528), or a combination of IGF-IR-53 and EGFR-528 oligonucleotides. EGFR (A) and IGF-IR (B) mRNA expressions of transfected DLD-1 cells were determined by quantitative RT-PCR. The relative expression of EGFR or IGF-IR mRNA was expressed as percent decrease compared with the Luc siRNA–transfected DLD-1 cells after normalization against endogenous PBGD and TBP. Columns, mean; bars, SD. *, P < 0.05; **, P < 0.01, statistically significant differences relative to Luc siRNA–transfected DLD-1 cells (Student’s t test).

C, cell lysates were analyzed by Western blotting for levels of EGFR and IGF-IRβ. Equal loading of total proteins was ensured by immunostaining against α-tubulin. EGFR-specific (D) and IGF-IRβ–specific (E) bands were densitometrically quantified and were given as percent increase or decrease compared with Luc siRNA–transfected DLD-1 cells. Columns, mean (n = 3); bars, SD. *, P < 0.05; **, P < 0.01, statistically significant differences in EGFR and IGF-IRβ proteins of DLD-1 cells transfected with the different EGFR- or IGF-IR–specific siRNAs compared with DLD-1 cells transfected with Luc-specific siRNA (Student’s t test).
the down-regulation of EGFR expression in DLD-1 cells, mRNA levels of EGFR were measured by quantitative RT-PCR after siRNA transfection (Fig. 2A). Forty-eight hours after transfection, EGFR-528 and EGFR-1246 siRNAs down-regulated EGFR mRNA levels to approximately 40% and 31% of control levels, respectively, whereas EGFR-2438 decreased EGFR mRNA levels only to 58%. The siRNA-mediated silencing of EGFR mRNA expression was significantly lower at 72 hours than at 48 hours after transfection (Fig. 2A).

Western blot analysis with an anti-EGFR antibody revealed that endogenous EGFR expression in siRNA-transfected DLD-1 cells was reduced to approximately 12% to 20% of levels observed in Luc-transfected cells at 48 hours after transfection and to ~6% to 12% at 72 hours after transfection (Fig. 2B and C). Of interest, transfection with EGFR siRNAs was not associated with a knockdown of the insulin receptor (data not shown). Furthermore, inhibition of endogenous EGFR expression by siRNA did not affect the levels of IGF binding protein (IGFBP)-2 and IGFBP-4 detected in supernatants of DLD-1 cells by 125I-IGF-I ligand blotting (Fig. 2D). A comparable down-regulation of EGFR by siRNAs was observed in Caco-2 cells as determined at both the mRNA and protein levels (Supplementary Fig. S1).5

Similar to EGFR, three different siRNAs (IGF-IR-53, IGF-IR-54, and IGF-IR-55) were used for IGF-IR silencing in DLD-1 and Caco-2 cells. In DLD-1 cells, IGF-IR mRNA expression was reduced to ~3% to 40% of levels of Luc-transfected cells at 48 hours and to about 2% to 5% at 72 hours after transfection, respectively (Fig. 3A). The siRNA-mediated silencing of IGF-IR decreased IGF-IRβ expression to ~6% to 8% of levels of control-transfected (Luc) DLD-1 cells. The blockade of IGF-IR expression by siRNA only weakly affected the level of insulin receptor (Fig. 3B), whereas an effect on IGFBP-2 and IGFBP-4 yields in supernatants of DLD-1 cells was not observed (Fig. 3D).

Similar results were observed in Caco-2 cells transfected with IGF-IR-specific siRNAs (Supplementary Fig. S2).5

**Dual Knockdown of EGFR and IGF-IR Expression in DLD-1 and Caco-2 Cells**

For down-regulation of both EGFR and IGF-IR expression in DLD-1 and Caco-2 cells, a combination of EGFR-528 and IGF-IR-53 siRNA oligonucleotides was used for transfection experiments. In DLD-1 cells, a simultaneous reduction of both EGFR and IGF-IR mRNA expression to approximately 4% to 2% was observed, respectively, compared with expression levels determined in control-transfected (Luc) cells by quantitative RT-PCR (Fig. 4A and B).

Western blot analysis with receptor-specific antibodies confirmed the results obtained by RT-PCR. Dual transfection of DLD-1 cells with EGFR-528 and IGF-IR-53 led to a simultaneous suppression of both EGFR and IGF-IRβ (Fig. 4C). Densitometric analysis of three independent experiments revealed that EGFR expression was depleted to approximately 6% to 8% of levels detected in Luc-transfected cells (Fig. 4D) and that IGF-IR expression was reduced to about 2% to 5% of levels observed in the controls at 72 hours after transfection (Fig. 4E). Of interest, EGFR knockdown was associated with a 2-fold increase of the IGF-IRβ protein 48 hours after transfection that declined to ~50% of control levels 72 hours after transfection (Fig. 4C and E). A comparable knockdown of both EGFR and IGF-IR was observed after transfection of Caco-2 cells with EGFR-528 and IGF-IR-53 oligonucleotides (Supplementary Fig. S3).5

**EGFR and IGF-IR Down-Regulation Is Associated with Diminished Cell Proliferation and Cell Survival**

In the following studies, the different siRNAs were used to study the effects of EGFR and IGF-IR knockdown on cellular functions of colorectal cancer cells such as cell proliferation and apoptosis. When cell proliferation was analyzed, siRNA-mediated blockade of either EGFR or IGF-IR was associated with moderate inhibitions of cell proliferation compared with Luc-siRNA–transfected control cells (Fig. 5A). In DLD-1 cells, EGFR and IGF-IR siRNA transfection led to a reduction of cell proliferation to approximately 70% to 80% of levels observed in Luc-transfected DLD-1 cells, respectively. Furthermore, IGF-IR-53 and EGFR-528 siRNA sequences were chosen for a dual silencing of both IGF-IR and EGFR in DLD-1 cells. These experiments showed that the double knockdown of IGF-IR and EGFR led to a further decrease of DLD-1 cell proliferation below the level observed for the silencing of the single receptors (Fig. 5B).

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5 Supplementary material for this article is available at Molecular Cancer Therapeutics Online [http://mct.aacrjournals.org/].
A comparable decrease of cell proliferation was observed after transfection of Caco-2 cells with IGF-IR– or EGFR-targeted siRNAs (Supplementary Fig. S4).5 Furthermore, transfection of DLD-1 cells with IGF-IR-53 or EGFR-528 siRNA alone was associated with approximately 15% and 11% increase of dead cells, respectively, whereas dual knockdown of both EGFR and IGF-IR resulted in an increase of dead cells to 23% compared with Luc-transfected DLD-1 cells (Fig. 6A). To further analyze the underlying mechanism of cell death, apoptosis was monitored by determining activation of caspase-3/caspase-7 (Fig. 6B). These experiments showed a ∼2-fold increase of apoptotic cells when DLD-1 cells were transfected with IGF-IR-53 or EGFR-528 siRNA oligonucleotides compared with Luc-transfected DLD-1 cells (Fig. 6B). Dual knockdown of both IGF-IR and EGFR was associated with an ∼10-fold increase of apoptosis compared with Luc-transfected DLD-1 cells.

In a second approach, the effect of EGFR and IGF-IR inhibition on cell proliferation and apoptosis was studied by means of pharmacologic tyrosine kinase inhibitors. The EGFR tyrosine kinase inhibitor erlotinib (Fig. 7A) and the

Figure 6. Dual knockdown of IGF-IR and EGFR increases apoptosis of DLD-1 cells. A, DLD-1 cells were transfected with IGF-IR-53 and/or EGFR-528 or luciferase (Luc) siRNA as indicated. After transfection, DLD-1 cells were plated in a 96-well plate. Seventy-two hours after transfection, cell viability was evaluated using the MultiTox-Fluor Multiplex Cytotoxicity Assay. The number of dead cells in Luc-transfected DLD-1 cells was defined as basal level. An increase of dead cells after IGF-IR-53 and/or EGFR-528 siRNA transfection was indicated as percent increase over the basal level of Luc-transfected DLD-1 cells. B, for the same approach, the rate of apoptosis as assessed by caspase-3/caspase-7 activation was determined. Columns, mean of triplicate values from two experiments; bars, SD. **, P < 0.01, statistically significant differences relative to Luc siRNA–transfected DLD-1 cells (Student’s t test).

Figure 7. Effect of EGFR and IGF-IR blockade by pharmacologic inhibitors on proliferation of DLD-1 cells. After starvation in serum-reduced medium for 24 h, DLD-1 cells were incubated in the presence or absence of the EGFR tyrosine kinase inhibitor erlotinib (A), the IGF-IR tyrosine kinase inhibitor NVP-AEW541 (B), or a combination of both inhibitors (C) at the concentrations indicated. Control cultures were maintained in serum-reduced medium. All cells received the same amount of DMSO. After different periods of incubation (24, 48, and 72 h), cell proliferation was determined using a nonradioactive assay. Columns, mean percent decrease compared with DLD-1 cells treated only with the solvent (control) from three independent experiments; bars, SD. *, P < 0.05; **, P < 0.01, statistically significant differences relative to control DLD-1 cells (Student’s t test).
IGF-IR tyrosine kinase inhibitor NVP-AEW541 (Fig. 7B) time- and dose-dependently inhibited the proliferation of DLD-1 cells. Simultaneous inhibition of EGFR and IGF-IR tyrosine kinase further impaired the proliferation of DLD-1 cells (Fig. 7C). Treatment of DLD-1 cells with the tyrosine kinase inhibitors was associated with a dose-dependent increase of dead cells (Fig. 8). NVP-AEW541 at a concentration of 1 μmol/L and erlotinib at a concentration of 10 μg/mL resulted in approximately 25% and 50% increase, respectively, of dead cells compared with control DLD-1 cells. NVP-AEW541 in combination with erlotinib increased the rate of dead cells >80% compared with control cells (Fig. 8).

Comparable effects on cell proliferation and apoptosis were observed when Caco-2 cells were treated with erlotinib and NVP-AEW541 (Supplementary Fig. S5).5

EGFR and IGF-IR Down-Regulation Is Associated with Diminished Signaling

We then assessed whether down-regulation of IGF-IR and EGFR by siRNA abolished IGF-I and EGF signaling in DLD-1 cells by determining the phosphorylation of AKT and ERK1/2. Treatment of DLD-1 cells with IGF-IR or EGFR resulted in phosphorylation of AKT (Ser473) and ERK1/2, with maximal effects observed 10 minutes after addition of growth factors (data not shown). Down-regulation of IGF-IR by IGF-IR-53 siRNA alone attenuated IGF-I- and EGF-stimulated activation of AKT and ERK1/2 more effectively than observed after transfection with IGF-IR-53 siRNA. Simultaneous down-regulation of both IGF-IR and EGFR by siRNA was associated with the strongest reduction of IGF-I- and EGF-stimulated phosphorylation of AKT and ERK1/2 (Fig. 9A).

NVP-AEW541 strongly reduced IGF-induced phosphorylation of AKT but showed no or weak effects on IGF-induced phosphorylation of ERK1/2 or EGF-stimulated phosphorylation of AKT and ERK1/2 (Fig. 9B). Erlotinib completely abrogated EGF-stimulated phosphorylation of AKT but showed no influence on AKT phosphorylation after IGF treatment. However, erlotinib completely inhibited IGF- and EGF-induced phosphorylation of ERK1/2. Therefore, erlotinib given in combination with NVP-AEW541 completely disrupted IGF- and EGF-dependent phosphorylation of AKT and ERK1/2 (Fig. 9B).

Figure 8. Effect of pharmacologic EGFR and IGF-IR blockade on apoptosis of DLD-1 cells. After starvation of DLD-1 cells for 24 h, cells were maintained in the presence or absence of the EGFR tyrosine kinase inhibitor erlotinib, the IGF-IR tyrosine kinase inhibitor NVP-AEW541, or a combination of both inhibitors. Control cultures were maintained in serum-reduced medium. All cells received the same amount of DMSO. Cell viability was evaluated using the MultiTox-Fluor Multiplex Cytotoxicity Assay. An increase of dead cells after treatment with NVP-AEW541 and erlotinib was indicated as a percent increase over the basal level of control DLD-1 cells. Columns, mean of triplicate values from two experiments; bars, SD. *, P < 0.05; **, P < 0.01, statistically significant differences relative to untreated DLD-1 cells (Student’s t test).

Figure 9. Effect of EGFR and IGF-IR blockade on activation of AKT and ERK1/2. A, DLD-1 cells were transfected with IGF-IR-53 and/or EGFR-528 or luciferase (Luc) siRNA as indicated. Seventy-two hours after transfection, serum-starved DLD-1 cells were stimulated with IGF-I (1 nmol/L) and/or EGF (100 ng/mL) for 20 min and immediately subjected to detergent lysis. Proteins (15 μg) extracted from whole-cell lysates were size-fractionated by SDS-PAGE and immunoblotted with antibodies raised against the phosphorylated forms of AKT and ERK1/2. Equal loading of proteins was shown by immunoblotting with antibodies directed against the nonphosphorylated form of AKT and ERK1/2 as well as α-tubulin. The positions of the molecular weight standards are indicated on the left. B, serum-starved DLD-1 cells were cultivated in the presence of the IGF-IR tyrosine kinase inhibitor NVP-AEW541 (NVP) and/or EGFR tyrosine kinase inhibitor erlotinib (Erl) for 24 h. Thereafter, DLD-1 cells were simultaneously stimulated with IGF-I (1 nmol/L) and/or EGF (100 ng/mL) for 20 min and were immediately proceeded for detergent lysis and Western blot analysis against the phosphorylated and nonphosphorylated forms of AKT and ERK1/2 as outlined.
Similar results on IGF-I- and EGF-stimulated signaling were observed in Caco-2 cells transfected with IGF-IR and/or EGF siRNA sequences or treated with tyrosine kinase inhibitors (Supplementary Fig. S7).5

Discussion

Inhibition of EGFR by antibody-induced receptor blockade is now well established in the treatment of patients with colorectal cancer. However, solid tumor cells may compensate strategies targeting EGFR by activating alternative mechanisms that are able to sustain signaling through survival pathways, thus limiting the efficacy of such therapeutic approaches. One of these candidate alternative pathways is the IGF-IR, which regulates important cell survival pathways (15, 30–38). Therefore, we investigated the effect of siRNA sequences to EGFR and IGF-IR in the colorectal cancer cell lines DLD-1 and Caco-2. By using this technology, the mRNA and protein levels of EGFR and IGF-IR in colorectal cancer cell lines were significantly decreased. The siRNA-mediated knockdown of EGFR and IGF-IR inhibited the proliferation of colorectal cancer cells and stimulated apoptosis at the same time. Furthermore, a combination of siRNAs to both EGFR and IGF-IR caused a stronger decrease of cell proliferation and induced cellular apoptosis more effectively than did siRNAs to either receptor alone. Pharmacologic inhibition of both IGF-IR and EGFR by the IGF-IR tyrosine kinase inhibitor NVP-AEW541 and the EGFR tyrosine kinase inhibitor erlotinib resulted in stronger effects on proliferation and apoptosis of colorectal cancer cell lines than the single receptor inhibition. In DLD-1 and Caco-2 cells, ligand-dependent activation of both EGFR and IGF-IR induced phosphorylation of AKT and ERK1/2, known major components of the downstream signaling pathways of both EGFR and IGF-IR. Combining the siRNA sequences to both receptors inhibited this phosphorylation more effectively than did siRNAs to either receptor alone. Similarly, combinational treatment of colorectal cancer cell lines with both NVP-AEW541 and erlotinib displayed stronger inhibition of phosphorylation levels of AKT and ERK1/2 than the single inhibitors did.

The recently developed siRNA technology is a powerful technique to inhibit specific gene expression pointing to the potential use of siRNA molecules to study gene function in carcinogenesis. In the present study, we used siRNA oligonucleotides to inhibit IGF-IR and EGFR expression. The data showed that the mRNA and protein levels of EGFR and IGF-IR in human colorectal cancer cell lines decreased significantly without major effects on expression of other genes such as the insulin receptor or IGFBPs, which might be the background for a more specific or effective approach.

At that time point, siRNA-mediated receptor knockdown decreased the protein levels of EGFR and IGF-IRβ to <10% of receptor levels observed in control-transfected DLD-1 cells. A comparable knockdown of EGFR and IGF-IR expression by siRNAs was reported in breast cancer cells (39, 40).

Similar to IGF-IR, EGFR is considered to be involved in the development of different types of cancers. Several studies have shown that overexpression of EGFR is correlated with advanced disease stage, low patient survival rate, development of tumor metastasis, and acquisition of chemoresistance (41, 42). Comparable with IGF-IR, the EGFR signaling pathway is also involved in cellular transformation, tumorigenesis, and tumor vascularization. Expression of IGF-IR and EGFR was assessed retrospectively in tumor specimens from 87 patients with advanced stages of colorectal cancer by immunohistochemistry. Coexpression of IGF-IR and EGFR was present in tumors from 75% of the patients providing a morphologic/anatomic basis for receptor interactions (43). However, it is unclear how IGF-IR and EGFR cooperate with each other. In normal human mammary cells, the EGFR tyrosine kinase is required for proper IGF-IR signaling (25). Vice versa, a functional IGF axis is required for the actions of EGFR, including the ability to induce chronic ERK activation (44). In addition, evidence for a direct physical interaction of IGF-IR with EGFR was presented very recently. In different cancer cell lines including breast cancer cell lines, a heterodimerization of IGF-IR and EGFR was observed (40). Further investigations revealed that siRNA-directed EGFR depletion increased ubiquitinylation and degradation of IGF-IR, pointing to a role of EGFR in modifying the stability of IGF-IR protein. Although not investigated experimentally in the present study, a similar mechanism might also be relevant for DLD-1 and Caco-2 cells because siRNA-mediated EGFR knockdown was associated with a significant reduction of IGF-IR protein in both cell lines 72 hours after transfection.

In addition, IGF-IR has been shown to interact with ErbB2/HER2 in MCF7 breast cancer cells (27, 45). Resistance to anti–HER-2 therapy by trastuzumab in HER-2-overexpressing metastatic breast cancer was caused by the presence of IGF-IR/HER2 hybrid receptors that bypassed the blocked HER-2-dependent pathway via the alternative up-regulated IGF-IR signaling pathway (27, 43). In accordance with these data, in the present study the effect of single EGFR or IGF-IR knockdown on cell proliferation and apoptosis of DLD-1 cells was quite moderate although the two receptors were significantly inhibited at both the mRNA and protein levels. However, dual silencing of both EGFR and IGF-IR revealed more prominent effects on cell proliferation and apoptosis than single receptor knockdown. These findings again point to interactions between EGFR and IGF-IR that might be involved in primary and secondary resistance mechanisms. Future studies are necessary to decode the precise nature of these interactions, which might be the background for a more specific or effective treatment.

IGF-IR is linked with at least three survival signals that are able to protect cancer cells from apoptosis, namely, the phosphoinositide 3-kinase/AKT and mitogen-activated protein kinase/ERK signaling pathways and a third one that results in the mitochondrial translocation of Raf-1, the
so-called 14-3-3 pathway (46). Simultaneous inactivation of two of these pathways is required to inhibit the IGF-IR capacity of protecting cells from apoptotic injuries (47). In the present study, Western blot analysis showed that combination transfection with IGF-IR– and EGFR-specific siRNA sequences was associated with the strongest reduction in levels of AKT and ERK1/2 phosphorylation. A complete abrogation of AKT and ERK1/2 phosphorylation was obtained by combinatorial treatment with IGF-IR– and EGFR-specific tyrosine kinase inhibitors as well. It is likely that blockade of these two survival pathways is directly involved in the inhibition of cell growth and induction of apoptosis observed in DLD-1 and Caco-2 cells after knockdown or inhibition of both receptors. However, single IGF-IR knockdown by IGF-IR siRNA or NVP-AEW541 alone inhibited IGF-I–dependent phosphorylation of AKT, but had no effect on IGF-I– or EGFR-dependent phosphorylation of ERK1/2. On the other hand, inhibition of EGFR by siRNA oligonucleotides was followed by reduction or complete inhibition in case of erlotinib of IGF- and EGF-stimulated ERK1/2 phosphorylation. These data point to a relevant role of EGFR in ligand-dependent ERK1/2 phosphorylation rather than of IGF-IR. Similar results were published very recently in the colon cancer cell lines FET, CBS, and GEO (48).

In patients with colorectal cancer, primary resistance to anti-EGFR therapy has been observed in tumors bearing mutations in the k-ras gene (49, 50). In another study including almost 600 patients with colorectal cancer, mutations in k-ras, B-type Raf, or phosphoinositide 3-kinase (PI-3K) genes were significantly associated with poor survival (51), pointing to the importance of the signaling network of tyrosine kinase receptors. In preclinical (52, 53) and clinical studies of patients with lung cancer and glioblastoma (36, 37, 54), resistance to anti-EGFR therapy was discovered as well. Resistance to the EGFR inhibitor AG1478 was associated with an up-regulation of IGF-IR levels in a glioblastoma model (55). Conversely, IGF-IR overexpression was found to correlate with decreased efficacy of EGFR targeting, suggesting the importance of IGF-IR signaling in resistance to EGFR inhibitors (55, 56). IGF-IR mediated resistance to anti-EGFR therapy in primary human glioblastoma cells through continued activation of the phosphoinositide 3-kinase/AKT pathway (55). Cotargeting IGF-IR and EGFR greatly enhanced both spontaneous and radiation-induced apoptosis in a glioblastoma model (55). IGF-IR signaling through phosphatidylinositol 3-kinase/AKT could be a novel and potentially important mechanism of resistance to anti-EGFR therapy (57).

As already shown for breast cancer, data of the present study suggest that EGFR- and IGF-IR–mediated signaling pathways could play an important role in determining the biological behavior of colorectal cancer. Overexpression of IGF-IR might result in increased resistance to EGFR inhibition through the signaling activity of IGF-IR (46). Several anti–IGF-IR compounds such as NVP-AEW541 (58–60) or bispecific antibodies targeting both IGF-IR and EGFR (61) are now being developed, and clinical trials to test the hypothesis that the efficacy of EGFR inhibition treatments is enhanced by IGF-IR targeting are necessary. The data presented here support further research into colorectal cancer therapeutic strategies combining EGFR inhibition with anti–IGF-IR agents.

In summary, we have shown that the additional inhibition of IGF-IR by either siRNA or NVP-AEW541 treatment augments the proapoptotic effects of EGFR inhibition in colorectal cancer cells. With the development of novel inhibitors that are able to discriminate IGF-IR from the insulin receptor (58, 62, 63), a clinical application of combination therapy targeting EGFR and IGF-IR will likely be feasible in the near future. Our results show that inhibiting IGF-IR is a promising strategy to block such an escape pathway and thus enhance the effect of EGFR inhibition.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


32. She QB, Sobti D, Basso A, Moosser MM. Resistance to gefitinib in PTEN-null HER-overexpressing tumor cells can be overcome through restoration of PTEN function or pharmacologic modulation of constitutive phosphatidylinositol 3'-kinase/Akt pathway signaling. Clin Cancer Res 2003;9:4340–46.


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Dual silencing of insulin-like growth factor-I receptor and epidermal growth factor receptor in colorectal cancer cells is associated with decreased proliferation and enhanced apoptosis

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