Tumorigenesis and Neoplastic Progression

Modification of the Primary Tumor Microenvironment by Transforming Growth Factor α -Epidermal Growth Factor Receptor Signaling Promotes Metastasis in an Orthotopic Colon Cancer Model

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The transforming growth factor α (TGF α)/epidermal growth factor receptor (EGFR) signaling pathway appears to play a critical role in colon cancer progression, but the cellular and molecular mechanisms that contribute to metastasis remain unknown. KM12C colon cancer cell clones expressing high (C9) or negligible (C10) levels of TGF α were implanted into the cecal walls of nude mice. C9 tumors formed autocrine and paracrine EGFR networks, whereas C10 tumors were unable to signal through EGFR. The tumor microenvironment of C9, but not C10, contained cells enriched in vascular endothelial growth factor (VEGF) A, interleukin-8, and matrix metalloproteinases-2 and -9 and had a high vascular surface area. C9 tumors recruited a macrophage population that co-expressed F4/80 and lymphatic vessel endothelial hyaluronic acid receptor and produced VEGFC. The mean lymphatic density of C9 tumors was threefold higher than that of C10 tumors. C9, but not C10, tumor cells metastasized to regional lymph nodes in all mice and to the liver in 5 of 10 mice. Forced expression of TGF α in C10 tumor cells led to the generation of autocrine and paracrine EGFR signaling, macrophage recruitment, enhanced blood and lymphatic vascular surface areas, and increased lymphatic metastasis. Collectively, these data show that activation of TGF α -EGFR signaling in colon cancer cells creates a microenvironment that is conducive for metastasis, providing a rationale for efforts to inhibit EGFR signaling in TGF α -positive colon cancers. (*Am J Pathol* 2008, 173:205–216; DOI: 10.2353/ajpath.2008.071147)

Colon cancer is the fourth most common malignancy in humans and is responsible for more than 500,000 deaths annually.¹ Surgical resection is effective for treating early-stage colon cancer: It results in a cure in approximately 90% of patients with stage I (American Joint Committee on Cancer tumor/node/metastasis classification system) tumors.^{2,3} However, at diagnosis, many patients have metastatic foci in regional lymph nodes (stage III) or distal tissues (stage IV), and the likelihood that these individuals will die as a result of their disease is dramatically increased. The overall 5-year survival rate for patients with stage IV colon cancer is less than 10%.^{4,5} Consequently, there is an urgent need for an improved understanding of the cellular and molecular factors that promote colon cancer metastasis.

Accumulating evidence suggests that the transforming growth factor α (TGF α)/epidermal growth factor receptor (EGFR) signaling pathway plays a critical role in colon cancer progression. EGFR is a member of the ErbB family of tyrosine kinase receptors that transmits a growth-inducing signal to cells that have been stimulated by an EGFR ligand (eg, TGF α and EGF).^{6,7} In normal tissues, the availability of EGFR ligands is tightly regulated to ensure that the kinetics of cell proliferation precisely match the tissues' requirements for homeostasis. In cancer, however, EGFR is often perpetually stimulated because of the sustained production of EGFR ligands in the tumor microenvironment^{8,9} or as a result of a mutation in EGFR itself that locks the receptor in a state of continual activation.¹⁰ Aberrant expression of TGF α or EGFR by tumors typically confers a more aggressive phenotype and is thus often predictive of poor prognosis.^{11–14} Not

Supported in part by SPORE in Prostate Cancer grant CA902701 from the National Cancer Institute.

Accepted for publication March 31, 2008.

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surprisingly, EGFR has emerged as a principal target for therapeutic intervention.

Measurements of EGFR expressed in human colon cancer cells in culture indicate that metastatic cells may express as much as five times more EGFR than do non-metastatic cells.¹⁵ Studies evaluating the distribution of EGFR and TGF α in colorectal biopsy samples have concluded that the receptor-ligand pair is a characteristic feature of more advanced tumors.^{16–20} Colon cancer cells secrete TGF α in response to environmental stress (eg, hypoxia) to signal their cell surface EGFR to initiate a sequence of cell survival programs.²¹ Prevention of EGFR signaling can have a profound effect on tumor progression in that we²² and others²³ have shown that in preclinical models of colon cancer, the inclusion of an EGFR inhibitor to therapy significantly reduces the mass of the primary tumor and, more importantly, decreases the incidence of lymphatic metastasis. Cetuximab, a chimeric monoclonal antibody that blocks ligand binding to EGFR, has demonstrated modest activity as a single agent in patients with metastatic colorectal cancer.²⁴ In a clinical trial evaluating cetuximab and irinotecan in patients with irinotecan-refractory colorectal cancer, investigators reported that the use of combined treatment resulted in a 22% response rate.²⁵

The aforementioned studies demonstrated that TGF α -EGFR signaling is one of the key regulatory pathways for tumors originating in the colon, but the cellular and molecular mechanisms whereby this signaling cascade contributes to metastatic disease remain unknown. Recent reports have emphasized that cancer metastasis is governed, to a large extent, by interactions that take place between tumor cells and the organ microenvironment.^{26,27} In the present study, we examine how the activation of autocrine and paracrine TGF α -EGFR signaling networks affects the tumor microenvironment in colon cancer and determine its effect on the formation of metastases.

Materials and Methods

Reagents

We used the following primary antibodies: anti-EGFR (SC03), anti-vascular endothelial growth factor (VEGF) A (A20), and anti-VEGFC (SC9047) (Santa Cruz Biotechnology, Santa Cruz, CA); anti-phosphorylated EGFR (Tyr1173) and anti-interleukin (IL)-8 (AHC0881) (Biosource International, Carlsbad, CA); anti-CD31 (MEK13.3) (BD PharMingen, San Diego, CA); anti-F4/80 (MCAP497) (Serotec, Raleigh, NC); anti-lymphatic vessel endothelial hyaluronic acid receptor (LYVE1) (103PA50AG and 10350PA50S) (Fitzgerald Industries, Concord, MA); anti-TGF α (GF10) (Oncogene, Boston, MA); anti-matrix metalloproteinase (MMP)-2 (AB807) and anti-MMP-9 (AB13458) (Chemicon, Billerica, MA); anti- α -smooth muscle actin (α -SMA) (AH1) (Dako, Carpinteria, CA); and anti-phosphorylated EGFR (Tyr1068) (Cell Signaling, Beverly, MA). Isotype control antibodies used in the study included goat

anti-mouse IgG (015-000-003), goat anti-rat IgG (012-000-003), and goat anti-rabbit IgG (011-000-003), all from Jackson ImmunoResearch Laboratories (West Grove, PA).

The following secondary antibodies were used for colorimetric immunohistochemical analyses: peroxidase-conjugated goat anti-rabbit IgG Fab₂ (111-036-047) and goat anti-rat horseradish peroxidase (HRP) IgG (112-035-167) (Jackson ImmunoResearch Laboratories); rat anti-mouse IgG2a HRP (MCA 421P) (Serotec); and goat anti-rabbit Alexa 488 (A-11034) and goat anti-rat Alexa 594 (A-11007) (Molecular Probes, Inc., Eugene, OR).

Colon Cancer Cell Lines and Culture Conditions

The KM12C parental colon cancer cell line originates from a primary human colon carcinoma and was maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), sodium pyruvate, nonessential amino acids, L-glutamine, a vitamin solution [all from Life Technologies (Grand Island, NY)], and a penicillin/streptomycin mixture (Flow Laboratories, Rockville, MD). Several clonal cell lines were isolated from KM12C parental cells using two double-limiting dilution steps (100, 10, 1, and 0.5 cells/well) in flat-bottom, 96-well microtiter plates. The clones were expanded, and the resulting populations were screened for TGF α production (see below). Two cell lines were selected for further examination. Specifically, a cloned line designated clone 9 (C9) was selected because it was found to constitutively secrete TGF α . A second cloned line, referred to as clone 10 (C10), did not produce detectable levels of TGF α and was chosen for comparative analysis. C9 and C10 cells were cultured in MEM containing 10% FBS. All cell lines were maintained at 37°C in a mixture of 5% CO₂ and 95% air and determined to be free of mycoplasma and pathogenic murine viruses [assayed by Science Applications International Co. (Frederick, MD)].

Reports suggest that mutations in the *K-ras* oncogene may contribute to progression of colon cancer by enhancing angiogenesis, invasion, and metastasis of tumors.²⁸ To rule out any possible confounding effects that a mutation in *K-ras* may have on our analysis, we determined the status of *K-ras* gene in KM12C cells. To detect any single-nucleotide polymorphisms (SNPs) across the *K-ras* gene of KM12C cancer cells, we used the Sequenom MALDI-TOF MassArray system (Sequenom, Inc., San Diego, CA). In brief, PCR and extension primers were designed using Sequenom Assay Design software, and the initial PCRs were performed in a 384-well format according to the manufacturer's instructions. PCR was performed in three different multiplex reactions that were pooled for genotyping. The primer extension reactions were conducted using iPLEX chemistry and then desalted and spotted on a SpectroChip (Sequenom) that were processed and analyzed with a Compact Mass Spectrometer equipped with MassArray software (Sequenom). All samples were assayed twice, and the spectra were visually inspected.

Transfection and Selection of KM12 Clone 10 Cells Expressing TGF α

Non-TGF α -expressing C10 cells were expanded, and a fraction was used to generate C10 cells that expressed TGF α . Sense TGF α plasmids containing a neomycin selection cassette were created using a stable mammalian transfection kit (Stratagene, La Jolla, CA). In brief, a 925-bp *Eco*R1 fragment containing the complete coding region of TGF α from pmt-TGF1 was ligated into a pcDNA3/neo expression vector. C10 tumor cells seeded onto 100-mm plates at a density of 1×10^6 cells/plate and monolayers (60 to 70% confluent) were transfected with TGF α plasmids or control pcDNA3 plasmids (*Eco*RI-*Eco*RI). The cultures were placed in a 37°C incubator for 12 hours and washed, and the medium was replaced with MEM containing 10% FBS. After a 24-hour incubation, 500 to 1000 μ g/ml G418 sulfate (Life Technologies) was added to the medium. The MEM/G418 medium was replaced every 72 hours until a resistant population had expanded. Enzyme-linked immunosorbent assay (ELISA) was used to determine the amount of TGF α expressed by C10-TGF α and C10-vector cells.

ELISA for TGF α Expression

To quantify the production of TGF α by KM12C, C9, C10, C10-TGF α , and C10-vector tumor cells, the cells were seeded on six-well plates at a density of 4×10^5 cells/well in MEM containing 10% FBS. The cells were allowed to stabilize for 24 hours, at which time the medium was aspirated and replaced with serum-free MEM. After a 48-hour incubation period, the medium was transferred to 1-ml conical vials and centrifuged at 3000 rpm for 3 minutes. The supernatant was collected and placed on ice. The cell number in each test well was determined by the trypan blue dye exclusion method, and the loading volume of each sample was adjusted accordingly. An ELISA kit measuring TGF α (R&D Systems, Minneapolis, MN) was used according to the manufacturer's directions, and the absorbance of the samples was compared with the standard curve.

Western Blot Analysis

To evaluate expression levels of EGFR and determine its activation status in KM12C parent, C9, C10, C10-TGF α , and C10-vector cells, we seeded cells in six-well plates at a density of 3×10^5 cells/well in MEM containing 10% FBS. The cells were allowed to stabilize for a 24-hour period, at which time the medium was aspirated and replaced with serum-free MEM for an additional 24 hours. After this incubation period, some cultured cells were stimulated with 10 ng/ml recombinant human TGF α (R&D Systems) for 15 minutes to evaluate expression of phosphorylated EGFR. Cells were washed with PBS and lysed with 0.1 ml of buffer [50 mmol/L Tris-HCL (pH 7.5), 50 mmol/L NaCl, 1% Triton X-100, 1 mmol/L Na₃VO₄, and protease inhibitors].

Protein concentrations were determined using the Bradford method (Bio-Rad Laboratories, Hercules, CA). Total protein (30 μ g) was resolved by 7.5% SDS-polyacrylamide gel electrophoresis under reducing conditions and transferred to polyvinylidene disulfide membranes. Membranes were blocked with 5% (w/v) nonfat dried milk in 0.1% Tween 20 (Sigma, St. Louis, MO) in PBS for 1 hour and incubated overnight with antibody directed against EGFR (SC03, 1:1000) and phosphorylated EGFR (Tyr1068, 1:1000). Immunodetection was performed using goat anti-rabbit HRP-conjugated antibodies (111-036-047, 1:3000). HRP activity was detected using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Animals and Orthotopic Implantation of Tumor Cells

Male athymic nude mice (NCI-nu) were purchased from the Animal Production Area of the National Cancer Institute Frederick Cancer Research and Development Center (Frederick, MD). The mice were housed and maintained 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 U.S. Department of Agriculture, the U.S. Department of Health and Human Services, and the National Institutes of Health. The mice were used, in accordance with institutional guidelines, when they were 8 to 12 weeks old.

To produce cecal tumors, we harvested KM12C parent, C9, C10, C10-TGF α , and C10-vector tumor cells from subconfluent cultures by briefly exposing the cells to a solution containing 0.25% trypsin and 0.02% EDTA. Cells were washed once in serum-free medium and resuspended in Hanks' buffered saline solution. Cells (5×10^5) in 50 μ l of Hanks' buffered saline solution were injected into the cecal walls of nude mice, as described previously.²²

Necropsy Procedures and Histological Studies

Five weeks after being injected with tumor cells, mice were euthanized with pentobarbital sodium (Abbot Laboratories, North Chicago, IL), and their body weights were recorded. After necropsy, cecal and peritoneal tumors were excised and weighed. For immunohistochemical and H&E staining procedures, a portion of the tumor tissue was fixed in formalin and embedded in paraffin. Another portion of tumor tissue was embedded in ornithine carbamyl transferase compound (Miles Laboratories, Elkhart, IN), rapidly frozen in liquid nitrogen, and stored at -80°C. All macroscopically enlarged mesenteric lymph nodes were harvested, and the presence of metastatic disease was determined by histological examination.

Immunohistochemical Analysis for TGF α , MMP-2, MMP-9, VEGFA, LYVE-1, IL-8, CD31, and α -SMA in Tumors

Paraffin-embedded tumors from the different mice were immunostained for expression of TGF α , MMP-2, MMP-9, VEGFA, LYVE-1, and IL-8. The sections were deparaffinized in xylene, treated with a graded series of alcohol [100, 95, and 80% (v/v) ethanol/double-distilled water], and rehydrated in PBS at pH 7.5. Endogenous peroxidase was blocked with 3% hydrogen peroxide in PBS. Antigen retrieval was necessary for IL-8 detection and was performed by incubating sections in a pepsin solution at 37°C for 20 minutes. Samples were incubated in a protein-blocking solution (5% normal horse serum and 1% normal goat serum in PBS) and then overnight at 4°C with the individual primary antibody in blocking solution. Primary antibodies were used at the following concentrations: 1:100 for determination of TGF α , VEGFA, and LYVE-1; 1:400 for α -SMA, MMP-2, and MMP-9; 1:800 for CD31 and 1:25 for IL-8. Control samples were incubated with the appropriate IgG isotype antibodies (mouse IgG for α -SMA and TGF α ; rat IgG for CD31; and rabbit IgG for VEGFA, LYVE-1, IL-8, MMP-2, and MMP-9). Slides were rinsed three times (5 minutes each) in PBS and then incubated with a 1:500 dilution of the corresponding peroxidase-conjugated IgG (MCA 421P rat anti-mouse IgG2a HRP for α -SMA and TGF α ; 112-035-167 goat anti-rat HRP for CD31; and 111-036-047 goat anti-rabbit HRP for VEGFA, LYVE-1, IL-8, MMP-2, and MMP-9) for 1 hour at ambient temperature. A positive reaction was detected by exposure to stable 3,3'-diaminobenzidine for 5 to 10 minutes. Slides were counterstained with Gill's hematoxylin. The procedure used to identify CD31 and α -SMA in tumors was performed as described above, with the exception that acetone-fixed frozen sections were used instead of paraffin-fixed sections.

Double Immunofluorescence Staining for CD31/EGFR and Phosphorylated EGFR

Frozen tissues used to identify CD31, EGFR, and phosphorylated EGFR were sectioned (8–10 μ m), mounted on positively charged slides, and air-dried for 30 minutes. Tissue fixation was performed using a protocol consisting of three sequential immersions in ice-cold solutions containing acetone, 50:50 (v/v) acetone:chloroform, and acetone (5 minutes each). Immunohistochemical procedures for CD31 were performed as described previously.²² In brief, samples were washed three times with PBS, incubated with protein blocking solution containing 5% normal horse serum and 1% normal goat serum in PBS for 20 minutes at room temperature, and then incubated with a 1:800 dilution of rat anti-mouse CD31 antibody (MEK13.3) for 18 hours at 4°C. After the samples were rinsed four times with PBS for 3 minutes each, the slides were incubated with a 1:1200 dilution of goat anti-rat Alexa 594 antibody (A-11007) for 1 hour. The samples were rinsed four times in PBS, placed in protein-blocking solution, and then incubated

with antibody directed against EGFR (SC03, 1:100) or phosphorylated EGFR (Tyr1173, 1:100) at 4°C overnight. Samples were washed in PBS and incubated with blocking solution for 10 minutes and then with a goat anti-rabbit Alexa 488 antibody (A-11034, 1:1200) for 1 hour. Control samples for this series of experiments were labeled with identical concentrations of isotype control antibodies (goat anti-rat IgG and goat anti-rabbit IgG) and the abovementioned secondary antibodies. All samples were rinsed and then briefly incubated with Hoechst stain (Polysciences, Inc., Warrington, PA) to visualize the cell nuclei. The slides were mounted with a glycerol/PBS solution containing 0.1 mol/L propyl gallate (Sigma) to minimize fluorescent bleaching. Immunofluorescent microscopy was performed using a Zeiss Axioplan fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a 100-W Hg lamp and narrow bandpass excitation filters. Representative images were obtained using a cooled charge-coupled device Hamamatsu C5810 camera (Hamamatsu Photonics, Bridgewater, NJ) and Optimas software (Media Cybernetics, Silver Spring, MD). Composite images were constructed with Photoshop software (Adobe Systems, Inc., Mountain View, CA). Blood vessels were identified by red fluorescence, and EGFR and phosphorylated EGFR were identified by green fluorescence. The presence of growth factor receptors or phosphorylated receptors on endothelial cells was detected by colocalization of red and green fluorescence, which appeared yellow.

Immunofluorescence Staining for Macrophages (F4/80), Lymphatic Vessels (LYVE-1), and VEGFC

Frozen tissues were used to identify macrophages (F4/80), lymphatic vessels (LYVE-1), and VEGFC protein. Frozen sections of cecal tumors from nude mice were mounted on slides and fixed in acetone solution, as described above. Samples were incubated in blocking solution for 15 minutes and then with antibody directed against either LYVE-1 (10350PA50S, 1:100) or VEGFC (SC9047, 1:100) at 4°C overnight. After being washed three times in PBS, samples were incubated with blocking solution and then for 1 hour with a 1:1200 dilution of a goat anti-rabbit Alexa 488 antibody (A-1034). The samples were then incubated with antibody against F4/80 (MCAP497, 1:100) at 4°C overnight. After being washed in PBS, the samples were blocked for 15 minutes and then incubated with goat anti-rat Alexa 594 (A-11007, 1:1200) antibody for 1 hour. To evaluate the degree of nonspecific labeling on tissues, control samples were incubated with goat anti-rabbit IgG and goat anti-rat IgG isotype control antibodies and then with Alexa 488 and Alexa 594 fluorescent antibodies. All samples were rinsed and incubated with Hoechst stain to visualize the nuclei. F4/80-positive cells were identified by red fluorescence, and LYVE-1 and VEGFC were identified by green fluorescence.

Determination of Microvascular Density, Lymphatic Vascular Density, and Blood Vessel Diameter

Microvessel density (MVD) is the measure of the number of blood vessels per high-power (microscope) field and has been shown to be a valuable prognostic indicator for a wide range of tumor types.²⁹ Lymphatic vascular density (LVD) refers to the number of lymphatic vessels associated with a given tumor and has been shown to correlate with tumor cell spread to regional nodes in some tumor models.³⁰ To determine the MVD and LVD in colon tumors expressing different levels of TGF α , we examined tumors microscopically to identify regions that stained intensely for CD31 (for MVD) or LYVE-1 (for LVD) by low-power (original magnification, $\times 40$ magnification) scanning of the section. The mean MVD or LVD was then quantified by counting the number of blood vessels or lymphatic vessels at high magnification (original magnification, $\times 100$) in a minimum of five microscopic fields for each tumor sample.

Slides stained with H&E were used to determine the mean diameter of the tumor-associated blood vessels. Tumors were viewed at high ($\times 100$) magnification using a Nikon Microphot-FXA photomicroscope (Nikon Instruments, Inc., Melville, NY) equipped with a Leica DFC320 digital camera (Leica Microsystems, Inc., Bannockburn, IL), and representative images from each tumor group were captured using PhotoShop software. The diameter of tumor-associated blood vessels in each group of tumors was measured using a stage micrometer that contained a scale etched with 10- μ m divisions. A minimum of 10 blood vessels from each tumor was included in the analysis.

Statistical Analysis

Differences in tumor weights among groups were assessed with the Mann-Whitney *U*-test. The Bonferroni correction was used to adjust for multiple comparisons and to control for the overall type I error rate of 0.05. For comparisons of microvascular density and lymphatic vascular density, Student's *t*-test was used. A *P* value of <0.05 was considered statistically significant.

Results

In Vitro Expression of TGF α and EGFR in KM12C Parental Colon Cancer Cells and Isolated Clones

ELISA was used to measure the amount of TGF α synthesized by KM12C parental tumor cells and the clonal populations isolated from parental cell lines. This initial screening proved useful for identifying cloned cells that constitutively secreted TGF α (C9 cells) and cloned cells that produced no detectable levels of TGF α (C10 cells) (data not shown). A second ELISA was performed to confirm the initial results and to compare TGF α measure-

Figure 1. Expression of TGF α and EGFR by human colon carcinoma cells. **A:** Representative ELISA measuring levels of TGF α protein in cell-free supernatants. Cells were cultured in serum-free medium for 48 hours before analysis, and the assay was conducted three times with medium collected from parallel cultures of cells. **B:** Western blot analysis of EGFR and phosphorylated EGFR on colon cancer cells. **C:** Corresponding densitometry analysis of the Western blots. The values represent the ratio of EGFR and phosphorylated EGFR protein levels to actin. Cells were serum-starved for 48 hours, and the basal level of EGFR phosphorylation was compared with that of cells stimulated with 10 ng/ml TGF α for 15 minutes. -, basal (unstimulated) conditions. The protein extracts for C10-TGF α and C10-vector cells were prepared 4 weeks after transduction. Actin was used as a loading control.

ments between KM12C parental cells and C9, C10, C10-TGF α , and C10-vector cells (Figure 1A). C9 tumor cells expressed an approximately 10-fold higher level of TGF α than did KM12C parental cells. TGF α -deficient C10 tumor cells that were engineered to produce TGF α cytokine did so at a slightly higher level than did parental KM12C cells. Control C10 vector cells that were transduced with only vector did not produce measurable amounts of TGF α . We did not detect any SNPs across the *K-ras* gene, indicating that these cells express wild-type *K-ras* (data not shown).

Western blot analysis was performed to determine the expression of EGFR in the colon cancer cell lines. All cell

Table 1. Tumorigenicity and Metastasis of Human Colon Carcinoma Cells Expressing Different Levels of TGF α in 10 Mice

Cell line	Mean body weight of mice (g) (range)	Tumor incidence	Mean weight of cecal tumors (g) (range)	Lymph node metastasis	Liver metastasis
KM12C parent	32.0 (27.5 to 36.3)	10/10	0.18 (0.09 to 0.60)	6/10	1/10
Clone 9	28.1 (23.9 to 35.2)	10/10	0.18 (0.10 to 0.68)	10/10	5/10
Clone 10	33.2 (29.1 to 36.4)	10/10	0.15 (0.05 to 0.48)	0/10	0/10
Clone 10-TGF α	33.7 (27.7 to 35.8)	10/10	0.17 (0.03 to 0.58)	5/10	1/10
Clone 10-vector	34.4 (31.1 to 35.9)	10/10	0.14 (0.03 to 0.48)	0/10	0/10

Tumor cells (5×10^5 cells) were injected into the cecum of nude mice, and mice were killed 5 weeks later. The table summarizes the clinical status of mice harboring tumors formed by KM12C parent, clone 9, clone 10, clone 10-TGF α , or clone 10-vector colon carcinoma cells.

lines expressed functional EGFR, as evidenced by their response to stimulation with 10 ng/ml TGF α (Figure 1, B and C). Expression levels of EGFR were highest in TGF α -secreting C9 tumor cells. In the absence of exogenous TGF α , EGFR was constitutively phosphorylated in C9, C10-TGF α , and to a lesser extent, KM12C parental tumor cells (Figure 1C).

Tumorigenicity and Metastasis of Human Colon Cancer Cells Expressing Different Levels of TGF α in Mice

To determine whether differential expression of TGF α by colon carcinoma cells affected their metastatic potential, we injected KM12C parental, C9, C10, C10-TGF α , and C10-vector tumor cells into the cecal walls of nude mice and evaluated the formation of metastases 5 weeks later. All cell lines produced tumors (100% incidence) in mice, and there were no significant differences in the size of the primary tumors among the mice (Table 1). Compared with KM12C parental tumors, TGF α -overexpressing C9 tumors had a significantly higher incidence of lymph node metastases; TGF α -deficient C10 tumors did not produce lymph node metastases. The colon tumors originating from C9 cells produced liver metastases in 50% of mice, but the tumors originating from C10 cells produced none. However, when C10 tumor cells were transfected with TGF α , they metastasized to the lymph nodes in 50% of mice and generated liver metastases with the same frequency as did parental cells (10%). The animal study was repeated twice, with similar results.

Expression of TGF α , EGFR, Phosphorylated EGFR, and CD31 in KM12C Parental Colon Cancer Cells and KM12C Clonal Cells Implanted Orthotopically in Mouse Colons

The expression patterns of TGF α and EGFR in colon tumors were identical to those found in tumor cells growing as monolayers. That is, the intensity of TGF α staining was most pronounced in tumors originating from C9, C10-TGF α , and KM12C parental tumor cells, whereas TGF α expression was virtually undetectable in tumors derived from C10 and C10-vector cells (Figure 2). All tumors exhibited robust staining for EGFR. However, EGFR was phosphorylated only in tumors expressing the EGFR ligand TGF α (KM12C parent, C9, and C10-TGF α

tumors). In addition, EGFR was phosphorylated in the endothelial cells of blood vessels supplying TGF α -expressing tumors, as determined by dual labeling with antibodies directed against phosphorylated EGFR and CD31. Taken together, these results suggest that tumor-secreted TGF α acts in an autocrine manner to activate EGFR expressed in tumor cells and in a paracrine fashion to communicate with the endothelial cells of blood vessels perfusing the tumor. We also observed that the blood vessels of the KM12C parent, C9, and C10-TGF α tumors had a larger diameter and were more numerous than the vessels perfusing C10 and C10-TGF α tumors (Table 2). TGF α -deficient C10 tumors contained the fewest number of blood vessels, and these vessels were characterized by small lumens.

Expression of VEGFA, IL-8, MMP-2, MMP-9, and α -SMA in KM12C Parental Colon Cancer Cells and KM12C Clonal Cell Lines Implanted Orthotopically in Mouse Colons

Because the tumors exhibited quantitative and qualitative differences in their vascular networks, we determined whether the expression of angiogenesis-related proteins differed between tumors that expressed TGF α and those that did not. We decided to focus our examination on the expression of VEGFA, IL-8, MMP-2, and MMP-9 (Figure 3). The decision to evaluate these proteins was based on the well documented role of VEGFA, MMP-2, and MMP-9 in tumor neovascularization^{31,32} and on the recent report identifying IL-8 as an important regulator of angiogenesis in the colon.³³ Our results indicate that the microenvironment of colon tumors that secrete TGF α (C9, C10-TGF α , and KM12C parental tumors) is characterized by an abundance of VEGFA, IL-8, MMP-2, and MMP-9. In contrast, the microenvironment of tumors that do not synthesize TGF α (C10 and C10-vector) is essentially devoid of these angiogenic proteins. Next, we determined whether any differences existed between the stroma of TGF α - and non-TGF α -expressing tumors. Immunofluorescence staining for α -SMA, a marker for myofibroblasts and pericytes, revealed that tumors resulting from the implantation of KM12C, C9, and C10-TGF α tumor cells contained a generous stromal compartment, whereas the stromal reaction in TGF α -deficient tumors was significantly attenuated.

Figure 2. Immunohistochemical analyses of TGF α , EGFR, and phosphorylated EGFR expression on tumor cells and tumor-associated endothelial cells in orthotopically implanted colon tumors. The pattern of tumor TGF α expression in the mouse colon is identical to the expression pattern observed *in vitro* on colon cancer cells. EGFR was present in all tumors (green) and was also detected on the tumor-associated vasculature of tumors that secreted TGF α (yellow). Expression of phosphorylated EGFR was restricted to tumors that expressed TGF α and localized to both tumor cells (green) and the supporting vascular network (yellow). The vascular networks that supplied TGF α -expressing tumors were more extensive than those of TGF α -deficient tumors and contained blood vessels of large diameter. Scale bars = 100 μ m.

Lymphatic Vasculature and Macrophage Recruitment in Colon Tumors

Because of the significant differences in lymphatic metastasis among the tumors, we examined the tumor-associated lymphatic vasculature by staining tumors for LYVE-1 (Figure 4). In all of the samples examined, lymphatic vessels were confined to the tumor interface. In no

tumor did we observe the presence of intratumoral lymphatic channels. We next determined the number of peritumoral lymphatic vessels. Compared with that in KM12C parental tumors, the number of tumor-associated lymphatic vessels was significantly increased in C9 tumors (27.5 versus 15.3, $P < 0.001$). C10 tumors had the fewest lymphatic vessels. The lymphatic vascular bed of these tumors was significantly reduced compared with that of KM12C tumors (8.3 versus 15.3, $P < 0.001$). The introduction of TGF α into C10 cells increased the intratumoral lymphatic vascular density equal to that of parental tumors, whereas the introduction of the empty vector into C10 cells did not significantly affect the number of lymphatic vessels (data not shown).

The tissues were stained with F4/80 to evaluate the macrophage content of the colon tumors (Figure 4). Macrophage recruitment to C10 tumors was reduced compared with other tumors. When samples were labeled with both LYVE-1 and F4/80, we noted that in several fields of C9 tumors, there was considerable overlap of the two signals (Figure 4, white arrows) suggesting that either the macrophage population in these tumors is positive for LYVE-1 or that the cells are transmigrating through the

Table 2. Mean Vascular Density and Mean Diameter of Tumor-Associated Blood Vessels in Orthotopic Colon Tumors Expressing Different Levels of TGF α *

Cell line	Mean number of blood vessels ($\pm$ SD)	Mean diameter (μ m) of blood vessels ($\pm$ SD)
KM12C parent	104 $\pm$ 22	31 $\pm$ 9
Clone 9	119 $\pm$ 26	36 $\pm$ 11
Clone 10	39 $\pm$ 5 [†]	20 $\pm$ 7 [†]
Clone 10-TGF α	96 $\pm$ 18	27 $\pm$ 18
Clone 10-vector	52 $\pm$ 10 [‡]	21 $\pm$ 12

*At least four tumors were analyzed in each tumor group.

[†] $P < 0.01$, compared with KM12C parental cells.

[‡] $P < 0.05$, compared with KM12C parental cells.

Figure 3. Immunohistochemical analyses of VEGFA, IL-8, MMP-2, MMP-9, and α -SMA expression in orthotopically implanted colon tumors. The microenvironment of TGF α -secreting tumors (KM12C parent, C9, and C10-TGF α) was enriched in VEGFA, IL-8, MMP-2, and MMP-9. Expression of the angiogenic proteins in tumors that did not express TGF α (C10 and C10-vector) was significantly attenuated. Expression of α -SMA reveals that the stromal reaction of tumors originating from KM12C, C9, and C10-TGF α carcinoma cells is considerably more intense than the response elicited by C10 and C10-vector tumors. Scale bars = 100 μ m.

lymphatic vessels. Because we noted significant differences in the LVDs of tumors, we also stained tumors for the known lymphatic endothelial cell mitogen, VEGFC.³⁴ VEGFC was only present in TGF α -expressing tumors, and tumor-associated macrophages were the predominant source of this growth factor (Figure 4, yellow).

Discussion

The communication networks that are established between tumor cells and non-neoplastic cells in the microenvironment of primary tumors play a critical role in determining the pathogenesis of metastasis.^{26,27} The results of studies in-

Figure 4. Immunofluorescent staining of LYVE-1, F4/80, and VEGFC in human colon carcinoma cells expressing different levels of TGF α . Lymphatic vessels are labeled with LYVE-1 (green) and macrophage cells with F4/80 (red). The number of tumor-associated lymphatic vessels was highest in C9 tumors and lowest in C10 tumors. Tumor recruitment of macrophages was also lowest in C10 tumors. Macrophage cells that localized to C9 tumors also expressed LYVE-1 (arrows) and produced abundant levels of the lymphatic endothelial cell growth factor VEGFC. Scale bars = 100 μ m.

investigating the signaling pathways that promote the growth and spread of cancer cells suggest that the information transmitted by means of TGF α -EGFR signaling is particularly important for the progression of colon tumors.^{22-25,35,36} To improve our understanding of how the EGFR signaling cascade affects the tumor microenvironment and influences metastasis, we generated colon cancer cell lines that express high or negligible levels of the EGFR ligand TGF α . When these tumor cells were implanted into the cecal walls of nude mice, the expression of TGF α by colon cancer cells led to marked alterations in the tumor microenvironment that were associated with increased metastasis.

One of the more striking differences between tumors that expressed TGF α and tumors that did not was that the former could activate autocrine EGFR regulatory loops while simultaneously signaling to the tumor-associated blood vessels in a paracrine fashion. We found that the highest TGF α -producing tumor cells (C9) also expressed the highest constitutive amount of EGFR and phosphorylated EGFR. Studies conducted on other cell types have shown that autocrine stimulation of the EGFR by TGF α results in up-regulated expression of the EGFR,^{37,38} but whether such a positive feedback mechanism is regulating EGFR expression in C9 colon cancer cells requires further investigation. In all TGF α -expressing tumors, phosphorylated EGFR was present in tumor-associated endothelial cells. This observation is consistent with recent data generated by our laboratory using a different orthotopic colon tumor model (HT-29 colon cancer cells)²² and suggests that phosphorylated EGFR is a characteristic feature of the blood vessels that perfuse colon tumors. In that study, we reported that 85% of clinical colon tumor specimens expressed TGF α and that phosphorylated EGFR was present in the adjoining endothelial cells. Phosphorylated EGFR has also been detected in the vasculature of tumors originating from the pancreas,³⁹ kidney,⁴⁰ skin,⁴¹ and lung,⁴² and one might predict that the receptor is in a state of continual activation in neoplasms that produce significant amounts of TGF α . To model this phenotype and determine the consequences of persistent EGFR phosphorylation on endothelial cells, we recently introduced a ligand-independent CD3-EGFR chimeric receptor into microvascular endothelial cells derived from mouse brain.⁴³ The results of that study revealed that sustained stimulation of EGFR in brain endothelial cells leads to activation of angiogenic programs and continuous cell division. The results of the present study suggest that a similar response occurs in vascular endothelial cells associated with TGF α -positive colon tumors, because the MVD of these tumors was significantly higher than that of non-TGF α -expressing tumors.

Two other angiogenic proteins, VEGFA and IL-8, were expressed in the microenvironment of tumors that produced TGF α . The expression levels of VEGFA and IL-8 were unremarkable in TGF α -deficient tumors. VEGFA is often regarded as the prototypical angiogenic protein because it can stimulate each of the cellular responses required for the generation of a new vascular bed (eg, migration, proliferation, protease production, and cell survival).^{44,45} Evidence that TGF α plays a role in regulat-

ing VEGFA expression comes from studies of the function of these two proteins in psoriasis.⁴⁶ The results generated from *in vitro* models of the skin disorder showed that keratinocytes respond to TGF α stimulation by up-regulating VEGFA expression.⁴⁶ Fibroblasts,⁴⁷ macrophages,⁴⁸ and endothelial cells⁴⁹ are some of the noncancer cells in the tumor microenvironment known to secrete VEGFA. Given the rather ubiquitous distribution of EGFR, it is conceivable that each cell type contributes to the VEGF pool in tumors through a TGF α -dependent signaling mechanism. VEGFA is also a potent stimulator of endothelial cell nitric oxide synthase,^{50,51} and the large-diameter blood vessels observed in TGF α -positive tumors may be secondary to VEGFA-induced nitric oxide formation.

Several lines of evidence suggest that some cells rely on TGF α -induced stimulation of EGFR to enhance their production of IL-8. For example, it has been shown that peripheral blood polymorphonuclear cells respond to ligand-induced activation of EGFR by up-regulating IL-8 expression.⁵² A separate report investigating the molecular basis for the airway neutrophilia that accompanies chronic obstructive pulmonary disease determined that bronchial epithelial cells release TGF α in response to cigarette smoke; the ensuing autocrine activation of EGFR in this cell population prompts cells to enhance their synthesis of IL-8.⁵³ On the basis of these results and the recent determination that IL-8 is a potent angiogenic factor for colon microvascular endothelial cells,³³ we determined whether IL-8 is preferentially expressed in TGF α -positive tumors. The pattern of IL-8 distribution was similar to that of VEGFA, with the most pronounced expression occurring in TGF α -expressing tumors. In aggregate, these data demonstrate that the extensive EGFR network (autocrine and paracrine) generated by TGF α -expressing colon cancer cells leads to a greater production of proangiogenic proteins (TGF α , VEGFA, and IL-8) in the microenvironment of primary tumors. Hence, therapies that inhibit EGFR activation may attenuate the expression of multiple angiogenic proteins.

Several other factors that promote angiogenesis and tumor cell invasion were also preferentially expressed in the microenvironment of TGF α -positive tumors. Specifically, we noted robust expression of two members of the MMP family, MMP-2 and MMP-9, in tumors that were positive for TGF α . Studies of the cellular origin of these two proteinases in cancer have determined that stromal cells are the predominant cellular source of MMP-2 and MMP-9 in colorectal tumors.⁵⁴ These proteolytic enzymes perform several key functions during angiogenesis (eg, increase the bioavailability of angiogenic proteins, degrade basement membrane barriers, and promote endothelial cell migration) and metastasis (eg, invasion and extravasation).⁵⁴ MMP-2 and MMP-9 levels were suppressed in TGF α -deficient tumors, illustrating yet another means by which agents that antagonize EGFR signaling can negatively affect the progression of TGF α -expressing colon tumors.

Our histopathological examination also revealed that TGF α -secreting tumor cells induce a more profound stromal reaction (ie, desmoplasia) than that evoked by TGF α -

deficient tumor cells. The stromal response of a tumor is typically evaluated by examining the expression levels of α -SMA, a characteristic marker for differentiated myofibroblasts and smooth muscle cells.⁵⁵ A reactive stroma signifies considerable cross talk between cancer cells and resident non-neoplastic cells, and recent evidence suggests that the reciprocal signaling that takes place between the two compartments frequently involves the transfer of information that stimulates tumor cell invasion and metastasis.⁵⁶ In fact, the results of some studies indicate that stromal cells may actually provide oncogenic signals to tumorigenic epithelial cells.⁵⁷ Myofibroblasts are one of the most common cell types found in the tumor stroma, and they contribute to angiogenesis and tumor cell invasion by secreting growth factors and MMPs that result in remodeling of the extracellular matrix. Our data suggest that a vigorous stromal response is a product of TGF α -EGFR signaling.

Macrophages are also capable of creating structural and biochemical imbalances in the extracellular matrix. The recruitment of macrophages is dramatically enhanced in tumors expressing TGF α . It is tempting to speculate that macrophages localize to TGF α -expressing tumors in response to the elevated VEGFA concentration gradient generated by these tumors, given that VEGFA has been identified as one of the primary factors that regulate macrophage content in tumors.^{58,59} A closer inspection of the tumor-infiltrating macrophages in TGF α -positive tumors showed that these cells express the lymphangiogenic growth factor VEGFC. The few macrophages present in the TGF α -negative tumors in our study did not express VEGFC, but they did so when tumor cells were transfected with TGF α transgenes and then implanted into the cecal walls of mice. These results add to the growing evidence that suggest that macrophages are a major source of VEGFC in pathological tissues and, therefore, function as central regulators of the lymphatic vascular surface area.^{60,61} The number of tumor-associated lymphatic vessels in the different tumors was determined by counting the number of vessels that were positive for LYVE-1. LYVE-1 is an integral membrane protein that functions as the receptor for the glycosaminoglycan hyaluronan. LYVE-1 is expressed on the luminal and abluminal surfaces of lymphatic vessels and is responsible for transporting hyaluronan into the lymph.⁶² LYVE-1 is also expressed by sinusoidal endothelial cells in the liver and spleen and by some macrophages.²⁶ Nonetheless, LYVE-1 is recognized as one of the most reliable markers to date for distinguishing lymphatic from blood vascular endothelium.^{26,63} We found that the number of lymphatic vessels in TGF α -expressing C9 tumors was threefold higher than that observed in TGF α -deficient (C10 and C10-vector) tumors, demonstrating that TGF α -EGFR signaling is an important cofactor for expansion of the tumor-associated lymphatic vascular network. When TGF α was introduced into C10 cells, the number of lymphatic vessels increased along with the number of lymphatic metastases.

Although the increase in lymphatic vascular surface area observed in the TGF α -overexpressing C9 tumors may help to explain, in part, the greater tendency of these

cells to spread to regional lymph nodes, the factors that promote the hematogenous spread of C9 tumor cells to the liver are less clear. Both KM12C parental tumors and C10-TGF α tumors expressed measurable levels of TGF α but metastasized to the liver in only 10% of mice. In addition, the expression patterns of proangiogenic cytokines in the primary tumor microenvironment of TGF α -expressing tumors were very similar, and there were no significant differences found in the vascular densities of these tumors. However, it is conceivable that the amount of TGF α secreted by a tumor cell plays an important role in determining the ability of that cell to form metastases in lymph nodes and liver. For example, the possibility exists that when C9 tumor cells enter the hepatic circulation, they produce TGF α at a sufficient level to stimulate the up-regulation of endothelial cell adhesion molecules on hepatic sinusoidal cells and to ensure their retention in the liver, whereas tumor cells that produce lesser amounts of TGF α are unable to activate the sinusoidal endothelium and, as a result, cannot generate the stable adhesive contacts necessary for extravasation. Supportive evidence for the involvement of TGF α in metastasis comes from a recent study that identified TGF α as a member of the gene set that identifies colorectal cancer cells that metastasize to the liver.⁶⁴ Alternatively, it has been known for some time now that a high vascular density increases the likelihood that tumor cells will enter the systemic circulation and reach distal organs of metastasis,⁶⁵ and we found that C9 tumors are perfused by a greater number of blood vessels than tumors that secrete lesser amounts of TGF α . Tumor blood vessels are considered to be inherently leaky, which makes them more susceptible to penetration by tumor cells,²⁶ but whether the slight increase in vascular density observed in C9 tumors (15 and 25% increase over KM12C and C10-TGF α , respectively) is responsible for their enhanced ability to form liver metastases is difficult to determine. Results from some,⁶⁶ but not all,⁶⁷ studies have demonstrated a positive correlation between microvessel density and liver metastasis.

In conclusion, the results of our study provide new insight into how the activation of TGF α -EGFR signaling in primary colon tumors contributes to the spread of tumor cells to the lymph nodes and liver. Our results indicate that TGF α -expressing tumor cells are more proficient in their ability to initiate metastases by virtue of their ability to communicate with resident nontumor cells. Therapeutic interventions that are designed to block EGFR signaling in TGF α -positive colon tumors will likely have a negative effect on a number of processes that are essential for metastasis.

Acknowledgments

We thank Ann M. Sutton for critical editorial review, Drs. Sun-Jin Kim and Dominic Fan for technical expertise, and Arminda Martinez for expert assistance with the preparation of the manuscript.

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