Research Article

Colon Cancer Cell–Derived Tumor Necrosis Factor-α Mediates the Tumor Growth–Promoting Response in Macrophages by Up-regulating the Colony-Stimulating Factor-1 Pathway

Karin Zins,[1] Dietmar Abraham,[1] Mouldy Sioud,[2] and Seyedhossein Aharinejad[1]

[1]Laboratory for Cardiovascular Research, Department of Anatomy and Cell Biology, Vienna Medical University, Vienna, Austria and
[2]Department of Immunology, Molecular Medicine Group, Norwegian Radium Hospital, Oslo, Norway

Abstract

The interplay between malignant and stromal cells is essential in tumorigenesis. We have previously shown that colony-stimulating factor (CSF)-1, matrix metalloprotease (MMP)-2, and vascular endothelial growth factor (VEGF)-A production by stromal cells is enhanced by CSF-1–negative SW620 colon cancer cells. In the present study, the mechanisms by which colon cancer cells up-regulate host factors to promote tumorigenesis were investigated. Profiling of tumor cell cytokine expression in SW620 tumor xenografts in nude mice showed increased human tumor necrosis factor (TNF)-α mRNA expression with tumor growth. Incubation of macrophages with small interfering (si) RNAs directed against TNF-α or TNF-α–depleted SW620 cell conditioned medium versus SW620 cell conditioned medium failed to support mouse macrophage proliferation, migration, and expression of CSF-1, VEGF-A, and MMP-2 mRNAs. Consistent with these results, human TNF-α gene silencing decreased mouse macrophage TNF-α, CSF-1, MMP-2, and VEGF-A mRNA expression in macrophages cocultured with human cancer cells. In addition, inhibition of human TNF-α or mouse CSF-1 expression by siRNA reduced tumor growth in SW620 tumor xenografts in mice. These results suggest that colon cancer cell–derived TNF-α stimulates TNF-α and CSF-1 production by macrophages, and that CSF-1, in turn, induces macrophage VEGF-A and MMP-2 in an autocrine manner. Thus, interrupting tumor cell–macrophage communication by targeting TNF-α may provide an alternative therapeutic approach for the treatment of colon cancer. [Cancer Res 2007;67(3):1038–45]

Introduction

Interactions between malignant cells and the tumor stroma are crucial events in the growth, invasion, and neovascularization of solid tumors. Macrophages are key effector cells in the tumor stroma, producing growth stimulators and inhibitors, proteolytic enzymes, and cytokines that modify the extracellular matrix and regulate angiogenesis (1). Tumor necrosis factor (TNF)-α is a key cytokine produced by both macrophages and malignant cells (2). In neoplastic tissues, TNF-α can induce tumor cell apoptosis (3); however, colon carcinoma cells frequently lose their sensitivity for the induction of apoptosis during tumor progression (4). Moreover, TNF-α may even promote tumor growth at lower levels (5).

TNF-α has been identified as an inducer of colony-stimulating factor (CSF)-1 production (6). CSF-1 has a regulatory effect on macrophages mediated through the *c-fms* proto-oncogene receptor tyrosine kinase (7), stimulates the proliferation, differentiation, and survival of the mononuclear cell lineage (8), and accelerates angiogenesis *in vivo* (9). Recent work suggests that CSF-1 is able to stimulate monocytes, precursors of macrophages, to secrete the biologically active vascular endothelial growth factor (VEGF), a key regulator of angiogenesis (10). Tumor-associated macrophages also facilitate the extracellular matrix breakdown by producing matrix metalloproteases (MMP), thereby enhancing tumor progression, invasion, and metastasis (11–13). Additionally, tumor cells can stimulate MMP production by macrophages via cytokines (14).

The mechanisms by which colon cancer cells up-regulate CSF-1 and MMPs produced by macrophages that result in promoting tumor growth are not clearly understood. This study addressed this issue by exploring the interplay between tumor cell–derived cytokines on cellular proliferation, migration, and gene expression regulation *in vitro* and in an established colon cancer xenograft model. The data suggest that the proinflammatory cytokine TNF-α is linked to tumor growth by acting as an autocrine growth factor for colon cancer cells and by up-regulation of CSF-1–dependent gene expression in macrophages in a paracrine manner.

Materials and Methods

cDNA arrays. Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized with 2 μg total RNA and an avian myeloblastosis virus-reverse transcriptase (Promega, Madison, WI). Total RNA was used to generate an [α-^{32}P]dCTP-labeled cDNA probe for cytokine human species-specific array analysis (GEArray Q series, SuperArray, Frederick, USA). Hybridizations were done according to the manufacturer's protocol with labeled cDNA probes from SW620 human colon cancer xenografts grown in nude mice (day 22) and SW620 colon cancer cells (15). The mRNA signals were detected and recorded in a Phosphorimager (Typhoon, Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) and quantified with ImageQuant software (Molecular Dynamics, Gladbeck, Germany).

Quantitative real-time reverse transcription-PCR. Samples were snap frozen in liquid nitrogen, homogenized, and processed for PCR as described earlier (16). The primer sequences for human molecules (VBC Genomics, Vienna, Austria) were (sense/antisense) β-2 microglobulin: 5′-GATGAGTATGCCTGCCGTGTG-3′/5′-CAATCCAAATGCGGCATCT-3′; TNF-α: 5′-AGGGACCTCTCTCTAATCAG-3′/5′-AGCTTGAGGGTTTGCTAC-3′; and VEGF-A: 5′-AGCCTTGCCGCCTTGCTGCTCTA-3′/5′-GTGCTGGCCTTGGTGAGG-3′. The primer sequences for mouse molecules were β-2 microglobulin: 5′-CCTCACATTGAAATCCAAATGC-3′/5′-CGGCCATACTGTCATGCTTAAC-3′; CSF-1: 5′-CATCTCCATTCCCTAAATCAAC-3′/5′-ACTTGCTGATCCTCCTTCC-3′; MMP-2: 5′-CCGATTATCCCATGATGAC-3′/5′-ATTCCCTGCGAAGAACAC-3′; VEGF-A: 5′-AGTACATCTTCAAGCCGTC-3′/

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Seyedhossein Aharinejad, Laboratory for Cardiovascular Research, Department of Anatomy and Cell Biology, Vienna Medical University, Waehringerstrasse 13, A-1090 Vienna, Austria. Phone: 431-4277-61119; Fax: 431-4277-61120; E-mail: seyedhossein.aharinejad@meduniwien.ac.at.

©2007 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-06-2295

Downloaded from http://aacrjournals.org/cancerres/article-pdf/67/3/1038/2579528/1038.pdf by guest on 17 July 2024

5′-GCAGGAACATTTACACGTC-3′; and TNF-α: 5′-CATGAGCACAGAAAG-CATGATC-3′/5′-CCTTCTCCAGCTGGAAGACT-3′. The species specificity of the human and mouse primers was tested in human tumor cells and mouse macrophages as described (17). LightCycler Data Analysis Version 3.1.102 (Roche, Mannheim, Germany) was used for PCR data analysis. The relative quantification of the signals was done by normalizing the signals of the different genes to β-2 microglobulin as described (15, 16). Measurements were done in duplicate.

Cell culture and analysis of siRNA effects *in vitro*. Human SW620 colon cancer cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM containing 10% FCS (15). CSF-1–dependent mouse macrophages (CRL-2470; American Type Culture Collection) were cultured in DMEM containing 10% FCS and 10% L-929 fibroblast medium containing mouse CSF-1 (18). Mouse CSF-1 and TNF-α small interfering (si) RNAs have been described previously (16, 19). Three siRNA sequences directed against human TNF-α were designed, synthesized, and tested for efficacy and optimal concentration *in vitro* (16). Cells were cultured until they reached 70% confluence, rinsed with PBS, re-fed with serum-free DMEM, and then transfected with 50 or 100 nmol/L siRNA using LipofectAMINE and Plus Reagent (Invitrogen) for 48 h according to the manufacturer's protocol. Untreated cells served as controls. Then, mRNA was isolated, and real-time reverse transcription (RT)-PCR was done as described above. The target sequence of the identified active human siRNAs was TNF-α: 5′-GCGTGGAGCTGAGAGATAA-3′; the target sequences for mouse siRNAs were CSF 1: 5′-GACCCTCGAGTCAACAGAG-3′; and TNF-α: 5′-GACAAC-CAACTAGTGGTGC-3′. The sequence of the scrambled siRNA was 5′-GAA-GCAGCACGACTTCTTC-3′. Chemically synthesized and purified siRNAs (Eurogentec, Philadelphia, PA) were used for all experiments. Experiments were done at least in triplicate.

Stimulation of colon cancer cells or macrophages with recombinant protein. Cells were rinsed and kept in serum-free medium for 3 h before treatment with 10 ng/mL recombinant human TNF-α (Chemicon, Temecula, CA) and recombinant 3×10^3 units/mL CSF-1 (Chiron, Emeryville, CA) for 3 h in serum-free DMEM. After 24 h incubation, RNA was isolated for real-time RT-PCR.

Treatment of mouse macrophages with conditioned medium of colon cancer cells. SW620 cells were cultured to 70% confluence (16) and then left untreated or transfected with human TNF-α siRNA (100 nmol/L) as described above. After 3 h, cells were supplemented with FCS and cultured for a further 24 h. At this time, the complete medium was replaced with serum-free medium, and the cells were incubated in serum-free DMEM for an additional 24 h, followed by the collection and filter sterilization of the conditioned medium from tumor cells. Mouse macrophages were seeded in six-well plates at a density of 10^6 cells/well. Following adhesion, the cells were rinsed and re-fed with 1 mL DMEM and 1 mL conditioned medium obtained from cancer cells, with conditioned medium in which the level of TNF-α was reduced or with the conditioned medium supplemented with 10 ng/mL recombinant TNF-α protein. After 24 h, RNA was isolated for real-time RT-PCR analyses. Experiments were done thrice.

ELISA. ELISA was done to detect TNF-α in the cell culture supernatant of SW620 or TNF-α siRNA–transfected SW620 cells using Quantikine Kit (R&D Systems, Minneapolis, MN). Two hundred microliters of supernatant was added to each well. The ELISA was done according to the manufacturer's protocol. The absorbance was detected using a microplate reader (Anthos, Salzburg, Austria) at 450 nm.

Cell proliferation assay. Human SW620 cells and mouse macrophages were seeded in 96-well plates at a density of 25,000 cells/well in DMEM supplemented with 10% FCS. Cells were transfected with 100 nmol/L mouse TNF-α siRNA (macrophages), 100 nmol/L mouse CSF-1 siRNA (macrophages), or 100 nmol/L human TNF-α siRNA (SW620 cells) with LipofectAMINE (Invitrogen). SW620 cells were stimulated with 10 ng/mL recombinant TNF-α, and mouse macrophages were stimulated with 60 μL conditioned medium or TNF-α–depleted conditioned from SW620 cells, 10 ng/mL recombinant TNF-α or 3×10^5 units/mL CSF-1 in serum-free medium. Three hours following transfection or stimulation, DMEM containing FCS was added to a final volume of 100 μL containing 10% FCS. Triplicate cultures were maintained for an additional 24 or 48 h for each treatment. Cell proliferation was determined using the WST-1 reagent (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol (20). Each experiment was repeated thrice.

Migration assay. Macrophages (1×10^5 in 500 μL DMEM with 1% FCS) were added to the top of each Boyden migration chamber (8-μm, 12-well plate format; BD Biosciences, Palo Alto, CA). One milliliter of conditioned medium from SW620 cells or from TNF-α siRNA–treated SW620 cells with 1% FCS, or recombinant CSF-1 or recombinant TNF-α in DMEM with 1% FCS, was used as chemoattractant in the lower chamber. DMEM with 1% FCS was used as control in the lower chamber. Cells were incubated at 37°C for 72 h. After incubation, nonmigrating cells were removed with the upper chamber. Migrating cells (lower chamber) were photographed under a microscope to determine the extent of cell migration. Each experiment was done in triplicate.

Coculture experiments. Human SW620 cells (5×10^5 cells/well) were cocultured with mouse macrophages (5×10^5 cells/well) in six-well plates, allowing direct cell-cell contact as described in the Supplementary Materials and Methods. To assess the role of factors secreted by cells, SW620 cells (5×10^5 cells/well) were seeded in the lower compartment of a cell culture chamber, and mouse macrophages (5×10^5 cells/well) were added to the upper compartment, with the two compartments being separated by a membrane (six-well plate with cell culture inserts, pore size of the membrane 1 μm; Greiner-Bio-One, Frickenhausen, Germany) that prevented direct cell-cell contact. Before siRNA treatment (100 nmol/L), cells were allowed to adhere for 24 h. Cocultures were treated with siRNA directed against human and mouse TNF-α. Three hours after transfection, cocultures were fed with DMEM medium containing 1% FCS. Cells were incubated for 48 h at 37°C, followed by RNA isolation for real-time RT-PCR. Experiments were done in triplicate.

Tumor model studies. The experiments done in this study were approved by the Institutional Animal Care and Use Committee at the Medical University of Vienna. Pathogen-free male BALB/*c-nu/nu* (nude) mice (Harlan-Winkelmann, Borchen, Germany), 5 weeks of age, were weighed and coded and randomly assigned to four experimental groups (n = 8) or to four groups of the time course study (n = 8). Mice were anesthetized (ketamine hydrochloride/xylazine at 55:7.5 mg/kg, s.c.) and 8×10^6 SW620 cells/100 μL Ringer solution were injected s.c. into their left flank as described (15). Animals of the experimental groups received human TNF-α siRNA, mouse CSF-1 siRNA or a combination of human TNF-α and mouse CSF-1 siRNA treatment that was initiated on day 8. The control group received scrambled siRNA. Mice were anesthetized, and 10 μg siRNA were injected intratumorally with LipofectAMINE (Invitrogen) in a volume of 20 μL. The treatment was cycled on days 11, 14, 17, and 20. On day 22, animals were sacrificed, and the tumors were isolated, weighed, and prepared for molecular analyses as described above (15, 16). One portion of the tissue was processed for paraffin embedding, and the remainder was processed for human cytokine array (control group), real-time RT-PCR and Western blotting (Supplementary Materials and Methods). Paraffin-embedded serial sections were stained with Ki-67 antibody (tumor proliferation assay; Dako, Glostrup, Denmark) or F4/80 (Caltag Laboratories, Burlingame, CA) antibody to evaluate the density of macrophages in the tumor. Digitalized images were generated, and morphometry was carried out (15, 16). Animals of the time course group were sacrificed on days 8, 15, 22, and 30, and the tumors were isolated, weighed, and prepared for molecular analyses as described above.

Statistical analysis. We used the Wilcoxon rank test to compare the data between the groups. All statistical tests were two-sided. Statistical tests were done with the use of SPSS software (version 10.0.7, SPSS Inc., Chicago, IL) Data are expressed as means ± SD. P values of <0.05 were considered to indicate statistical significance.

Results

Human and mouse TNF-α as well as mouse CSF-1 are up-regulated during colon cancer xenograft growth. To study the mechanisms by which colon cancer cells up-regulate macrophage

Downloaded from http://aacrjournals.org/cancerres/article-pdf/67/3/1038/2579528/1038.pdf by guest on 17 July 2024

CSF-1 production in the tumor microenvironment, we first profiled the tumor cell cytokine gene expression by using a human cytokine array. The expression of several human cytokine genes was clearly up-regulated in SW620 colon cancer xenografts on day 22 following cancer cell injection compared with SW620 cells, among them an 11-fold up-regulation of TNF-α (Fig. 1*A*). We selected human TNF-α for further analysis, because of its known potential to up-regulate CSF-1 in macrophages (6). Real-time RT-PCR confirmed the increased expression of human TNF-α and also showed significantly increased mouse TNF-α mRNA expression during the development of tumors from days 8 to 30. In addition, as reported previously (15–17), up-regulation of mouse stroma-derived CSF-1 accompanied tumor growth (Fig. 1*B*).

Autocrine regulation of TNF-α expression in colon cancer cells. To determine whether TNF-α regulates its own expression in cancer cells, we first examined the effects of human species-specific siRNA on TNF-α expression. Treatment of SW620 human colon cancer cells with the most effective TNF-α siRNA sequence tested reduced TNF-α mRNA expression significantly following treatment with 50 nmol/L siRNA, but this decrease was even more efficient following 100 nmol/L siRNA, indicating that the effect of the siRNA is dose dependent (Fig. 2*A*). Consistent with this, stimulation of SW620 cancer cells with 1, 10, and 20 ng/mL recombinant human TNF-α induced TNF-α gene expression in a dose-dependent manner (Fig. 2*A*). These experiments suggest that an autocrine loop is involved in TNF-α gene expression in colon cancer cells.

CSF-1 up-regulation by TNF-α in macrophages increases VEGF-A and MMP-2. To understand the potential connection between increased expression of TNF-α by cancer and host cells and host cell CSF-1 transcription, we analyzed the role of TNF-α in the activation of the CSF-1 and its downstream target genes, VEGF-A and MMP-2, in cultured mouse CRL-2470 macrophages. In agreement with previous work (15), we found that macrophages express the CSF-1 receptor (CSF-1R) and CSF-1, whereas SW620 cancer cells are negative for both CSF-1R and CSF-1 (data not shown). To investigate whether TNF-α regulates macrophage CSF-1, VEGF, and MMP-2 expression, we stimulated mouse macrophages with recombinant human TNF-α or inhibited macrophage TNF-α production by TNF-α siRNA. TNF-α stimulation significantly increased TNF-α, CSF-1, VEGF-A, and moderately increased MMP-2 mRNA expression, whereas TNF-α siRNA effectively reduced TNF-α and CSF-1, but not VEGF-A and MMP-2 gene expression as compared with untreated macrophages (Fig. 2*B*). We next studied the effect of CSF-1 by stimulating mouse macrophages with recombinant CSF-1 or inhibition of macrophage CSF-1 production by CSF-1 siRNA. CSF-1 treatment up-regulated its own expression, VEGF-A, and MMP-2 expression, whereas CSF-1 siRNA significantly reduced CSF-1 and moderately reduced VEGF-A, but not MMP-2 expression. Interestingly, both CSF-1 stimulation and inhibition up-regulated TNF-α expression (Fig. 2*B*). These data indicate that TNF-α increases VEGF-A and MMP-2 expression via CSF-1 up-regulation in cultured macrophages.

To study whether TNF-α production by cancer cells affects macrophages, we first treated mouse macrophages with serum-free conditioned medium from cancer cells. In macrophages, CSF-1, VEGF-A, and MMP-2 mRNA expression levels increased significantly compared with untreated controls (Fig. 2*C*). This effect on CSF-1 and VEGF-A expression was augmented by treating the cells with recombinant TNF-α in addition to the conditioned medium (Fig. 2*C*). To further investigate whether increased growth factor and MMP levels produced by macrophages was directly regulated by TNF-α released by colon cancer cells, SW620 cells were treated with siRNA during preparation of the conditioned

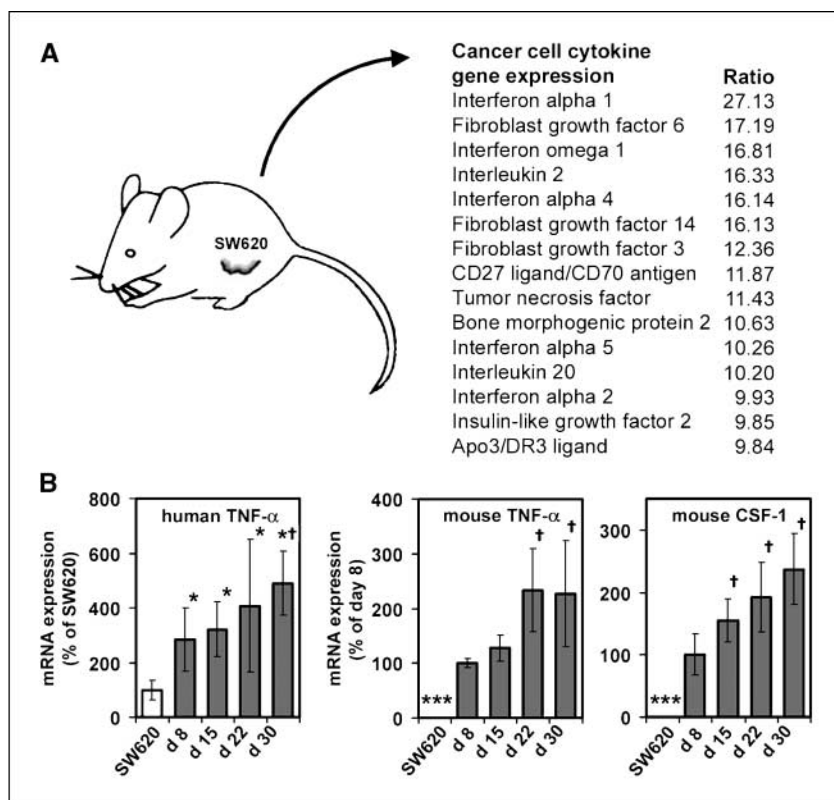

Figure 1. *A,* human cytokine array analysis of SW620 colon cancer tumors relative to the expression in SW620 cells. The ratio is the intensity of the gene expression in SW620 human colon cancer cells at day 22 following cell grafting into nude mice divided by the intensity of gene expression in cultured SW620 cells. *B,* expression of SW620 tumor cell–derived TNF-α mRNA and of host TNF-α and CSF-1 mRNA expression with tumor growth. Human and mouse TNF-α and mouse CSF-1 mRNA expression of human colon cancer xenografts in nude mice at 8, 15, 22, and 30 days (*d*) following tumor cell engraftment were compared with SW620 cells cultured *in vitro* (*white column*; ***, not detectable). mRNA expression was measured by real-time RT-PCR. Results were normalized to β-2 microglobulin levels and expressed as the percentage of expression in SW620 cells (*left*) or in day 8 tumors (*center* and *right*). *, $P < 0.05$, significantly different compared with the expression of SW620 colon cancer cells; †, $P < 0.04$, significantly different compared with expression on day 8.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/67/3/1038/2579528/1038.pdf by guest on 17 July 2024

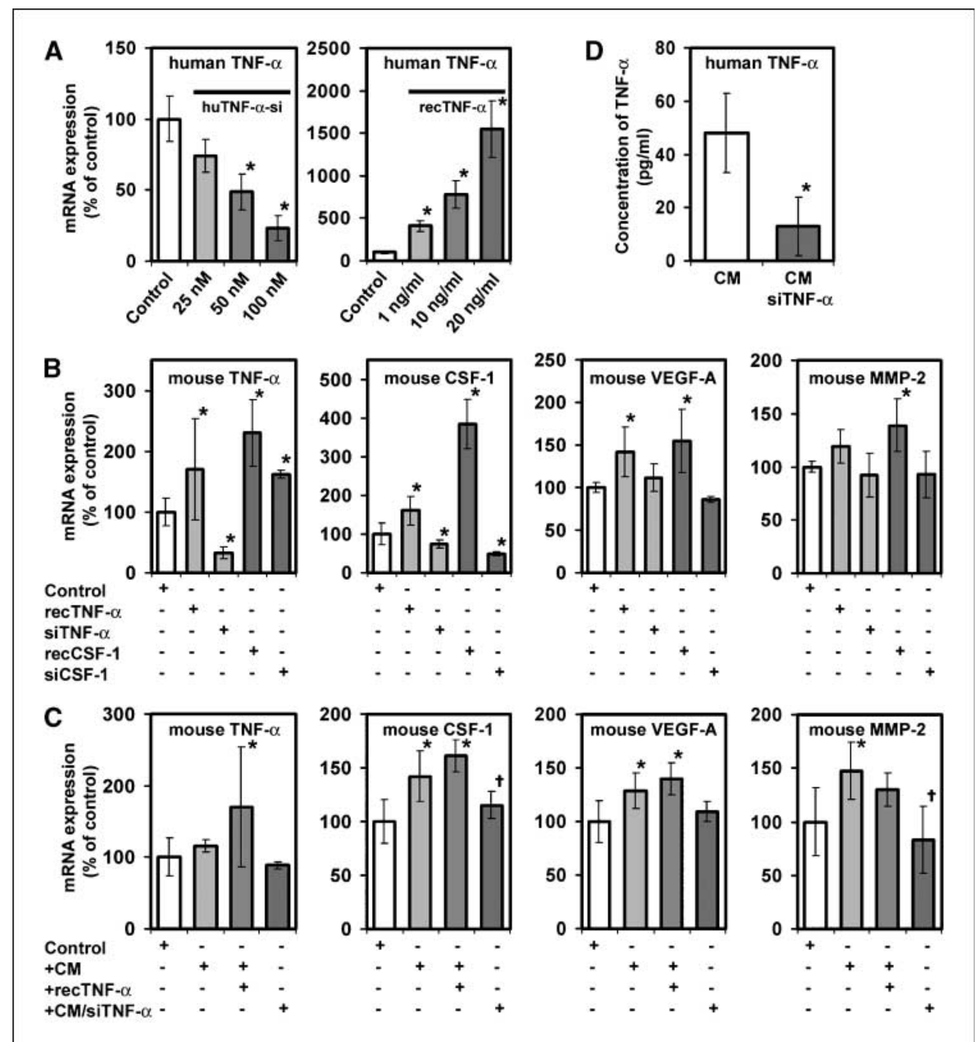

Figure 2. SW620 cancer cell–derived TNF-α up-regulates macrophage gene expression. *A,* siRNA directed against human TNF-α (*huTNF-α-si*) down-regulates TNF-α mRNA expression in SW620 colon cancer cells. *Left,* real-time RT-PCR measurement of TNF-α mRNA in human SW620 cell lysates from cells treated with 25, 50, and 100 nmol/L siRNA. *Right*, effect of treatment of SW620 cells with 1, 10, and 20 ng/mL recombinant TNF-α protein (*recTNF-α*). *, $P < 0.001$, significantly different compared with untreated controls. *B,* real-time RT-PCR mRNA measurements of macrophages treated with human recombinant TNF-α or human recombinant CSF-1 (*recCSF-1*), or treated with siRNAs against TNF-α (*siTNF-α*) or CSF-1 (*siCSF-1*). *, $P < 0.04$, significantly different from control. *C,* real-time RT-PCR of mRNA in CRL-2470 macrophages treated with SW620 cell conditioned medium (*CM*), conditioned medium supplemented with 10 ng/mL human recombinant TNF-α, or TNF-α–depleted conditioned medium (*CM/siTNF-α*). *, $P < 0.014$, significantly different from control; †, $P < 0.045$, significantly different compared with conditioned medium treatment. Results are normalized to β-2 microglobulin levels. *Columns,* means as percentage expression of control; *bars*, SD. *D,* measurements of TNF-α in SW620 supernatants (conditioned medium) using ELISA. The concentrations of TNF-α in the culture supernatants derived from SW620 transfected with TNF-α siRNA (*CM siTNF-α*) is significantly decreased compared with conditioned medium from untransfected SW620 cells. *, $P < 0.001$, significantly different compared with untransfected controls.

medium. In conditioned medium from SW620 cells transfected with TNF-α siRNA, TNF-α protein was significantly reduced compared with the conditioned medium from untreated SW620 cells (Fig. 2*D*). The macrophage up-regulation of CSF-1, VEGF-A, or MMP-2 observed with the conditioned medium was not observed in incubations with this TNF-α–depleted conditioned medium. In contrast, TNF-α mRNA expression in macrophages was not significantly affected by either TNF-α–depleted or untreated conditioned medium from SW620 cells (Fig. 2*C*). These experiments suggest that TNF-α released by cancer cells up-regulates the expression of CSF-1, VEGF-A, and MMP-2 in macrophages.

TNF-α promotes cancer cell and macrophage proliferation. To test whether TNF-α promotes cancer cell or macrophage proliferation, we first treated human SW620 colon cancer cells with recombinant TNF-α and with TNF-α siRNA directed against human or mouse TNF-α and determined the increase in mass of cell protein for up to 48 h. SW620 cell proliferation was significantly increased after 48 h of incubation with recombinant TNF-α and significantly decreased after inhibition of endogenous TNF-α production in cancer cells by treatment with TNF-α siRNA, compared with untreated control cells (Fig. 3*A*). These data indicate that a positive TNF-α autocrine feedback loop promotes colon cancer cell proliferation.

Next, we investigated whether TNF-α regulates macrophage proliferation. Comparable with the results obtained with SW620 cancer cells, recombinant TNF-α promoted macrophage proliferation, whereas TNF-α siRNA treatment reduced cell proliferation, compared with untreated control cells (Fig. 3*A*). To examine the role of cancer cell–derived TNF-α in macrophage proliferation, we treated macrophages with serum-free conditioned medium or TNF-α–depleted conditioned medium from SW620 cancer cells for up to 48 h. Conditioned medium stimulated and TNF-α–depleted conditioned medium decreased macrophage proliferation compared with controls (Fig. 3*A*). These data support the hypothesis that TNF-α production by cancer cells may lead to increased macrophage proliferation. The potential role of CSF-1 in mediating the proliferative stimulus on macrophages in response to TNF-α was tested by treating macrophages with recombinant human CSF-1, CSF-1 siRNA, and a combination of recombinant TNF-α and CSF-1 siRNA. CSF-1 stimulated, whereas CSF-1 siRNA treatment inhibited macrophage proliferation. Macrophage proliferation increased in response to treatment with recombinant TNF-α and was inhibited following the concomitant blockade of CSF-1 by siRNA (Fig. 3*A*). These results suggest that the proliferative effect of TNF-α on macrophages is mediated by CSF-1.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/67/3/1038/2579528/1038.pdf by guest on 17 July 2024

TNF-α promotes macrophage migration. Because macrophage infiltration is frequently observed in growing tumors, we compared the effects of cancer cell conditioned medium, TNF-α–depleted conditioned medium, and recombinant TNF-α and CSF-1 on macrophage migration in an *in vitro* migration assay. Conditioned medium from SW620 cancer cells and recombinant TNF-α, as well as recombinant CSF-1, significantly increased macrophage migration as compared with untreated controls (Fig. 3*B*). In contrast, treatment with TNF-α–depleted conditioned medium failed to stimulate macrophage migration, indicating a role for SW620 cancer cell–derived TNF-α in the recruitment of macrophages.

Enhanced activation of the CSF-1 target gene expression in macrophages is mediated by factors released by SW620 cancer cells. The foregoing results suggest that TNF-α secreted by cancer cells is likely to activate CSF-1 production in macrophages, thereby affecting macrophage gene expression, proliferation, and migration. To determine whether the presence of cancer cells affects the behavior of macrophages and vice versa, we did coculture experiments using SW620 cancer cells and mouse CRL-2470 macrophages allowing cell-cell contact. Coculturing increased the mRNA expression of human TNF-α and VEGF-A mRNA by the tumor cells and TNF-α, CSF-1, VEGF-A, and MMP-2 mRNA by mouse macrophages (Supplementary Fig. 1). To determine the mechanisms underlying these gene expression changes in both human cancer and mouse macrophages, we treated cocultured colon cancer cells and mouse macrophages with siRNA specifically directed against human TNF-α and mouse TNF-α. Inhibition of human TNF-α in SW620 colon cancer cells resulted in significantly decreased mRNA expression of mouse macrophage CSF-1, VEGF-A, and MMP-2 as compared with untreated cocultures. In addition, expression of TNF-α by mouse macrophages was also decreased. Likewise, inhibition of mouse macrophage TNF-α resulted in decreased mRNA expression of mouse macrophage CSF-1 and VEGF-A. However, human cancer cell–derived TNF-α and mouse MMP-2 gene expression were not affected by mouse macrophage TNF-α inhibition. To analyze whether direct cell-cell contact between cancer cells and macrophages plays a role in this context, we cocultured these cells in cell culture chambers, where the cell lines were separated by a membrane allowing only the exchange of secreted factors released by the cells. The obtained results were quite similar to those obtained in cocultures with cell-cell contact described above (Fig. 4).

These results suggest that TNF-α expression by SW620 colon cancer cells leads to the induction of macrophage TNF-α and CSF-1, and CSF-1, in turn, increases macrophage VEGF-A and MMP-2 expression. Furthermore, cancer cell–derived TNF-α seems to play a major role in this context, whereas macrophage TNF-α effects are less pronounced. Importantly, the effect of TNF-α is likely to be enhanced when macrophages and cancer cells are cocultured, although direct cell-cell contact is not necessary because TNF-α increased VEGF-A and MMP-2 expression in macrophages to a much lesser extent when cultured alone (Fig. 2).

TNF-α and CSF-1 blockade suppresses colon cancer growth. We next examined the human SW620 colon cancer xenografts to validate the *in vitro* findings *in vivo* and to investigate the role of

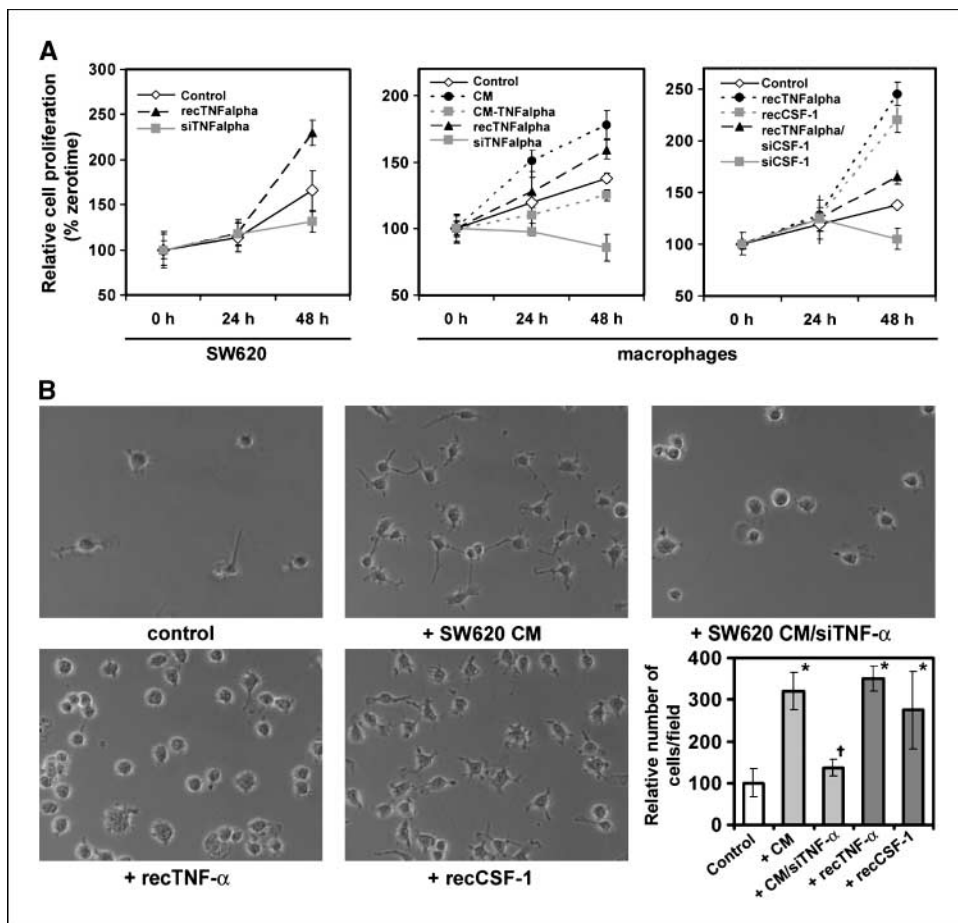

Figure 3. *A,* effects of TNF-α and siRNAs against TNF-α on SW620 cancer cell and macrophage proliferation and of SW620 cell conditioned medium, TNF-α–depleted conditioned medium, CSF-1, siRNAs against CSF-1, and combined TNF-α and siRNAs against CSF-1 on macrophage proliferation. Relative cell densities of SW620 cancer cells and macrophages up to 48 h following treatment with recombinant TNF-α (*recTNFalpha*), siRNA against TNF-α (*siTNFalpha*; *left* and *middle*), and of macrophages following treatment with SW620 cell conditioned medium, TNF-α–depleted conditioned medium, recombinant CSF-1, and siRNA against CSF-1 (*middle* and *right*) were measured using the WST-1 reagent. *Points*, means of three independent experiments; *bars*, SD. *B,* TNF-α increases macrophage migration. Representative images and quantification of migrated macrophages from an *in vitro* migration assay. Macrophages treated with SW620 cell conditioned medium, TNF-α–depleted conditioned medium, recombinant TNF-α, and recombinant CSF-1 or control medium and cell migration measured in a Boyden incubation chamber. Data were collected from five individual consecutive fields of view (10× objective) of each of the three replicate Boyden chambers. *, $P < 0.001$, significantly different from control; †, $P < 0.001$, significantly different compared with conditioned medium treatment.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/67/3/1038/2579528/1038.pdf by guest on 17 July 2024

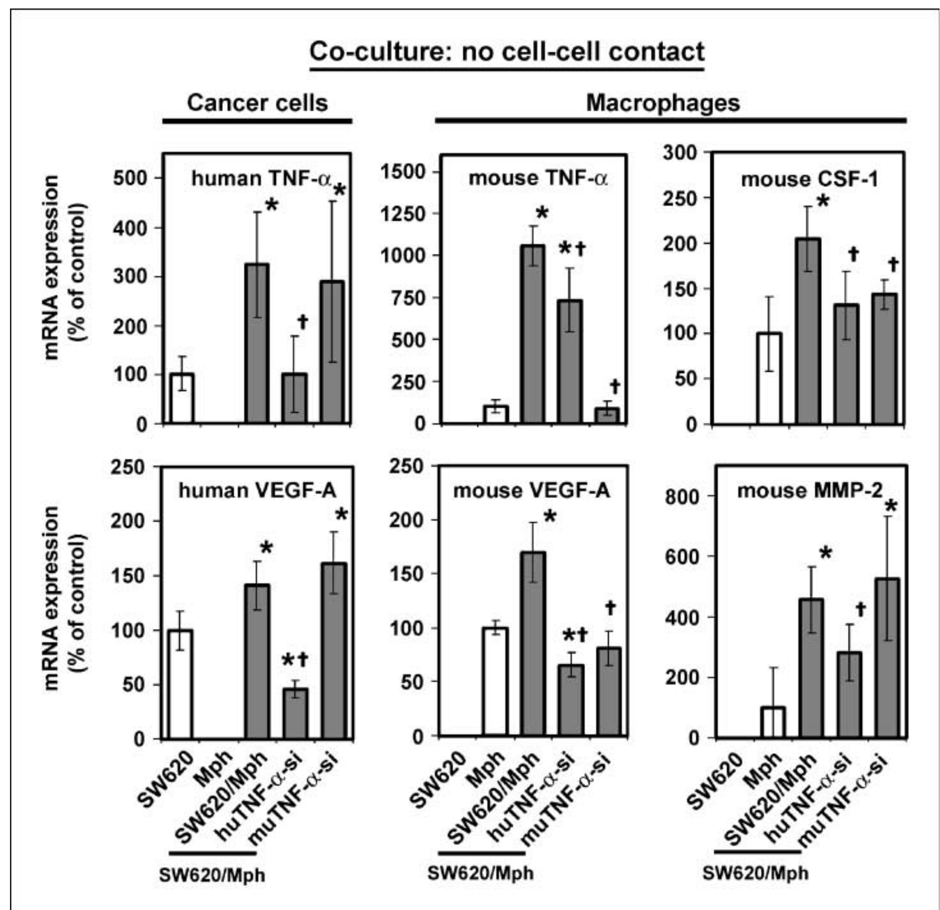

Figure 4. Cancer cells up-regulate macrophage gene expression. Cocultured SW620 colon cancer cells and mouse CRL-2470 macrophages in a cell culture chamber without cell-cell contact. Human TNF-α, human VEGF-A and mouse TNF-α, CSF-1, VEGF-A, and MMP-2 mRNA expression were measured by real-time RT-PCR in RNA from cultured SW620 cells, cultured CRL-2470 macrophages, or from cocultured human SW620 colon cancer cells and mouse CRL2470 macrophages (*Mph*) following treatment with human TNF-α siRNA (*huTNF-α-si*) or mouse TNF-α siRNA (*muTNF-α-si*). Results were normalized to β-2 microglobulin mRNA levels and expressed as a percentage of the levels in RNA from the cultured SW620 cells (*Cancer cells*) or CRL-2470 macrophages (*Macrophages*). *, significantly different from SW620 cancer cells (human genes; $P < 0.01$) and macrophages (mouse genes; $P < 0.005$); †, $P < 0.01$, significantly different from cocultured SW620 cells and macrophages.

TNF-α expression by cancer cells in regulating CSF-1 and its downstream effects *in vivo*.

Mice bearing human colon cancer xenografts were treated with siRNA against human TNF-α, mouse CSF-1, a combination of human TNF-α and mouse CSF-1, or scrambled siRNA (control). The mean tumor weight was markedly reduced in mice treated with human TNF-α (886 ± 44 mg), mouse CSF-1 (712 ± 50 mg), or combined human TNF-α/mouse CSF-1 (672 ± 111 mg) siRNA compared with control mice (1343 ± 139 mg; Fig. 5*A*). Thus, treatment with human TNF-α siRNA, mouse CSF-1 siRNA, or combined human TNF-α/mouse CSF-1 siRNA suppressed tumor growth by 34%, 47%, and 50%, respectively. In addition, cell proliferation, assessed by staining with Ki67 antibody, and the number of tumor-infiltrating macrophages, assessed by staining with mouse macrophage–specific F4/80 antibody, were reduced following treatment with human TNF-α siRNA, mouse CSF-1 siRNA, or combined human TNF-α/mouse CSF-1 siRNA (Fig. 5*B* and *C*). Blocking human TNF-α reduced mRNA levels of human and host (mouse) TNF-α, host CSF-1, MMP-2, and VEGF-A mRNA levels in tumor lysates (Supplementary Fig. 2*A*). Similarly, following host CSF-1 blockade, mouse CSF-1, MMP-2, and VEGF-A, but not mouse and human TNF-α mRNA expression, declined. In contrast, neither TNF-α nor CSF-1 blockade affected human VEGF-A expression by cancer cells (Supplementary Fig. 2*A*). Moreover, the significant reduction of total VEGF-A and MMP-2 protein expression in the tumor tissue following CSF-1 and combined TNF-α and CSF-1 blockade further supports the importance of host cell gene expression for tumor growth (Supplementary Fig. 2*B*). These experiments indicate that the blockade of tumor-derived TNF-α or host CSF-1 down-regulates host CSF-1, VEGF-A, and MMP-2 and significantly suppresses the growth of colon cancer xenografts in mice. Notably, the combination of TNF-α and CSF-1 blockade resulted only in a moderate additive suppression of colon cancer growth. Finally, these results indicate that the blockade of TNF-α in cancer cells suppresses tumor growth by targeting macrophages via CSF-1 blockade.

Discussion

The cross-talk between different cell types within the tumor plays a pivotal role in tumor growth. Tumor cells and stromal cells produce factors that modify the local extracellular matrix, stimulate migration, and promote cell proliferation and survival (21). The interactive signaling between cancer cells and their microenvironment determines the phenotype of the tumor. Cancer cells themselves can alter their adjacent stroma by producing stroma-modulating growth factors to form a supportive environment for tumor progression (22).

We have shown previously that SW620 colon cancer cells up-regulate CSF-1 in the tumor tissue, and that CSF-1 blockade suppresses tumor growth in mice associated with decreased host tissue MMP-2 and VEGF-A expression. Because the SW620 cancer cells are CSF-1 negative, the CSF-1 blockade targeted tumor stromal cell CSF-1 (15). In the current study, we show that up-regulated host macrophage CSF-1 is mediated by TNF-α secreted by colon cancer cells. Moreover, in our studies using TNF-α–containing and TNF-α–depleted conditioned medium from

Downloaded from http://aacrjournals.org/cancerres/article-pdf/67/3/1038/2579528/1038.pdf by guest on 17 July 2024

colon cancer cells, TNF-α successfully stimulated macrophage migration and proliferation.

TNF-α can exert various biological effects, including regulation of apoptosis, cell proliferation, and inflammation. Although TNF-α is capable of initiating a tumor apoptotic response, these pathways are frequently deactivated within tumor cells (5, 23). In some circumstances, TNF-α can provide a survival signal for the cancer cell, and hence, it has been referred to as a tumor-promoting factor (5). In line with this, our data show that SW620 colon cancer cell proliferation is, at least partly, promoted by TNF-α in an autocrine feedback loop. Another potential tumor-promoting effect of TNF-α has been shown in TNF-α–deficient mice, which were resistant to skin carcinogenesis (24, 25). Overexpression of TNF-α increased metastatic activity of tumor lines (25), and pretreatment of animals from an experimental fibrosarcoma model with TNF-α increased lung metastases (26). Thus, the present study confirms previous observations indicating that increases in TNF-α concentrations within the tumor microenvironment promote cancer spread. In concert with these reports, others have confirmed tumor and stromal expression of TNF-α in breast, ovarian, colorectal, and prostate cancers (23). Our *in vitro* and *in vivo* data show that TNF-α promotes the migration and recruitment of macrophages to the tumor. The ability of macrophages to move to the site of the growing tumor plays an important role for cancer development and cancer cell invasion (27). A number of tumor-derived chemoattractants are thought to ensure this ongoing recruitment, including CSF-1 and VEGF

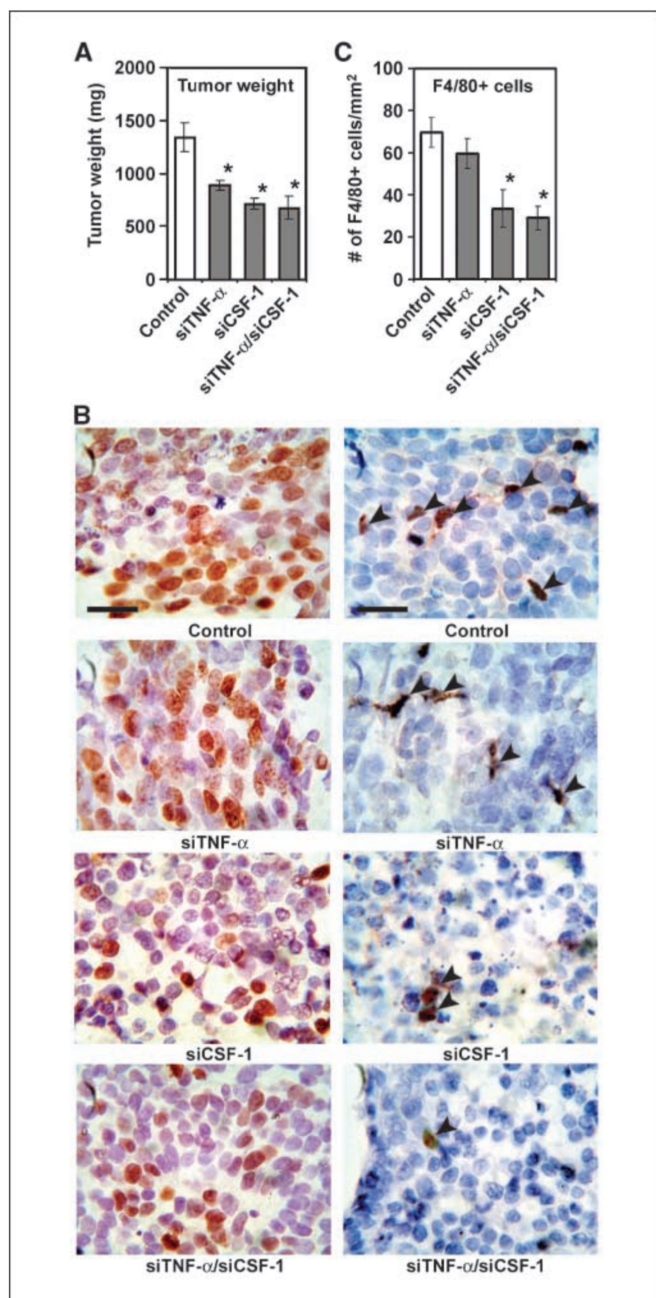

Figure 5. Effect of human TNF-α and mouse CSF-1 siRNA treatment on tumor growth and macrophage recruitment. *A,* gene silencing of human TNF-α suppresses tumor growth. Mean colon cancer xenograft tumor weights on day 22 of control tumors treated with scrambled siRNA and tumors treated with siRNAs to mouse CSF-1, to human TNF-α, or a combination of human siTNF-α and mouse siCSF-1. *, $P < 0.001$, significantly different from scrambled siRNA (*Control*) day 22. *B,* representative immunohistochemistry images of tumor tissue sections in a control mouse on day 22 (*Control*) and in mice treated with siTNF-α, siCSF-1 or combined siTNF-α/siCSF-1, stained with Ki67 antibody (*left*) or with antibody to the mouse macrophage marker protein F4/80 (*right*). *C,* quantitative histomorphometric analysis showing the density of F4/80-positive cells per square millimeter. Cell proliferation and the number of macrophages in the tumor are reduced after TNF-α, CSF-1 blockade, or TNF-α/CSF1 blockade. *Arrowheads,* macrophages stained positively with F4/80 antibody inside the colon cancer xenografts. *Bar*, 20 μm. *, $P < 0.01$, significantly different from scrambled siRNA (*Control*) day 22.

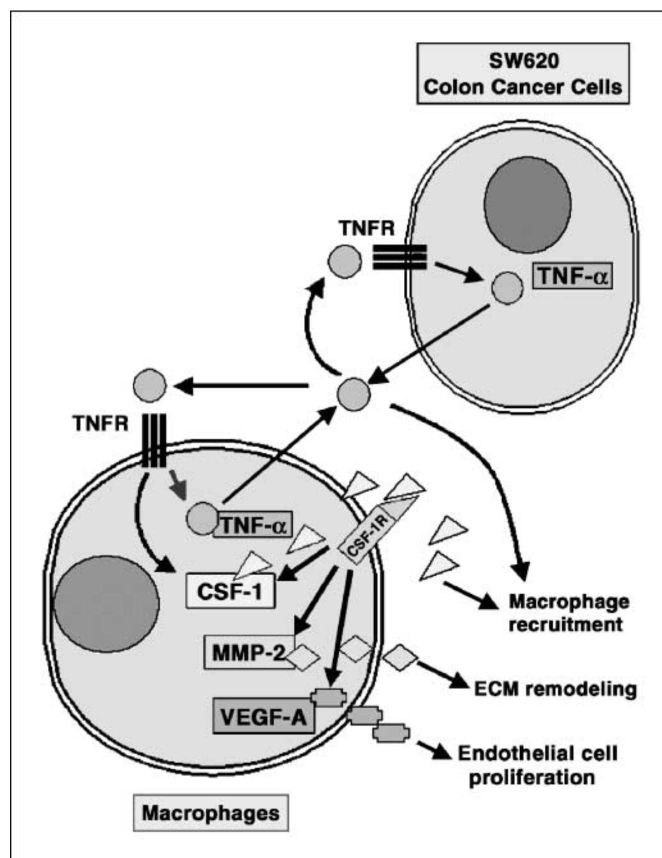

Figure 6. Proposed model for cross-talk between colon cancer cells and host macrophages. Cancer cells activate stromal macrophages by secreting TNF-α, which can act in autocrine and paracrine manners. TNF-α induces the expression of CSF-1 in macrophages and its own expression in cancer cells and macrophages. Secreted CSF-1, in turn, leads to increased MMP-2 and VEGF-A expression by macrophages via signaling through its receptor (CSF-1R). TNF-α as well as CSF-1 recruits macrophages, and CSF-1 promotes tumor angiogenesis via the angiogenic factor VEGF-A and modifies the extracellular matrix (*ECM*) by the enzyme MMP-2. *TNFR,* TNF-α receptor.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/67/3/1038/2579528/1038.pdf by guest on 17 July 2024

(16, 27), and our data show that both are up-regulated in response to tumor-derived TNF-α. Because both CSF-1 (28) and TNF-α (29) can trigger the classic mitogen-activated protein kinase and extracellular signal-regulated kinase (ERK) pathway, it is possible that TNF-α might exert its effects by activating this CSF-1 pathway in macrophages, or alternatively, that the TNF-α and CSF-1 signaling converge at the level of ERK in macrophages. However, the fact that TNF-α leads to the up-regulation of CSF-1 expression in macrophages and that there is no additional effect of TNF-α/CSF-1 siRNA treatment over CSF-1 siRNA treatment alone on tumor weight or mouse CSF-1, VEGF-A, or MMP-2 mRNA expression favors the hypothesis that these effects of TNF-α are mediated through the elevation of mouse CSF-1 production by macrophages or other stromal cells (see below). In any case, TNF-α/CSF-1 signaling leads not only to increased macrophage proliferation and chemotaxis towards the source of TNF-α and CSF-1, but also to the expression of proteinases and angiogenic factors, which are altogether essential events for successful tumor growth and invasion.

Consistent with these observations, macrophages seem to be involved in the onset and maintenance of tumor angiogenesis (27), and TNF-α may modulate tumor stromal cells in several ways to promote angiogenesis. Various factors can mediate the proangiogenic effects of TNF-α, including VEGF and basic fibroblast growth factor, but TNF-α can also promote further tumor remodeling by the induction of MMPs (30). In line with this, our data provide evidence that colon cancer cell–derived TNF-α is pivotal in this autocrine and paracrine network. TNF-α, by initiating and sustaining the production of CSF-1, causes increased VEGF-A and MMP-2 levels in cultured macrophages. Importantly, cocultivation of colon cancer cells with macrophages leads to enhanced host CSF-1, VEGF-A, and MMP-2 expression in macrophages following the up-regulation of colon cancer-derived TNF-α, indicating the importance of secreted factors up-regulated by tumor cell–stromal cell interactions in promoting tumor growth. We have previously shown the functional importance of CSF-1 up-regulation in animal models of colon cancer and breast cancer, in which the blockade of host CSF-1 suppressed tumor growth (15–17). In line with our previous findings, the present study shows that the blockade of tumor cell TNF-α suppresses tumor growth and impairs macrophage CSF-1, VEGF-A, MMP-2 expression, and macrophage infiltration, comparable to the effects observed for host CSF-1 blockade. As indicated above, combined TNF-α and CSF-1 blockade did not significantly improve the tumor-suppressive effect, supporting the hypothesis that both TNF-α and CSF-1 blockades exert their effects, at least partly, via the same pathway. In accordance with our results, cocultivation of breast cancer cell lines with macrophages led to TNF-α–dependent MMP induction in the macrophages associated with enhanced invasiveness of the malignant cells (14).

Together, we provide evidence that the interaction between macrophages and colon cancer cells promotes tumor growth. In particular, TNF-α derived from colon cancer is an autocrine growth factor for cancer cells, increases macrophage migration, and stimulates CSF-1 production by stromal macrophages and possibly other cell types. CSF-1, in turn, induces VEGF-A and MMP-2 overexpression in macrophages in an autocrine manner, thereby modulating angiogenesis and colon cancer growth (Fig. 6).

Acknowledgments

Received 6/22/2006; revised 10/2/2006; accepted 11/13/2006.

Grant support: Austrian Research Foundation grant S9412-B11 (S. Aharinejad), European Commission "Anti-tumor-targeting" grant LSHC-CT-2005-518178 (S. Aharinejad), and the Norwegian Cancer Society (M. Sioud).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/67/3/1038/2579528/1038.pdf by guest on 17 July 2024

References

1. Wyckoff J, Wang W, Lin EY, et al. A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. Cancer Res 2004;64:7022–9.
2. Cox GW, Melillo G, Chattopadhyay U, Mullet D, Fertel RH, Varesio L. Tumor necrosis factor-α–dependent production of reactive nitrogen intermediates mediates IFN-γ plus IL-2–induced murine macrophage tumoricidal activity. J Immunol 1992;149:3290–6.
3. Lejeune FJ, Ruegg C, Lienard D. Clinical applications of TNF-α in cancer. Curr Opin Immunol 1998;10:573–80.
4. Petak I, Danam RP, Tillman DM, et al. Hypermethylation of the gene promoter and enhancer region can regulate Fas expression and sensitivity in colon carcinoma. Cell Death Differ 2003;10:211–7.
5. Balkwill F. Tumor necrosis factor or tumor promoting factor? Cytokine Growth Factor Rev 2002;13:135–41.
6. Oster W, Lindemann A, Horn S, Mertelsmann R, Herrmann F. Tumor necrosis factor (TNF)-α but not TNF-β induces secretion of colony stimulating factor for macrophages (CSF-1) by human monocytes. Blood 1987;70:1700–3.
7. Sherr CJ, Rettenmier CW, Sacca R, Roussel MF, Look AT, Stanley ER. The c-fms protooncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. Cell 1985;41:665–76.
8. Pixley FJ, Stanley ER. CSF-1 regulation of the wandering macrophage: complexity in action. Trends Cell Biol 2004;14:628–38.
9. Aharinejad S, Marks SC, Jr., Bock P, et al. CSF-1 treatment promotes angiogenesis in the metaphysis of osteopetrotic (toothless, tl) rats. Bone 1995;16:315–24.
10. Eubank TD, Galloway M, Montague CM, Waldman WJ, Marsh CB. M-CSF induces vascular endothelial growth factor production and angiogenic activity from human monocytes. J Immunol 2003;171:2637–43.
11. Liotta LA, Steeg PS, Stetler-Stevenson WG. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell 1991;64:327–36.
12. Stetler-Stevenson WG, Aznavoorian S, Liotta LA. Tumor cell interactions with the extracellular matrix during invasion and metastasis. Annu Rev Cell Biol 1993;9:541–73.
13. Lynch CC, Matrisian LM. Matrix metalloproteinases in tumor-host cell communication. Differentiation 2002;70:561–73.
14. Hagemann T, Robinson SC, Schulz M, Trumper L, Balkwill FR, Binder C. Enhanced invasiveness of breast cancer cell lines upon co-cultivation with macrophages is due to TNF-α dependent up-regulation of matrix metalloproteases. Carcinogenesis 2004;25:1543–9.
15. Aharinejad S, Abraham D, Paulus P, et al. Colony-stimulating factor-1 antisense treatment suppresses growth of human tumor xenografts in mice. Cancer Res 2002;62:5317–24.
16. Aharinejad S, Paulus P, Sioud M, et al. Colony-stimulating factor-1 blockade by antisense oligonucleotides and small interfering RNAs suppresses growth of human mammary tumor xenografts in mice. Cancer Res 2004;64:5378–84.
17. Paulus P, Stanley ER, Schafer R, Abraham D, Aharinejad S. Colony-stimulating factor-1 antibody reverses chemoresistance in human MCF-7 breast cancer xenografts. Cancer Res 2006;66:4349–56.
18. Stanley ER. The macrophage colony-stimulating factor, CSF-1. Methods Enzymol 1985;116:564–87.
19. Sorensen DR, Leirdal M, Sioud M. Gene silencing by systemic delivery of synthetic siRNAs in adult mice. J Mol Biol 2003;327:761–6.
20. Liu SQ, Saijo K, Todoroki T, Ohno T. Induction of human autologous cytotoxic T lymphocytes on formalin-fixed and paraffin-embedded tumour sections. Nat Med 1995;1:267–71.
21. Liotta LA, Kohn EC. The microenvironment of the tumour-host interface. Nature 2001;411:375–9.
22. Mueller MM, Fusenig NE. Friends or foes—bipolar effects of the tumour stroma in cancer. Nat Rev Cancer 2004;4:839–49.
23. Szlosarek PW, Balkwill FR. Tumour necrosis factor α: a potential target for the therapy of solid tumours. Lancet Oncol 2003;4:565–73.
24. Moore RJ, Owens DM, Stamp G, et al. Mice deficient in tumor necrosis factor-α are resistant to skin carcinogenesis. Nat Med 1999;5:828–31.
25. Malik ST, Naylor MS, East N, Oliff A, Balkwill FR. Cells secreting tumour necrosis factor show enhanced metastasis in nude mice. Eur J Cancer 1990;26:1031–4.
26. Orosz P, Echtenacher B, Falk W, Ruschoff J, Weber D, Mannel DN. Enhancement of experimental metastasis by tumor necrosis factor. J Exp Med 1993;177:1391–8.
27. Lewis CE, Pollard JW. Distinct role of macrophages in different tumor microenvironments. Cancer Res 2006;66:605–12.
28. Bhatt NY, Kelley TW, Khramtsov VV, et al. Macrophage-colony-stimulating factor-induced activation of extracellular-regulated kinase involves phosphatidylinositol 3-kinase and reactive oxygen species in human monocytes. J Immunol 2002;169:6427–34.
29. Wajant H, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. Cell Death Differ 2003;10:45–65.
30. Leber TM, Balkwill FR. Regulation of monocyte MMP-9 production by TNF-α and a tumour-derived soluble factor (MMPSF). Br J Cancer 1998;78:724–32.