Blockade of Insulin-like Growth Factor I Receptor Function Inhibits Growth and Angiogenesis of Colon Cancer

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ABSTRACT

Purpose and Experimental Design: Insulin-like growth factors (IGFs) I and II and their principle receptor, IGF-I receptor (IGF-IR), are frequently expressed in human colon cancers and play a role in preventing apoptosis, enhancing cell proliferation, and inducing expression of vascular endothelial growth factor (VEGF). To elucidate the in vitro and in vivo effects of IGF-IR in human colon cancer growth and angiogenesis, HT29 cells were transfected with a truncated dominant-negative (DN) form of IGF-IR or vector alone.

Results: IGF-I increased VEGF expression in parental and vector-transfected cells, whereas IGF-I induction of VEGF mRNA and protein was abrogated in IGF-IR DN cells. The IGF-IR DN cells demonstrated inhibited growth in both monolayer culture and soft agar (P < 0.05). s.c. injections of IGF-IR DN cells in nude mice led to significantly decreased tumor growth (P < 0.05). Immunohistochemical analyses revealed that IGF-I DN tumors demonstrated decreased tumor cell proliferation, VEGF expression, and vessel count and increased tumor cell apoptosis (P < 0.05 for all parameters compared with controls). Furthermore, IGF-IR DN-transfected cells yielded significantly decreased tumorigenicity and growth in the liver.

Conclusions: These studies demonstrate that the IGF ligand-receptor system plays an important role in multiple mechanisms that mediate human colon cancer growth including regulation of VEGF and angiogenesis.

INTRODUCTION

A variety of tumor systems including colon cancers demonstrate altered expression of the IGF-1 and IGF-II and their principle receptor, IGF-IR. Colorectal carcinomas have a 10–50-fold increase in the levels of IGF-I and IGF-II when compared with adjacent uninvolved colonic mucosa (1–3). The highest source of IGF-I is the liver, which is also the most frequent site of metastasis from colon cancer. However, the biological effect of IGF-I in malignant progression has not been fully elucidated. Studies have shown that IGF-I may prevent apoptosis in numerous tumor cell systems (4, 5). In addition, it may play a role in induction of cell proliferation (6, 7), although this is a subject of debate (8). Recently, a possible role of IGF-I in contributing to tumor angiogenesis has been suggested because IGF-I induces VEGF (9, 10). VEGF (or VEGF-A) is a fundamental regulator of angiogenesis and exists as several isoforms due to alternate splicing (121-, 145-, 165-, 189-, and 206-amino acid isoforms; Refs. 9–11). Over the last 10 years, there has been an explosive interest in understanding the complex regulatory mechanisms of tumor angiogenesis because growth and metastasis of tumors depend on their ability to induce growth of new blood vessels (12–14).

The role of IGF-I in malignant growth is at times difficult to define because IGFBPs may modulate the biological effects of IGF (reviewed in Ref. 15). These factors can bind IGF-I and either enhance or inhibit its ability to bind to its receptor (dependent upon the specific IGFBP), thus affecting its biological activity. However, VEGF induction by IGF-I is most likely not affected by IGFBPs because a truncated IGF-I lacking the binding site for IGFBPs does not alter VEGF induction (9, 16).

The IGF-IR, a tyrosine kinase receptor, is the major receptor for both IGF-I and IGF-II and consists of two α and two β subunits (4). The α subunits are entirely extracellular chains, containing a cysteine-rich domain responsible for ligand binding (4, 17). The β subunits display a highly hydrophobic transmembrane domain, which divides the β subunit into an extracellular and an intracellular region containing a tyrosine kinase domain (4, 17). Several authors have demonstrated that the IGF-IR is crucial for maintaining normal growth and development (17, 18). Homozygous IGF-IR knockout mice exhibit severe growth deficiency and invariably die at birth (18). In human colon cancer, IGF-IR is not only present but is frequently overex-

Received 4/5/02; revised 6/21/02; accepted 7/2/02.

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1 Supported in part by the Dr. Mildred Scheel Stiftung für Krebsforschung, Deutsche Krebshilfe (N. R.), NIH Grant T-32 (9599) to S. A. A., NIH Grant CA74821 (to L. M. E.), and NIH Core Grant CCSG CA16672 (to L. M. E.).

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3 The abbreviations used are: IGF, insulin-like growth factor; BrdUrd, bromodeoxyuridine; Erk, extracellular signal-regulated kinase; FBS, fetal bovine serum; HRP, horseradish peroxidase; IGFBP, IGF-binding protein; IGF-IR, IGF-I receptor; IRS, insulin receptor substrate; MAPK, mitogen-activated protein kinase; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; VEGF, vascular endothelial growth factor; DN, dominant-negative; rtPCR, reverse transcription-PCR; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
pressed (1, 2). However, the biological significance of IGF-IR in colon cancer has not been elucidated.

To further our understanding of the role of the IGF-IR in human colon cancer growth, we stably transfected human colon cancer cells with a truncated IGF-IR (IGF-IR DN) lacking the cytosolic kinase domain. IGF-IR DN forms a nonfunctional heterodimer with endogenous IGF-IR (19, 20). Our studies demonstrate that inducible IGF-I signaling was strongly impaired in vitro and that VEGF induction by IGF-I was nearly completely abolished in IGF-IR DN transfectants. Growth of IGF-IR DN-transfected cells was significantly impaired in both monolayer and soft agar cultures. Subcutaneous growth of IGF-IR DN-transfected cells in nude mice was significantly impaired, and this was associated with increased apoptosis and decreased proliferation and blood vessel formation. In addition, IGF-IR DN cells injected directly into the liver formed significantly fewer tumors than controls. These results suggest that the IGF-IR plays a multifunctional role in human colon cancer growth, suggesting that this receptor would be a rational target for antineoplastic therapy.

MATERIALS AND METHODS

Materials

Recombinant human IGF-I was purchased from R&D Systems, Inc. (Minneapolis, MN). Antibodies for immunohistochemistry and Western blot analysis were monoclonal mouse anti-BruUrd (Becton Dickinson Labware, Franklin Lakes, NJ), monoclonal mouse anti-PCNA clone PC10 (DAKO A/S, Glostrup, Denmark), monoclonal rat anti-mouse CD31 (PharMingen, San Diego, CA), polyclonal rabbit anti-phospho-p44/42 MAPK (Cell Signaling Technology, Beverly, MA), monoclonal mouse anti-human VEGF recognizing the 165-, 189-, and 206-amino acid splice variants (PharMingen), monoclonal mouse anti-IGF-I and anti-IGF-II (Upstate Biotechnology, Lake Placid, NY), HRP-conjugated goat antirat IGF and Texas Red-conjugated goat antirat IGF (Jackson Research Laboratories, West Grove, PA), HRP-conjugated rat antiserum IgG (Serenity, Harlan Bioproducts for Science, Inc., Indianapolis, IN), HRP-conjugated goat antimouse IgG (Jackson Research Laboratories), HRP-conjugated antimouse IgG (PharMingen), monoclonal mouse anti-phospho-tyrosine (Cell Signaling Technology), rabbit anti-IRS-1 (Upstate Biotechnology), polyclonal rabbit anti-IGF-IRα (Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal rabbit anti-IGF-IRβ (Santa Cruz Biotechnology), polyclonal rabbit anti-MAPK (Oncogene Research Products, Cambridge, MA), and HRP-conjugated goat antirabbit IgG (Bio-Rad Laboratories, Hercules, CA).

Cell Lines and Culture Conditions

HT29 human colon cancer cells were purchased from American Type Culture Collection (Manassas, VA) and maintained as described previously (9). All in vitro experiments were performed at 50–60% confluence, and cells were used at passages 3–8 after their receipt from the supplier.

Stable Transfections

A mutated IGF-IR truncated at position 952 in the β subunit transmembrane region (IGF-IR DN) was the generous gift of Dr. D. Prager [Cedars-Sinai Medical Center-University of California Los Angeles School of Medicine, Los Angeles, CA (19, 20)]. The IGF-IR DN was subcloned into pcDNA3 vector containing the neomycin (G418; Life Technologies, Inc.) resistance gene. The resulting vector was stably transfected into human HT29 colon cancer cells using FuGENE 6 transfection reagent (Roche Diagnostics Corp., Indianapolis, IN) following the manufacturer’s protocol. As controls, cells were transfected with the pcDNA3 vector only (mock transfectants). After 48 h, the medium was replaced by medium containing 400 μg/ml G418. Clones were expanded and characterized as described below. To confirm successful transfection, cell extracts of parental and transfected cells were analyzed by nondenaturing Western blot analysis using anti-IGF-IRα and anti-IGF-IRβ antibodies.

VEGF Induction by IGF-I in Transfected Cells

Parental and transfected cells were grown to 50–60% confluence in standard medium as described above, and medium was changed to 5% FBS-containing medium overnight. Cells were then incubated in the presence or absence of IGF-I (100 ng/ml) in serum-free medium for 24 h. Total RNA was extracted, and VEGF mRNA expression was determined by Northern blot analysis and semiquantitative RT-PCR as described previously (21, 22). The cDNA probes used were as follows: a human VEGF-specific 204-bp cDNA probe (a generous gift of Brygida Berse, Harvard Medical School, Boston, MA; Ref. 23); and a glyceraldehyde-3-phosphate dehydrogenase probe (purchased from American Type Culture Collection). The VEGF probe identifies all alternatively spliced forms of its mRNA transcripts. In a separate experiment designed to evaluate IGF-I induction of VEGF protein, conditioned medium was harvested after 48 h from cells growing in 1% FBS-containing medium with or without IGF-I (100 ng/ml). The VEGF concentration was determined using an ELISA kit for human VEGF (R&D Systems) as reported previously (Ref. 24) [the ELISA kit recognizes the VEGF165 and VEGF121 isoforms of the protein because the antibodies used were raised against the NH2-terminal portion of VEGF165 (25)].

Effect of IGF-IR DN Transfection on IGF-I Induction of Signaling

Previous experiments have shown that VEGF is induced in HT29 colon cancer cells via the Erk1/2 pathway but not through Akt,4 which is consistent with the findings of others (26). Parental and transfected cells were grown to 50% confluence. Cells were then incubated in 1% FBS-containing medium overnight, and IGF-I (100 ng/ml) was added for 10 min. Cells were harvested, and protein was extracted. For IRS-1 immunoprecipitation, cell lysates containing 300 μg of protein were incubated with anti-IRS-1 and protein A/G-agarose (Santa Cruz Biotechnology) in rotating tubes overnight at 4°C. Finally, Western blot was performed for phosphorylated tyrosine and IRS-1 as described previously (24). For determination of Erk1/2 activity induced by IGF-I, 50–60% confluent cells were incubated over-

4 Y. D. Jung and L. M. Ellis, unpublished data.
night with 1% FBS-containing medium. IGF-I (100 ng/ml) was added for 15 min, and cell lysates were harvested. Western blot analysis was performed using antibodies against phosphorylated and total forms of p44/42 (Erk1/2).

Effect of IGF-IR Stop Transfection on Monolayer Cell Growth

MTT Assay. Cells (10^4) were plated in 96-well plates. After cell attachment, medium was changed to 10% FBS-containing medium for the indicated times. MTT (Sigma, St. Louis, MO) was added to a final concentration of 0.5 mg/ml, and cells were incubated for 90 min. Medium and MTT were removed, DMSO was added for 1 min, and absorption was read at 570 nm.

BrdUrd Labeling. Cells (2 × 10^4) were plated on 2-chamber glass slides (Becton Dickinson Labware) and incubated with 10% FBS-containing medium for 36 h. BrdUrd was added to a final concentration of 10 μM, and cells were incubated for 30 min at 37°C. Finally, cells were fixed with acetone and chloroform, and immunohistochemistry was performed as described below.

Effect of IGF-IR Stop Transfection on Three-dimensional Cell Growth

Six-well plates were plated with semisolid medium containing MEM, 0.6% Bacto-agar, 10% FBS, and penicillin-streptomycin with or without IGF-I (100 ng/ml). Cells suspensions were then plated in semisolid medium consisting of MEM with 0.3% Bacto-agar, 10% FBS, and penicillin-streptomycin with or without IGF-I (100 ng/ml). After incubation for 20 days at 37°C, colonies larger than 50 μm in diameter were counted.

In Vivo Studies

Animals. Eight-week-old male nude mice (Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD) were acclimated for 1 week while caged in groups of five. The mice were housed and maintained in laminar flow cabinets under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, the United States Department of Health and Human Services, the NIH, and The University of Texas M. D. Anderson Cancer Center Animal Care and Use Committee.

Tumor Cell Injection and Therapy. For in vivo injection, HT29 parental and transfected human colon cancer cells were grown to 50% confluence and incubated in G418-free medium for 24 h to improve cell viability. Cells were harvested from culture flasks after a brief trypsinization and transferred to serum-free HBSS. Single-cell suspensions of >90% viability, as determined by trypan blue dye exclusion (200 μl containing 10^6 cells), were injected s.c. through a 27-gauge needle in the right flank. All mice were sacrificed when ≥3 mice in any group (n = 10 mice/group) had tumors that exceeded 1.2 cm in diameter. In another experiment to determine the role of IGF-IR on growth in the liver, the most common site of colon cancer metastasis, cell suspensions (50 μl containing 10^6 cells) were prepared as described. Cells were injected beneath the capsule into the left lobe of the liver of nude mice using a 30-gauge needle via a midline incision. Mice were sacrificed when ≥3 mice became moribund in any group (n = 10 mice/group).

Necropsy Procedures and Histological Studies. Mice were weighed and sacrificed by cervical dislocation after sedation with methoxyflurane. Tumors were measured, excised, weighed, and processed for immunohistochemical analyses. For evaluation of tumor growth in the liver, livers were excised and weighed, and tumors were measured. If no tumor was visible, the liver was serially sectioned at 1-mm intervals to search for small tumor growth within the parenchyma. A section of each tumor was fixed in formalin and embedded in paraffin, and another section was embedded in OCT compound (Miles Inc, Elkhart, IN), rapidly frozen in liquid nitrogen, and stored at −70°C.

Standard Immunohistochemistry. Paraffin-embedded tissues were sectioned and stained for H&E, PCNA, IGF-I, IGF-II, and VEGF. Immunohistochemical staining was performed as described previously (27, 28) using stable diamino benzidine (Research Genetics, Huntsville, AL) or AEC (Biogenex Laboratories, San Ramon, CA) as substrates. Sections were counterstained with Gill’s 3 hematoxylin (Sigma) and mounted with Universal Mount (Research Genetics). Negative controls underwent the exact same procedure with omission of the primary antibody. For analysis of VEGF expression, the signal was amplified using the TSA Biotin System (NEN Life Science, Boston, MA), and counterstaining was omitted. Standard immunohistochemistry for CD31 (endothelial cells) was done on frozen tissue sections as described previously (29). For BrdUrd labeling, cells were permeabilized with 1% Triton X-100 followed by treatment with 2 N HCl at 37°C before performing standard immunohistochemistry.

Immunofluorescence Staining for Phosphorylated Erk1/2, CD31, and TUNEL. For immunofluorescence staining, frozen tissue was fixed in cold acetone and chloroform, washed with PBS, and incubated with primary and secondary antibodies as described previously (28–30). The TUNEL assay was performed with a commercial kit (Promega, Madison, WI) according to the manufacturer’s protocol. Immunofluorescence microscopy was done on an epifluorescence microscope equipped with narrow bandpass excitation filters (Chroma Technology Corp., Brattleboro, VT) to individually select for green, red, and blue fluorescence. Images were captured with a C5810 Hamamatsu camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan) mounted on a Zeiss Axioplan microscope (Carl Zeiss Inc.) using Optimas image analysis software (Media Cybernetics, Silver Spring, MD). Images were further processed with Adobe Photoshop software (Adobe Systems, Mountain View, CA). CD31 and TUNEL double-staining was performed where endothelial cells were identified by red fluorescence (CD31 positive), and DNA fragmentation was detected in tumor and endothelial cells by localized green (TUNEL-positive) and yellow (TUNEL- and CD31-positive) fluorescence, respectively. Greater than 95% of cells in these tumor specimens are tumor epithelial cells. Therefore, quantification of tumor cell apoptosis was made under the assumption that the majority of green-stained cells were tumor cells. This was confirmed by observing the relative amount of apoptotic events.
in non-CD31/TUNEL-positive cells versus CD31/TUNEL-positive cells.

Quantification of PCNA, TUNEL, VEGF, Microvessel Density, and BrdUrd Labeling. For evaluation of immunohistochemical staining in tumors grown in mice, only the tumor edge was examined to avoid geographic bias. Necrotic areas were excluded. The number of PCNA-positive cells was assessed in three areas containing the highest PCNA expression at \( \times 100 \) magnification (0.22 mm\(^2\)). For determination of TUNEL expression in tissue sections, we counted the number of apoptotic events in five random fields at \( \times 100 \) magnification and divided that number by the total number of cells per field. The intensity of VEGF staining was graded using computer-assisted image analysis with Optimas software. The analysis was based on converting the image into a grayscale image and measuring the absorbance. Four distinct areas with high VEGF expression were evaluated at \( \times 100 \) magnification. To validate the image analysis system, all sections were also semiquantitatively scored subjectively as described previously (27, 31) with no significant differences between the objective and subjective techniques. Vessels were counted in four quadrants at \( \times 50 \) magnification according to the method described by Weidner et al. (32). For evaluation of BrdUrd staining in vitro, the percentage of BrdUrd-labeled cells was determined in 10 random fields at \( \times 200 \) magnification.

Densitometric Quantification and Statistical Analysis. Densitometric analysis was performed (Image Quant software, Molecular Dynamics, Sunnyvale, CA) to quantify the results of Northern blot analyses in the linear range of the film. Glycer-aldehyde-3-phosphate dehydrogenase mRNA was used as an internal control for loading of Northern blots. All analyses were done using InStat statistical software (GraphPad Software, San Diego, CA), with \( P < 0.05 \) considered to be significant. For comparing tumor-associated parameters, the nonpaired Student’s \( t \) test was used unless otherwise specified. If the SDs of two Gaussian distributions were significantly different, the alternate Welch \( t \) test was used.

RESULTS

VEGF Induction by IGF-I in Human Colon Cancer Cells Transfected with a Truncated IGF-IR. HT29 cells were transfected with a truncated IGF-IR (IGF-IR DN) or the empty vector (pcDNA3). Successful transfection was confirmed by performing nondenaturing Western blot analysis. Using antibodies directed against the \( \alpha \) and \( \beta \) chain of the IGF-IR, IGF-IR DN cells demonstrated strong additional bands indicating the 300-kDa mutant homodimer and the 360-kDa wild-type/mutant hybrid, respectively (as described in Ref. 19). Transfection of the empty vector or IGF-1 DN construct had no gross apparent effect on cell morphology. Incubation with IGF-I increased VEGF mRNA expression 8–10-fold in parental and pcDNA3-transfected cells, in contrast to a only slight induction in IGF-IR DN-transfected cells (Fig. 2A). Although induction of all isoforms of VEGF mRNA were attenuated in the IGF-IR DN-transfected cells, the band corresponding to the VEGF\(_{189}\) splice variant was decreased to the greatest degree. These results were confirmed by performing quantitative rTqPCR (data not shown). The transfected clone (clone 15) showing the least VEGF induction by IGF-I was used for additional experiments. A second clone (clone 9) with a moderate decrease in IGF-IR function was also studied to confirm the results reported below. As determined by ELISA, the baseline and IGF-I-induced VEGF protein in conditioned medium was significantly lower in IGF-IR DN-transfected cells as compared with controls (\( P < 0.001; \) Fig. 2B).

Effect of Altered IGF-IR Function on Intracellular Signaling. Immunoprecipitation and Western blot analysis demonstrated that IGF-IR DN-transfected cells exhibited only a minor increase in IRS-1 phosphorylation after treatment with IGF-I. In addition, the total amount of IRS-1 was also reduced in IGF-IR DN cells (Fig. 3A). We further determined the effect of IGF-IR DN transfection on downstream signaling, specifically activation of Erk1/2. Parental and pcDNA3-transfected HT29 cells showed a strong increase in Erk1/2 phosphorylation after IGF-I treatment. In contrast, IGF-I treatment of IGF-IR DN-transfected cells produced no major induction of Erk1/2 phosphorylation (Fig. 3B).

Monolayer Growth of Cells with Impaired IGF-IR Function. We used the MTT assay to investigate the monolayer growth rates of IGF-IR DN-transfected cells. Cells with impaired IGF-IR function demonstrated significantly slower growth rates compared with controls (Fig. 4A) that were proportional to their decrease in IGF-IR function (clone 9; data not shown). To confirm the effect of IGF-IR on regulating cell proliferation in response to ligand, we also labeled growing HT29 cells by BrdUrd staining. Cells transfected with DN
IGF-IR demonstrated significantly fewer BrdUrd positively stained cells when compared with controls (Fig. 4B).

**Soft Agar Growth of Cells with Impaired IGF-IR Function.** To investigate three-dimensional anchorage-independent growth of IGF-IR DN-transfected cells, cells were plated in soft agar with and without IGF-I supplementation, and colonies were counted after 20 days. IGF-IR DN-transfected cells formed few, small colonies, in sharp contrast to control cells, which formed multiple colonies with larger diameter (P < 0.05). Supplementation with IGF-I led to the development of even more colonies in the control cells, whereas IGF-IR DN-transfected cells failed to respond to IGF-I (Fig. 5).

**Tumor Formation of IGF-IR DN-transfected Cells in Nude Mice.** To study the effect of IGF-IR on tumor formation *in vivo*, parental, pcDNA3-transfected, and IGF-IR DN-transfected HT29 cells were injected s.c. into nude mice. After 25 days, mice were sacrificed due to the fact that control tumors exceeded 1.2 cm in diameter. Mice injected with pcDNA3-transfected cells formed the largest tumors compared with parental (P > 0.05) and IGF-IR DN-transfected cells (P < 0.001). These mice also weighed significantly less, indicating more advanced systemic disease (P < 0.05; data not shown). In contrast, IGF-IR DN-transfected cells developed significantly smaller and lighter tumors compared with both control groups (Fig. 6, A and B). Tumor size was proportional to the degree of inhibition of IGF-IR activity because the transfected clone with intermediate inhibition of IGF-IR function (clone 9) developed tumors that were smaller than mock transfecants (P < 0.05) but larger than tumors grown from the transfected cell line (clone 15) with the greatest decrease of IGF-IR function (data not shown).

The s.c. grown tumors were further analyzed by immunohistochemistry to determine the effect of IGF-IR DN transfection on proliferation, apoptosis, and angiogenesis (Fig. 7). There was no significant difference between HT29 parental and pcDNA3-transfected cells (controls) in any of the above parameters. In contrast, IGF-IR DN-transfected tumors demonstrated a decrease in PCNA labeling (proliferation) compared with controls (Fig. 8A). In addition, overall apoptosis was enhanced in IGF-IR DN-transfected HT29 cells (Fig. 8B). Furthermore, as determined by immunofluorescence staining, the phosphorylation status of Erk1/2 was decreased in IGF-IR DN-transfected cells as compared with controls (data not shown).

The effect of IGF-IR on tumor angiogenesis in tumor xenografts was also investigated. Because IGF-IR DN transfection nearly abrogated VEGF induction by IGF-I in monolayer cultures (Fig. 2), we performed VEGF staining on the HT29 tumors grown in nude mice using an antibody that recognizes the VEGF165, VEGF189, and VEGF206 splice variants. Using computer-assisted image analysis, tumor cells with impaired IGF-IR function demonstrated significantly less VEGF staining intensity than controls (Fig. 9A). Finally, vessels were highlighted by staining sections with an antibody to CD31. IGF-IR DN-transfected tumors had significantly fewer vessels than control groups (Fig. 9B). Immunohistochemistry demonstrated the expression of IGF-1 and IGF-II in all tumor specimens (data not shown).
Tumor Formation of DN-transfected Cells after Direct Liver Injections. To underline the biological significance of our findings, mock and IGF-IR DN-transfected cells were injected directly into the liver (note that in contrast to some other colon cancer cell lines, these cells do not form liver metastases after splenic injection). After 30 days, mice in the control group became moribund, and all mice were sacrificed. Tumor formation was significantly impaired in IGF-IR DN transfectants. Only 3 of 10 mice demonstrated small liver tumors grown from IGF-IR DN transfectants, whereas mock transfectants formed tumors in 7 of 8 mice (*, \( P < 0.05 \); Student's t-test). As a gross measure of hepatic tumor burden, the total liver weight was significantly less in mice injected with IGF-IR DN-transfected cells than in controls [IGF-IR DN transfectants, 1.54 ± 0.05 g; mock transfectants, 1.78 ± 0.05 g (mean ± SE); *P < 0.05].

**DISCUSSION**

The IGF-IR is a member of the large family of protein tyrosine kinases that play a pivotal role in normal and abnormal proliferative processes (4, 17, 33). Upon activation, the IGF-IR stimulates synthesis of RNA and DNA, cell proliferation, differentiation, and survival (4, 17, 34). Several studies indicate that the number of IGF-IRs on cells is critical in effecting the cell phenotype (35, 36). IGF-IR appears to be crucial for maintaining normal growth and development because IGF-IR knockout mouse embryos suffer generalized organ hypoplasia and invariably die at birth (18, 37). Furthermore, a minimal level of IGF-IR seems to be required for tumor growth and survival (38). When IGF-IR is activated above a certain threshold, it leads to growth in serum-free medium and to the establishment of a transformed phenotype (39). The importance of the IGF-IR has been demonstrated in many cell types including colon cancer (1, 40, 41). However, its effects on the various processes in colon cancer progression remain to be elucidated.

In addition to the established functions of the IGF-IR listed above, preliminary evidence suggests that it might also be involved in regulating angiogenesis. Our laboratory previously demonstrated that IGF-I increases VEGF expression in human
colon carcinoma cells (9). This observation is of particular interest because the sites of colon cancer growth express IGF-I constitutively. The organ with the highest levels of IGF-I, the liver (15), is also the primary organ of colon cancer metastasis. In addition to a paracrine effect, IGF-I and IGF-II may also act in an autocrine manner because colon mucosa and colon cancers themselves synthesize significant amounts of IGF-I (1).

To evaluate the importance of IGF-IR in human colon cancer and its contribution to tumor angiogenesis, we stably transfected HT29 colon cancer cells with a truncated IGF-IR lacking the cytosolic tyrosine kinase domain that formed heterodimers with wild-type IGF-IR, thereby impairing its function (19, 20). Incubation with IGF-I led to induction of VEGF mRNA in parental and pcDNA3-empty vector transfected cells, whereas this induction was nearly completely abrogated in IGF-IR DN-transfected cells. Similar results were also reported by Reiss et al. (45), who transfected various cancer cell lines with an IGF-IR truncated at residue 486 in the α subunit that led to a moderate decrease in monolayer growth but marked inhibition of colony formation in soft agar.

To evaluate the various effects of IGF-IR DN transfection of colon cancer cells on tumor formation in vivo, we injected nude mice s.c. with IGF-IR DN-transfected, pcDNA3-transfected, or parental HT29 cells. IGF-IR DN-transfected cells formed significantly smaller tumors than did controls (weight and volume), findings consistent with those of previous studies in other tumor systems (20, 45). However, we observed a decrease of tumor volume dependent on the level of IGF-IR activity, suggesting a dose-dependent effect. To further evaluate the distinct effects of IGF-IR DN transfection on the processes involved in tumor growth, we analyzed the s.c. grown tumors by immunohistochemical techniques. By PCNA labeling, we confirmed the in vivo antiproliferative effect of IGF-IR DN transfection that was demonstrated in our in vitro studies. In addition, cells transfected with IGF-IR DN showed decreased levels of phosphorylated Erk1/2 in vivo, again consistent with our find-
Fig. 7  Immunohistochemical analysis of parental and transfected HT29 cells grown in mice. Parental and transfected (pcDNA3 and IGF-IR DN) cells were grown s.c. in nude mice. Tumors were harvested and stained by immunohistochemistry for H&E, PCNA, VEGF, CD31, and TUNEL. Representative areas are shown (×50 magnification; for VEGF, ×200 magnification).
The antiapoptotic effect of the IGF-IR has been addressed previously (37–39) and was confirmed in colon cancer growth in vivo because tumors grown from HT29 cells transfected with IGF-IR DN exhibited enhanced apoptosis compared with control tumors.

The studies reported above are novel because they are the first to address the role of IGF-IR on tumor angiogenesis in vivo. In addition to evaluation of the antiproliferative and proapoptotic effects of IGF-IR DN transfection in human HT29 colon cancer cells, we determined the effect of IGF-IR DN on tumor angiogenesis. Because we have previously shown that IGF-I induces VEGF in vitro (9), we hypothesized that inhibition of the IGF-IR alters human colon cancer angiogenesis in nude mice. IGF-I and IGF-II are expressed by colon cancer cells, including HT29 cells (46), and therefore may act in an autocrine/paracrine loop in s.c. tumor formation. Because the most active angiogenic site in tumors is the tumor-normal interface (31), we assessed VEGF expression and microvessel density in this region in s.c. tumors in nude mice. IGF-IR DN-transfected cells expressed significantly less VEGF and produced significantly fewer vessels than control tumors. In addition, expression and activity of other angiogenic factors may also be mediated through the IGF-IR because the vessel count in tumors from IGF-IR DN-transfected cells was decreased to a greater degree than that of VEGF expression in some tumor areas. The contribution of IGF-I-induced angiogenesis may be even more important in organs with constitutive IGF-I expression such as the liver, which is the most frequent distant metastatic site for colon cancer. Therefore, we injected mock and IGF-IR DN-transfected cells directly into the liver of nude mice. Mice injected with IGF-IR DN cells demonstrated significantly less tumorigenicity and decreased liver weights.

Our data demonstrate the critical role of IGF-IR function in colon cancer cells in various processes involved in tumor growth both in vitro and in vivo. Furthermore, this report is the first to demonstrate that inhibition of IGF-IR function may be an indirect means of inhibiting VEGF expression and angiogenesis, similar to principles established in targeting the epidermal growth factor receptor (28). Due to the multiple functions of the IGF-IR, this receptor appears to be a promising target for therapy of human colon cancer (47, 48). Additional studies are clearly needed to further develop a more fundamental understanding of the complex IGF system including the IGF-IR and to evaluate its value and selectivity as a target in anticancer treatment.
ACKNOWLEDGMENTS

We thank W. Pagel from the Department of Scientific Publications and Rita Hernandez and Stephanie Ramirez from the Department of Surgical Oncology at The University of Texas M. D. Anderson Cancer Center for editorial assistance.

REFERENCES


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*Clin Cancer Res* 2002;8:3259-3269.

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