Implication of STAT3 Signaling in Human Colonic Cancer Cells during Intestinal Trefoil Factor 3 (TFF3) – and Vascular Endothelial Growth Factor–Mediated Cellular Invasion and Tumor Growth

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Abstract

Signal transducer and activator of transcription (STAT) 3 is overexpressed or activated in most types of human tumors and has been classified as an oncogene. In the present study, we investigated the contribution of the STAT3s to the proinvasive activity of trefoil factors (TFF) and vascular endothelial growth factor (VEGF) in human colorectal cancer cells HCT8/S11 expressing VEGF receptors. Both intestinal trefoil peptide (TFF3) and VEGF, but not pS2 (TFF1), activate STAT3 signaling through Tyr705 phosphorylation of both STAT3α and STAT3β isoforms. Blockade of STAT3 signaling by STAT3α/β phosphorilation or STAT3 inhibitory peptide abrogates TFF- and VEGF-induced cellular invasion and reduces the growth of HCT8/S11 tumor xenografts in athymic mice. Differential gene expression analysis using DNA microarrays revealed that overexpression of STAT3α3 down-regulates the VEGF receptors Flt-1, neuropilins 1 and 2, and the inhibitor of DNA binding/differentiation (Id-2) gene product involved in the neoplastic transformation. Taken together, our data suggest that TFF3 and the essential tumor angiogenesis regulator VEGF165 exert potent proinvasive activity through STAT3 signaling in human colorectal cancer cells. We also validate new therapeutic strategies targeting STAT3 signaling by pharmacologic inhibitors and RNA interference for the treatment of colorectal cancer patients. (Cancer Res 2005; 65(1): 195-202)

Introduction

During the early stages of the neoplastic progression in human colorectal mucosa, local invasion of the tumor stroma by epithelial cancer cells is controlled by inappropriate activation of the src, ras, and met proto-oncogenes and of several components of the Wnt/β-catenin pathways (1–4). Acquisition of the invasive phenotype by cancer cells is also controlled by the dysregulation of several growth factor receptors activated by hepatocyte growth factor, leptin, stem cell factor, transforming growth factor-β, and epidermal growth factors (2, 5–8). Tumor angiogenesis is induced by multiple proangiogenic factors secreted by endothelial, stromal, and cancer cells, such as vascular endothelial growth factor (VEGF; ref. 9).

Recent studies have suggested a causal link between local inflammation and the occurrence of gastrointestinal tumors. Among the local mediators of inflammatory processes, trefoil factors (TFF) TFF1 (pS2), TFF2 (SP), and TFF3 (ITF) constitute a new family of regulatory peptides involved in mucosal protection and repair of the gastrointestinal tract (10). These peptides are overexpressed during tumor progression and promote tumor cell invasion and angiogenesis (11, 12). We have shown recently that the proinvasive activity of TFFs is controlled by src- and Rho-dependent signaling pathways, suggesting that the signal transducers and activators of transcription (STAT) 3 might be involved in TFF signaling (13). Indeed, STAT3 is activated by several cytokines, growth factors, and oncogenes involved in cellular transformation and cancer cell invasion, including hepatocyte growth factor, leptin and stem cell factor, the src family kinases, and the small GTPases Rac1 and RhoA (6, 14–17).

STATs are latent transcription factors activated by phosphorylation of a conserved tyrosine residue. Experimental and clinical data have revealed the oncogenic potential of STAT3 through overexpression and constitutive activation in a variety of human malignancies, including leukemia, melanoma, head and neck squamous cell carcinoma, breast, prostate, ovarian, and colorectal carcinoma (18, 19). Accordingly, STAT3 exerts antiapoptotic and mitogenic effects (20, 21). The constitutively active STAT3C mutant displays a transforming activity scored by colony growth in soft agar and by tumor formation in nude mice (20). STAT3C also induces tumor angiogenesis by activating the promoter of the gene coding VEGF (22). Interestingly, VEGF and STAT3 function through a reciprocal signaling loop in endothelial cells because VEGF activates STAT3 (23). STAT3β is a naturally occurring splice variant of STAT3, which lacks the COOH-terminal transactivation domain and functions as a dominant-negative element for STAT3-mediated transcription (24). Accordingly, src oncogenic transformation is impaired by STAT3β, and overexpression of STAT3β leads to cell and tumor growth inhibition, apoptosis, and suppression of colony formation (17, 25, 26).

In this study, we investigated the possible implication of STAT3 signaling in the proinvasive activity of TFF1, TFF3, and VEGF, because we have shown recently that several human colonic cancer cells, including HCT8/S11 cells, express specific and functional receptors and coreceptors for VEGF.4 In addition, we examined the

impact of STAT3 inhibition on cell invasion and tumor growth after overexpression of the splice variant STAT3β in human colon cancer cells, ablation of both isoforms of STAT3 by RNA interference, and use of pharmacologic agents targeting STAT3α and STAT3β phosphorylation.

Material and Methods

Plasmids and Reagents. The expression vectors encoding pCEFmyc-STAT3α, pYN3218-STAT3β, pcDNA3-pS2, pCR-TFF3, and the GAS-luciferase vector were provided by Drs. Pfefler, Schaefer, Emami, Podolsky, and Dusanter, respectively. Purified recombinant human TFF1 and TFF3 were provided by Drs. Thim and Westley. Human recombinant VEGF165 was from R&D Systems (Lille, France). STAT3 inhibitor peptide was from Calbiochem (France Biochem, Meudon, France). Cucurbitacin was provided by Dr. Schultz (National Cancer Institute, Bethesda, MD). The VEGF receptor (VEGFR) tyrosine kinase inhibitor ZD1939 was provided by Dr. Ryan (AstraZeneca, Macclesfield, United Kingdom).

Cell Culture, Transient Transfections, and Luciferase Reporter Gene Assays. Human colorectal cancer cells HCT8/S11 and embryonic kidney HEK-293T cells were cultured as described (27). HEK-293T and HCT8/S11 cells were transfected with 0.25 μg GAS-luciferase reporter construct using the LipofectAMINE Plus Reagent (Invitrogen, Cergy Pontoise, France). Where indicated, the transfection mixture was combined with 1 μg of TFF1, TFF3, STAT3α, or STAT3β expression vectors, or vectors were pretreated for 1, 3, or 6 hours with cucurbitacin (1 μmol/L). Luciferase assays were done using the Luciferase Reporter Assay System kit (Promega, Charbonnières, France). For analysis of STAT3 phosphorylation, HEK-293T cells were transfected with either the empty control vector pCEFmyc, the STAT3α or STAT3β expression plasmids, or the TFF1 or TFF3 plasmids. Cells were then grown for 24 hours in a medium containing 0.1% serum and lysed in SDS buffer. Alternatively, cells transfected with STAT3α or STAT3β were grown for 24 hours in a 0.1% serum medium and then treated by TFF1 (0.1 μmol/L), TFF3 (0.1 μmol/L), or VEGF (100 ng/mL) in fresh low-serum medium.

Construction of Small Interfering RNA Plasmids. Small interfering RNA oligonucleotide for STAT3 was designed using the Target Finder program (Ambion, Austin, TX). The following sequence was used to construct the small interference oligonucleotide for STAT3 vectors in pSUPER (Oligoengine, Seattle, WA): forward 5′-GATTGACCTAGGACCCAC-3′.

Western Blot Analysis. Proteins were resolved on 8% SDS-PAGE gel and the blots were probed with the rabbit polyclonal antibodies directed against phospho-STAT3 (Cell Signaling, Ozyme, Saint-Quentin en Yvelines, France), the COOH-terminal domain of STAT3α (C-20), the conserved NH2-terminal domain of STAT3α and STAT3β (K-15, Santa Cruz, Tebu, Le Perray en Yvelines, France), or the mouse monoclonal antibody (AC15) against J-actin (Sigma, Saint-Quentin-Fallavier, France).

Gene Expression Profiling in HCT8/S11-pcDNA3 and HCT8/S11-STAT3β Cells by cDNA Microarray Analysis. Microarrays were prepared using a collection of 5,760 cDNA clones selected from a normalized infant brain library (28). The 3' and/or 5' ends of these cDNA clones have been sequenced previously (29). RNA was extracted from pcDNA3 and STAT3β-transfected HCT8/S11 cells and reverse transcribed using the SuperScript II reverse transcriptase (Invitrogen) in the presence of oligo(dT) and either cyanine 3-DUTP (red dye) or cyanine 5-DUTP (green dye) (Perkin-Elmer Life Science, Courtaboeuf, France). Hybridizations were done overnight at 42°C. Slides were scanned with an Axon 4000B fluorescence laser scanning instrument (Axon, Foster City, CA). Image analysis and the calculation of average foreground signal adjusted for local channel-specific background were done using the GenePix Pro version 4.0 software (Axon). Cy5/Cy3 intensity ratios from each gene were calculated and globally normalized to make the median value of the log2 ratio equal to 0. Genes were considered as significantly modulated according to the criteria of 2-fold cutoff ratio.

RNA Isolation and Reverse Transcription-PCR Amplification. Total RNA was extracted using the Trizol reagent (Invitrogen). Reverse transcription-PCR (RT-PCR) analyses were done with the SuperScript One-Step RT-PCR kit (Invitrogen). Fragments of the human Flt-1, neuropilin-1 (np-1), neuropilin-2 (np-2), Id-2, and STAT3 cDNAs were amplified using the following primers: Flt-1: 5′-GCACCTGGTTGTTGGTGAC-3′ and 5′-CTGTCTGGTCCCTGCTCG-3′, np-1: 5′-ACATGAGTGGCAGGTCAT-3′ and 5′-ATGTGACATTCAAGGCTTGTG-3′, np-2: 5′-TGTACGGACA-GACCTCAAC-3′ and 5′-CGAACAACTGTTGACTCC-3′, Id-2: 5′-TCCTTGCAAGCTCTGAGAG-3′ and 5′-CCATTCAACTGTCCTCC-3′, and STAT3: 5′-ATTTCAACACTTGAGTTG-3′ and 5′-ATTGTGCTCAG-CATGTTG-3′. PCR products were resolved on a 2% agarose gel stained with ethidium bromide. Basal mRNA level of each gene was normalized to 1. Results presented are representative of three independent experiments.

Cell Invasion, Proliferation, Apoptosis, and Tumor Growth. Invasion assays were done as described previously (27). For cell proliferation assays, HCT8/S11 cells were seeded in six-well plates at a density of 100,000 cells per well and treated with cucurbitacin. DNA synthesis was measured with a 4-hour pulse with 1 μCi/mL [3H]thymidine and liquid scintillation counting. Alternatively, cells were seeded for 24 hours in 60-mm dishes at a density of 400,000 cells per well. Control and treated cells were then harvested and either counted using a cell counter (Beckman Coulter, Villepinte, France) or fixed with 70% ethanol in PBS and submitted to fluorescence-activated cell sorting analysis for the determination of apoptosis.

Parental and stably transfected HCT8/S11 cells were injected s.c. into the lateral flank of 6-week-old athymic nu/nu mice (3 × 106 cells in each xenograft, 8 animals in each group, Elevage Janvier, Le Genest, France). Tumor volume (V) was calculated using the formula: V = 0.4 × a × b2 where a is the length and b is the width of the tumor. Where indicated, animals were randomized into two groups, and one group was treated with cucurbitacin (1 mg/kg/d) diluted in DMSO 20% and water. Throughout this study, procedures were conducted in conformity with institutional guidelines (European Economic Community’s Council Directive 86/609, OJ L 358, 1, December 12, 1987; NIH Guide for Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985).

Statistical Analysis. Data are means ± SE of three independent experiments. The differences between experimental values was assessed by the unpaired Student’s t test and P < 0.05 versus control was considered statistically significant.

Results

TFF3 and VEGF Activate STAT3 Signaling. On ligand stimulation, STAT3 is phosphorylated at Tyr705 dimerizes, and translocates to the nucleus to transactivate target genes. To determine whether the trefoil peptides TFF1 and TFF3 activate STAT3 signaling, we transiently transfected HEK-293T epithelial cells with expression vectors encoding either STAT3α or its splice variant STAT3β. Cotransfection of TFF3, but not TFF1, vector increased basal levels of STAT3 phosphorylation at Tyr705 in both isoforms with no impact on the total amount of STAT3 protein (Fig. 1A). We next examined the transcriptional activity of STAT3 in TFF1- and TFF3-transfected HEK-293T cells using the GAS reporter construct containing three STAT consensus elements upstream of the luciferase gene. As shown in Fig. 1B, TFF3 increased STAT3-dependent transcription 2.3-fold, whereas TFF1 had no significant effect (Fig. 1B). We further examined the effects of the addition of the trefoil peptides TFF1 and TFF3 on the activation status of STAT3 as immediate cellular responses. For this purpose, STAT3-transfected HEK-293T cells were treated for 5 to 30 minutes with 0.1 μmol/L TFF1 or TFF3. TFF3 induced a time-dependent activating phosphorylation of STAT3α, reaching maximal levels at 5 to 10 minutes, and a persistent activation of STAT3β (Fig. 1C). In agreement with our data in Fig. 1A, no STAT3α/β activation was observed in response to TFF1 stimulation. We also treated HCT8/
S11 cells with VEGF_{165} (100 ng/mL), because we detected the expression of functional proinvasive VEGFR in human colonic cancer cells. Moreover, a recent report showed that STAT3 is essential for VEGF-induced migration and morphogenesis in human endothelial cells. In a manner identical to TFF3, VEGF_{165} induced prompt activation of STAT3α in HCT8/S11 cells (5–10 minutes) that returned to basal levels 15 to 30 minutes after stimulation (Fig. 1C). Interestingly, phosphorylation of the STAT3β isoform by VEGF_{165} was also induced at 5 minutes and persisted up to 30 minutes after stimulation (Fig. 1C, bottom). We next investigated the impact of STAT3α or STAT3β transient expression on STAT3 transcriptional activity. As expected, STAT3α activated the GAS-luciferase reporter gene 2.2-fold, whereas STAT3β reduced by 50% STAT3-mediated transcription in HCT8/S11 cells (Fig. 1D).

Blocking STAT3 Signaling Abrogates Cellular Invasion and Leads to Tumor Growth Inhibition. According to our recent data (13), both VEGF and TFF induce the invasiveness of HCT8/S11 cells in collagen gels (Fig. 2B). As expected, the KDR/Flt-1 tyrosine kinase inhibitor ZD4190 abrogated the proinvasive activity of VEGF_{165}. Interestingly, ZD4190 was ineffective on TFF1- or TFF3-induced invasion (data not shown). Therefore, our data indicate that TFFs are not acting through VEGF activation or transactivation. To determine the contribution of STAT3 to the invasive phenotype induced by TFFs and VEGF_{165}, we established HCT8/S11 cell lines stably transfected with STAT3α or STAT3β. Expression of the STAT3 transgens was detected by Western blot analyses in individual clones and pools of clones. As shown in Fig. 2B, cellular invasion is not induced or potentiated by overexpression of STAT3α in control cells or HCT8/S11 cells exposed to the proinvasive agents TFF1, TFF3, or VEGF. Most interestingly, blockade of STAT3 signaling by STAT3β, which lacks the STAT3 transactivation domain, abrogated the invasive behavior of HCT8/S11-STAT3β cells treated by these proinvasive agents. These encouraging results led us to investigate the possible impact of STAT3β on the growth of human tumor xenografts. For this purpose, parental and STAT3α- or STAT3β-transfected HCT8/S11 cells were xenografted in nude mice. Activation of STAT3 signaling by stable expression of STAT3α in HCT8/S11 cells substantially increased tumor size (2.0-fold) after 6 weeks following xenografts (Fig. 2C, left). Conversely, disruption of STAT3 signaling by STAT3β

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**Figure 1.** Induction of STAT3 tyrosine phosphorylation and transcriptional activation by TFF3 and VEGF_{165}. A, Western blot analysis of Tyr705-phosphorylated STAT3 in kidney epithelial HEK-293T cells cotransfected with expression vectors encoding STAT3α or STAT3β either alone or combined with the TFF1 and TFF3 vectors. B, STAT3-mediated transcription in HEK-293T cells transfected with the empty control vector pcDNA3 and the vectors encoding TFF1 or TFF3. STAT3-dependent transcriptional activity was determined by cotransfection with the GAS-luciferase reporter construct. *, P < 0.05 versus control. C, Western blot analysis of the Tyr705-phosphorylated STAT3 and β-actin levels in HEK-293T cells transfected with STAT3α or STAT3β and treated for the indicated times with the TFF1, TFF3, or VEGF_{165} peptides. D, impact of STAT3α and STAT3β transient expression on STAT3-dependent transcription in HCT8/S11 cells. *, P < 0.05 versus control.

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led to a remarkable reduction (67%) of tumor growth in HCT8/S11-STAT3
$x$ xenografts (Fig. 2, right).

**Disruption of STAT3 Signaling by STAT3**$\beta$ **Is Associated with the Down-regulation of the Genes Encoding VEGFR and Id-2.**

To better understand the mechanisms underlying the antitumoral effects of STAT3$\beta$, we searched for differentially expressed genes in parental and HCT8/S11-STAT3$\beta$ cells by a cDNA microarray analysis. Among the genes regulated by STAT3$\beta$, we identified np-2, a receptor for class 3 semaphorin and for VEGF, as well as the inhibitor of DNA binding/differentiation (Id-2), a helix-loop-helix protein that lacks the DNA binding domain and functions as a negative regulator of basic helix-loop-helix transcription factors. We then confirmed by RT-PCR that the levels of both Id-2 and np-2 transcripts were reduced by 62% to 66%. This validation was extended to the expression of other VEGF receptors (i.e., VEGFR-1/Flt-1 and np-1). Similarly, np-1 and Flt-1 gene expression was also reduced by 52% and 68%, respectively (Fig. 3).

**Depletion of STAT3$\alpha$/$\beta$ Proteins by RNA Interference Abrogates TFF- and VEGF-Induced Cellular Invasion.** We next investigated the impact of STAT3 depletion on the invasive properties of parental HCT8/S11 cells using RNA interference. Our STAT3 interference vector was introduced into HCT8/S11 cells, generating a stable HCT8/S11-siSTAT3$\beta$ cell line. RT-PCR experiments revealed that STAT3 transcripts decreased by 28% and 67% in HCT8/S11-siSTAT3$\beta$ pool and clone 2, respectively (Fig. 4A). Both isoforms of STAT3 proteins were depleted by 65% and 80% in silenced STAT3 cells pool and clone 2, respectively (Fig. 4B). As expected, STAT3 transcriptional activity was significantly reduced by 31% and 50% in pool and clone 2, respectively, compared with the stably transfected control HCT8/S11-pSUPER cell line (Fig. 4C). We next investigated the functional impact of STAT3 protein depletion on cellular invasion. Figure 4D shows that siSTAT3 abrogates TFF1-, TFF3-, and VEGF-induced invasiveness in the HCT8/S11-siSTAT3$\beta$ cell line.

**Blocking STAT3 Signaling by Cucurbitacin and a STAT3 Dimerization Disrupter Abrogates TFF3- and VEGF-Mediated Cellular Invasion.** We examined the effects of pharmacologic agents known to inhibit STAT3 phosphorylation on HCT8/S11 invasion. The STAT3 phosphorylation inhibitor cucurbitacin was recently described to impede the Janus-activated kinase/STAT3 signaling pathway in human cancer cells (30). As shown in Fig. 5A, treatment of HCT8/S11 cells with cucurbitacin for 24 hours abrogates the invasive potential of TFF1, TFF3, and VEGF in a dose-dependent manner. Total inhibition of cellular invasion was

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**Figure 2.** Inhibition of cellular invasion and tumor growth by overexpression of the dominant negative variant STAT3$\beta$. A, Western blot analysis of STAT3$\alpha$ or STAT3$\beta$ expression in stably transfected HCT8/S11-STAT3$\alpha$ cells (pool, CI 3, and CI 6) and HCT8/S11-STAT3$\beta$ cells (pool 1, pool 2, and CI 9). B, collagen invasion assays done on parental and STAT3$\alpha$- or STAT3$\beta$-transfected colonic cancer cells HCT8/S11 treated for 24 hours with TFF1 (0.1 μmol/L), TFF3 (0.1 μmol/L), or VEGF$\beta$ (100 ng/mL). C, tumor growth assay in nude mice injected s.c. with HCT8/S11, HCT8/S11-STAT3$\alpha$, or HCT8/S11-STAT3$\beta$ cells. *, $P < 0.05$ versus HCT8/S11.
Figure 3. Down-regulation of the transcripts encoding VEGFR and Id-2 in HCT8/S11-STAT3β cells. Semiquantitative RT-PCR analysis of Flt-1, np-1, np-2, and Id-2 transcript levels in the HCT8/S11-pcDNA3 and HCT8/S11-STAT3β cell lines. Basal mRNA level of each gene was normalized to 1. Representative of three independent experiments.

Figure 4. Inhibition by STAT3 RNA interference of STAT3-mediated transcription and cellular invasion induced by TFF1, TFF3, and VEGF165. A, RT-PCR analysis of STAT3 transcript levels in HCT8/S11 cells stably transfected with either the empty pSUPER vector or pSUPER expressing a small interfering RNA targeting STAT3α and STAT3β (siSTAT3pool and siSTAT3cl2). B, Western blot analysis of endogenous STAT3α and STAT3β in whole cell lysates prepared from HCT8/S11-pSUPER and HCT8/S11-siSTAT3-transfected cells (siSTAT3pool and clone 2). C, STAT3-mediated transcription in HCT8/S11-pSUPER (lane 1) and HCT8/S11-siSTAT3 cells (lanes 2 and 3; pool and clone 2, respectively). Cells were transfected with the GAS-luciferase reporter construct. *, P < 0.05 versus control. D, collagen invasion assays done on HCT8/S11-pSUPER and HCT8/S11-siSTAT3 cells treated for 24 hours with TFF1, TFF3 (0.1 μmol/L), or VEGF165 (100 ng/mL).
observed at 1 \( \mu \text{mol/L} \) cucurbitacin. Alternatively, HCT8/S11 cells were pretreated with 1 \( \mu \text{mol/L} \) cucurbitacin for shorter periods (1, 3, or 6 hours), washed, and then seeded on type I collagen in cucurbitacin-free medium for the 24-hour invasion assay. TFF3-induced cellular invasion is totally inhibited by the 3- to 6-hour pretreatment (Fig. 5B). Under these conditions, STAT3 transcriptional activity is partly impaired (Fig. 5C).

We next used a STAT3-derived phospho-hexapeptide corresponding to the region encompassing the Tyr705 residue interacting with the SH2 domain of another STAT monomer (31). This phosphopeptide disrupts the formation of STAT3 dimers, affecting both \( \alpha \) and \( \beta \) isoforms. As expected, the STAT3 inhibitory peptide inhibited both TFF3- and VEGF-induced HCT8/S11 cell invasiveness (Fig. 5D).

**Impact of Cucurbitacin on HCT8/S11 Cell Survival, Proliferation, and Growth of HCT8/S11 Tumor Xenografts.** Inhibition of STAT3 activity by pretreatment of HCT8/S11 cells for 1 to 6 hours with cucurbitacin (1 \( \mu \text{mol/L} \)) is associated with a very weak induction of apoptosis from 3.7\% (control HCT8/S11 cells) to 5.3\% to 7.8\% in cucurbitacin-treated cells and inhibition of cell proliferation (Fig. 6A). Similarly, both thymidine incorporation and HCT8/S11 cell counts indicate that 6-hour pretreatment with cucurbitacin had a modest impact on HCT8/S11 cell proliferation, which was reduced by 37\% to 38\% (Fig. 6B and C). Prolonged treatment of HCT8/S11 cells for 24 hours with 1 \( \mu \text{mol/L} \) cucurbitacin results in a significant induction of cell death (23\% of apoptotic cells) and inhibition of cell proliferation by 93\% (Fig. 6D and E). Consistent with these data, we observed that cucurbitacin (1 mg/kg/d) reduced the growth of HCT8/S11 xenografts in the nude mice (Fig. 6F). When HCT8/S11 tumors grew to 70 to 100 mm\(^3\) volume at day 21 after s.c. injections, athymic nude mice were treated daily with cucurbitacin for an additional period of 3 weeks. Treatment of mice with cucurbitacin (1 mg/kg/d) resulted in a significant decrease (60\%; \( P < 0.05 \)) of tumor volume.

**Discussion**

In the present study, we show that intestinal TTF (TFF3) and VEGF function as regulators of colon cancer cell invasion through STAT3-dependent mechanisms. Both peptides are locally produced and secreted by human colonic cancer cells and are also involved in cell proliferation, survival, invasion, and angiogenesis (9, 11). Our data favor the conclusion that TFF3 and VEGF act through autocrine self-activation loops involving STAT3 signaling. The proinvasive activity of VEGF in the present study is consistent

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*Figure 5.* Inhibition of cellular invasion and STAT3-dependent transcription by cucurbitacin and the STAT3 inhibitory peptide. A, invasion assays done on HCT8/S11 cells incubated for 24 hours with the proinvasive factors TFF1, TFF3 (0.1 \( \mu \text{mol/L} \)), and VEGF\(_{165} \) (100 ng/mL) either alone or combined with increasing concentrations of cucurbitacin (1 nmol/L to 10 \( \mu \text{mol/L} \)). B, invasion assays done on HCT8/S11 cells pretreated for 1-6 hours with cucurbitacin (1 \( \mu \text{mol/L} \)), washed, and then incubated for 24 hours in the presence of TFF3 (0.1 \( \mu \text{mol/L} \)). C, STAT3-mediated transcription in HCT8/S11 cells transfected with the GAS-luciferase reporter construct and pretreated for 1-6 hours in the presence or absence (control) of cucurbitacin (1 \( \mu \text{mol/L} \)). \* \( P < 0.05 \) versus control. D, invasion assays done on HCT8/S11 cells incubated for 24 hours with TFF3 (0.1 \( \mu \text{mol/L} \)) and VEGF\(_{165} \) (100 ng/mL) either alone or combined with the STAT3 inhibitory peptide (0.1 mmol/L).
with the expression of several VEGFR in HCT8/S11 human colonic cancer cells, including Flt-1, np-1, and np-2. Other human colorectal cancer cells such as HT-29 and PCMsrg cells also express Flt-1 and neuropilins. Neuropilins np-1 and np-2 are receptors for the angiogenic and proinvasive factor VEGF165 (32). Although neuropilins bind VEGF, their short intracellular tail does not allow signal transduction. Cellular effects of neuropilins are mediated through their association with other membrane-bound proteins. Np-1 binds both Flt-1 (VEGFR-1) and KDR (VEGFR-2), whereas np-2 binds Flt-1 to transduce VEGF signals (33, 34). These VEGFR are not detected in normal colonic epithelial crypts (35), suggesting that their ectopic induction is connected with the neoplastic progression in the colorectal mucosa.

In human colorectal cancer cells, both TFF3 and VEGF are connected to STAT3 signaling as proven by their ability to induce phosphorylation and transcriptional activation of STAT3. This conclusion is also supported by the ability of several STAT3 inhibitors, including STAT3β, STAT3 interfering RNAs, and curcumin, to reverse the invasive phenotype determined by TFF and VEGF. In contrast, no phosphorylation of STAT3 was induced by TFF1 in response to external addition of the TFF1 peptide and forced expression of TFF1. Interestingly, TFF1-induced cellular invasion was also abolished by inhibition of STAT3 signaling. One can postulate that the invasive phenotype promoted by TFF1 is STAT3 dependent, although TFF1 does not directly activate STAT3. Moreover, both TFF3 and VEGF genes are directly up-regulated by STAT3 (22, 36). It is possible that TFF3 activates STAT3 phosphorylation and transcriptional activity, thereby inducing transcription of its own gene. Earlier reports also indicated that VEGF activates STAT3, which in turn up-regulates VEGF gene transcription. Interestingly, we found that blockade of STAT3-mediated transcription by overexpression of STAT3<sup>β</sup> leads to a dramatic decrease in the transcript levels of VEGF-1 (Flt-1) and VEGF coreceptors np-1 and np-2, suggesting that these genes are also functioning as transcriptional targets of STAT3 in human colorectal cancer cells.

Inhibition of STAT3 transcription and consequent loss of VEGFR and coreceptors renders cells insensitive to the addition of VEGF as shown by the blockade of VEGF-induced cellular invasion in HCT8/S11-STAT3<sup>β</sup> cells. Moreover, the powerful action of curcumin against tumor growth in the present study can be related to a direct inhibition of cancer cell proliferation, invasiveness, and tumor neovascularization by adjacent endothelial cells.

Consistent with the observations made by Schaefer et al. (37) regarding the activation of STAT3 by EGF, we found that phosphorylation of the splice variant STAT3β on Tyr<sup>705</sup> on cell stimulation by TFF3 or VEGF is more stable than phosphorylation of the STAT3α isoform. It is conceivable that STAT3α undergoes a more rapid dephosphorylation than STAT3β. Because STAT3α is

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largely predominant (≥90%) over STAT3β in colon cancer cell lines (data not shown), it can be hypothesized that on ligand stimulation both isoforms are phosphorylated and dimerize, producing preferentially STAT3α homodimers for the transcriptional activation of STAT3-dependent genes. Subsequently, after dephosphorylation of STAT3α, the persistence of phosphorylated forms of STAT3β results in the inhibition of STAT3-dependent transcription. Indeed, overexpression of STAT3β in HCT8/S11 cells favors the formation of STAT3β-containing dimers and results in a 50% decrease in STAT3 transcription levels. Consequently, forced expression of STAT3β and depletion of both STAT3α and STAT3β isoforms by RNA interference are associated with reduced cellular invasion or tumor growth. In addition, down-regulation of the Id-2 mRNA levels was observed in the HCT8/S11-STAT3β cell line. The Id-2 gene, a target of the oncogenic Wnt/b-catenin pathway, is found overexpressed in colon cancer (38). Id-2 in turn inhibits the helix-loop-helix transcription factors through heterodimeric interactions (39). The balance between ID proteins and other helix-loop-helix factors determines the commitment to either proliferation or differentiation. Thus, a high level of ID proteins inhibits cell differentiation and promotes proliferation by inducing the G1-S transition. Conversely, depletion of Id-2 in colorectal cancer cells leads to growth arrest (40). It is therefore reasonable to consider that Id-2 down-regulation induced by forced expression of STAT3β can be responsible for the tumor growth inhibition observed here when STAT3 signaling is inhibited.

In conclusion, we propose to consider STAT3 signaling and STAT3-regulated genes as potential molecular targets for the design of new therapeutic agents against colon cancer progression. The anticancer activity of drugs and RNA interference vectors targeting TF3 and VEGFR signaling should be reexamined in view of their potential impact on human colonic cancer cells themselves besides their classic actions on morphogenesis of the tumor vasculature, cancer growth, and metastasis.

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References

**STAT3, TFF3, VEGFR in Colon Cancer Cells**

In the article on *STAT3, TFF3, VEGFR in colon cancer cells* in the January 1, 2005, issue of *Cancer Research* (1), the first and last names of the contributors were transposed. The list of authors should have read Christine Rivat, Sylvie Rodrigues, Erik Bruyneel, Genevieve Piétu, Amelie Robert, Gerard Redeuilh, Marc Bracke, Christian Gespach, and Samir Attoub. The affiliation for Drs. Rivat, Rodrigues, Redeuilh, Gespach, and Attoub is now designated as INSERM U673. The affiliation for Dr. Bracke should have been Laboratory of Experimental Cancerology, University Hospital, Gent, Belgium. The correct corresponding author information is as follows: Dr. Samir Attoub, INSERM U673, Hôpital Saint–Antoine, 75571 Paris Cedex 12, France. Phone 33-1-49284648; Fax: 33-1-44749318; E-mail: attoub@st-antoine.inserm.fr.

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