Oncogenic events regulate tissue factor expression in colorectal cancer cells: Implications for tumor progression and angiogenesis

Joanne L. Yu¹, Linda May¹, Vladimir Lhotak¹, Siranoush Shahrzad², Senji Shirasawa³, Jeffrey I. Weitz¹, Brenda L. Coomber², Nigel Mackman⁴, and Janusz W. Rak¹,*

¹ Henderson Research Centre, Experimental Thrombosis Research, McMaster University, Hamilton, ON, Canada
² Department of Biomedical Sciences, University of Guelph, Guelph, ON, Canada
³ Department of Pathology, International Medical Center of Japan, Tokyo, Japan
⁴ Departments of Immunology and Vascular Biology, The Scripps Research Institute, La Jolla, CA, USA

* To whom correspondence should be addressed at: Henderson Research Centre, 711 Concession Street, Rm. 216, Hamilton, ON, Canada, L8V 1C3. E-mail: jراك@thrombosis.hhscr.org.

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ABSTRACT

Tissue factor (TF) is the primary cellular initiator of blood coagulation, and a modulator of angiogenesis and metastasis in cancer. Indeed, systemic hypercoagulability in cancer patients and TF overexpression by cancer cells are both closely associated with tumor progression, but their causes have been elusive. We now report that in human colorectal cancer cells, TF expression is under control of two major transforming events driving disease progression (activation of K-ras oncogene and inactivation of the p53 tumor suppressor), in a manner dependent on MEK/MAPK and PI3K. Furthermore, the levels of cell-associated as well as circulating (microvesicle-associated) TF activity are linked to the genetic status of cancer cells. Finally, RNA interference experiments suggest that TF expression is an important effector of the K-ras dependent tumorigenic and angiogenic phenotype in vivo. Thus, this study establishes a causal link between cancer coagulopathy, angiogenesis and genetic tumor progression.
INTRODUCTION

Cancer is believed to arise and progress towards increasing malignancy as a result of cumulative genetic ‘hits’ sustained by the tumor cell genome. Paradigmatic in this regard is the development of colorectal carcinoma (CRC), where sequential transition through clinical stages of the disease is paralleled by a series of well-characterized alterations in proto-oncogenes and tumor suppressor genes\(^1\). In this tumor type, activation of mutant K-\(\text{ras}\) and subsequent inactivation/loss of \(\text{p53}\) are key changes, which drive many interrelated aspects of the malignant phenotype including aberrant mitogenesis and survival\(^2\). Moreover, both of these genetic alterations are thought to contribute to proangiogenic properties of affected cancer cells\(^3\); \(^4\), and thereby enable them to exploit the host vascular system to advance malignant growth and metastasize \textit{in vivo}\(^5\).

The involvement of the vascular system in malignancy encompasses not only angiogenesis, but also systemic hypercoagulability. Blood clotting abnormalities are detected in up to 90\% of patients with metastatic disease, and thrombosis represents the second most frequent cause of cancer-related mortality\(^6\). Cancer coagulopathy is often linked to upregulation of tissue factor (TF), the primary cellular initiator of the blood coagulation cascade\(^7\); \(^8\). Interaction of coagulation factor VIIa (fVIIa) with TF on the cell surface leads to activation of factor X (fXa) and generation of thrombin, with subsequent involvement of platelets and formation of a fibrin clot\(^9\). Remarkably, as a member of the class II cytokine receptor family, TF is also capable of transducing intracellular signals and regulating gene expression\(^10\); \(^11\). Interestingly, elements of the coagulation/fibrinolytic system in general\(^12\), and TF in particular,
have been implicated in regulation of angiogenesis\textsuperscript{13, 14}, as well as tumor growth\textsuperscript{15} and metastasis\textsuperscript{16} in various experimental settings. This is consistent with the observed upregulation of TF in human malignancies, and its elevation with advancing disease\textsuperscript{17, 18}. For instance, in human colorectal cancer (CRC), TF positivity correlates with clinical stage, histological grade, poor prognosis, and vascularity\textsuperscript{19-21}. Collectively, these observations suggest that TF is not only an important element of cancer-related coagulopathy, but is also a correlate and indeed a likely determinant of malignant behaviour of tumor cells.

In this context two main questions remain unanswered. First, what causes the upregulation of TF in human cancer cells, with subsequent increase in their malignancy? Second, how are the consequences of TF expression related to the phenotypic changes induced by underlying ‘cancer causing’ genetic alterations? Here we report that in CRC, TF expression by cancer cells is under directly linked to their genetic status, e.g. activation of the K-\textit{ras} oncogene, and loss of \textit{p53} are involved in TF regulation. These transforming alterations influence the level and activity of TF not only on the cell membrane, but also on the surface of microvesicles shed by cancer cells into the circulation. We therefore suggest that both local and systemic hypercoagulability in cancer may have a hitherto unappreciated genetic cause or component. Finally, we demonstrate that TF is required for expression of the K-\textit{ras} dependent tumorigenic and angiogenic phenotype of CRC cells \textit{in vivo}, but not for cellular transformation \textit{in vitro}.
METHODS

Cell lines and reagents.

The human colorectal cancer (CRC) cell lines HCT116 and DLD-1, and their K-\textit{ras} deleted sublines (HKh-2, DKs-8, DKO-1, DKO-3) have been previously characterized\textsuperscript{22}. The \textit{p53}\textsuperscript{-/-} subline of HCT116 (379.2)\textsuperscript{23} was kindly provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore). The 528\textit{ras+mTF} cell line was produced by transfecting transformed murine 528\textit{ras} fibroblasts\textsuperscript{24} with a full-length mouse TF expression vector, to produce cells expressing high levels of mTF (unpublished data). The A549 human non-small cell lung carcinoma cell line was purchased from ATCC. All cell lines were maintained in DMEM (HyClone) with 10\% fetal bovine serum (Gibco BRL/Invitrogen). For determination of cell growth in monolayer culture, cells were seeded into 24-well plates in complete medium. At each time point, cells (3 wells per cell line) were detached by trypsinization and cell number determined using a hemocytometer. The dominant negative H-Ras-N17 mutant expression construct\textsuperscript{25,26} was a generous gift from Dr. Abhijit Guha (University of Toronto, Toronto, ON). PD98059, LY294002 and AG1296 were purchased from Biomol Research Laboratories Inc. CI-1033 was made available by Pfizer Inc., courtesy of Dr. Christian Marsolais. All inhibitor stock solutions were prepared in DMSO.

Flow cytometric detection of cell surface TF and cell sorting.

For detection of surface TF antigen, cells were detached with 2 mM EDTA to obtain a single cell suspension. 1.5x10\textsuperscript{6} cells were washed in PBS with 1\% FBS and 0.1\% sodium azide, and stained for 30 min at 4\degree C with a monoclonal antibody against human TF (American Diagnostica). After
washing, samples were incubated with Alexa Fluor 488 goat anti-mouse secondary (Molecular Probes) for 30 min at 4°C, washed, and fixed in 1% paraformaldehyde before being acquired on a FACScalibur (BD Biosciences). For cell sorting experiments, 2x10^7 HKh-2 cells were stained for TF as described, and a sterile sort performed on a FACStar Plus (BD Biosciences). Gates were set to collect the most TF-positive and TF-negative viable cells; 3.5x10^5 cells from each side of the expression spectrum were collected and cultured in complete media containing penicillin-streptomycin (Gibco BRL/Invitrogen).

**Analysis of RNA and protein expression.**

Trizol (Gibco BRL/Invitrogen) was used to isolate total RNA from cells or homogenized tumor tissue. Northern blotting was performed as described previously24, using VEGF, TF, TSP-1 or TSP-2 cDNA fragments as probes. For Western blotting, cells were lysed with NP-40 lysis buffer (1% NP-40, 10% glycerol, 20 mM Tris-HCl pH 7.5, 137 mM NaCl, 100 mM NaF, 1 mM sodium vanadate, 1 mM PMSF), supplemented with complete protease inhibitor cocktail (Roche). Protein was quantified by Bradford assay (Bio-Rad), resolved by SDS-PAGE and transferred to Immobilon-P membrane (Millipore). Membranes were probed with rabbit anti-human TF IgG (American Diagnostica) followed by goat anti-rabbit secondary antibody (Jackson ImmunoResearch Labs). To confirm equal loading, membranes were probed with anti-ERK1/2 antibody (Upstate). The IMUBIND Tissue Factor ELISA kit (American Diagnostica) was used to quantify TF protein levels in plasma or conditioned medium.
TF activity assay.

TF activity was measured as described previously27. Standard curves were prepared using different dilutions of rabbit brain thromboplastin (Thromboplastin C Plus; Dade Behring), and 1 U of TF activity was defined as the activity of the 1/10^4 dilution of thromboplastin standard. Cell number was used to normalize the TF activity. Detection of TF activity in conditioned media was performed similarly, by adding the TBS reaction mixture to 100 µL of media (or supernatant, or resuspended microvesicles after centrifugation).

Animals and tumor analysis.

All in vivo experiments were performed in 6-8 week-old SCID mice (5-10 per group; Charles River). Briefly, 1 to 5x10^6 CRC cells were injected s.c. in 0.2 mL PBS. Blood was collected from mice by cardiac puncture, into 1/10 vol 3.8% sodium citrate. Platelet-free plasma was prepared by centrifugation at 2000 x g for 15 min, 2000 x g for 5 min, and 16000 x g for 5 min and stored at -80°C until use. Tumor growth was monitored by measurements with Vernier calipers, and tumor volume (mm³) estimated using the standard formula (length x width² x 0.52). Animal studies were approved by the Animal Research Ethics Board at McMaster University.

Preparation of microvesicles from conditioned media.

Cells were washed and fresh complete medium added for 24 h. Conditioned medium was removed and centrifuged to eliminate debris (500 x g for 15 min, then 800 x g for 20 min), and then ultracentrifuged for 90 min at 4°C (100 000 x g) to pellet microvesicles, which were resuspended in PBS.
Immunohistochemistry.

Tumors were excised and fixed in 10% formalin. Deparaffinized 4 µm sections were stained with hematoxylin and eosin (H&E) for analysis of gross morphology, or immunostained for TF after microwave antigen retrieval. Sheep anti-human TF IgG was used as primary antibody (Affinity Biologica ls Inc., Ancaster, Canada), followed by Alexa Fluor 488 donkey anti-sheep IgG (Molecular Probes).

K-ras PCR-RFLP.

Mutations in codon 13 of the K-ras gene were detected by PCR-RFLP analysis. DNA was extracted using a DNeasy Tissue Kit (QIAGEN Inc.). PCR was performed in a reaction volume of 50 µL containing 250 ng DNA, 1X reaction buffer, 0.2 mM dNTP, 1.5 mM MgCl₂, 0.2 µM of each primer, and 2.5 units of Taq polymerase (Invitrogen). As described in Schimanski et al., the primers were RAS A (sense) 5’-ACTGAATATATAACTTTGTGGTGTCATGGAGCT-3’ and RAS B (antisense) 5’-TTATCTGTATCAAAGAATTGCCTGCACCA-3’. Amplification was performed in a thermocycler (Progene, Techne Ltd.), and consisted of 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. Two rounds of PCR were performed to obtain a very clean 166 bp product. Ten µL of the PCR reaction was digested with XcmI (10 U; New England Biolabs) for 20 hours at 37°C, in a total volume of 40 µL. When codon 13 is wild type, the PCR product contains a restriction site for XcmI, and digestion yields bands of 138 and 28 bp. If there is a mutation in either of the first two bases of codon 13, the mutant PCR fragment will not be cut by XcmI, and will remain at its original size of 166 bp. Bands were visualized by electrophoresis on a 3.5% agarose gel. DNA extracted from the whole blood of a healthy donor was used as the wild-type K-ras codon 13 GGC-Gly control. DNA from HCT116 cells
(heterozygously GAC mutated in codon 13)\textsuperscript{29} was used as the control for known K-\textit{ras} mutation. Amplified products were confirmed by sequencing.

**TF promoter activity assays.**

Cells grown in 6-well plates were transiently transfected with the dominant negative H-Ras-N17 plasmid (1.5 µg) and 0.5 µg pGL2-hTF (full length: -2102 to +121 TF promoter sequence cloned into the pGL2 luciferase reporter plasmid), using Lipofectamine 2000 (Invitrogen). Empty pcDNA3.1 vector was used as a control for H-Ras-N17 transfection. LacZ expression vector (pcDNA3.1-LacZ, 0.25 µg) was co-transfected to monitor transfection efficiency. Luciferase activity was determined 24 hours after transfection using the Luciferase Assay System (Promega) and TD-20/20 luminometer (Turner Designs), according to supplied protocols. Alternatively cells transfected with pGL2-hTF were treated with PD98059 (50 µM) or LY294002 (20 µM) for 2-48 hours prior to read out. Values were normalized to the β-galactosidase activity measured in the lysates.

**TF gene silencing by siRNA.**

The siRNA expression plasmid (pSN-TF) was constructed by annealing and cloning the following siRNA oligonucleotides into the pSuppressorNeo (pSN) vector (Imgenex, San Diego, CA): 5’-TCGAGGCAGCTCCAGGCACACTAACATTCAAGAGATTTGTAGTGCTGAAGCGC TTTTT-3’ (forward) and 5’-CTAGAAAAAGCGCTTCAGGCACACTACAAAATCTCTTGAGATTG GTAGTGCTGAAGCGC-3’ (reverse). These oligonucleotides utilize a previously validated siRNA sequence\textsuperscript{30}, and encode the sense siRNA sequence followed by a short spacer, the reverse complement of the sense strand, and a transcriptional termination signal. Thus, transcription of
pSN-TF generates hairpin RNA that are processed into siRNA in the cell. As a control, oligonucleotides incorporating a siRNA sequence known to be inactive in TF silencing\textsuperscript{30} were used to construct a control expression vector (pSN-77): 5’-TCGAGTGGAGACCCCTGCCTGGCCTTCAGAGAGGCCAGGGGTCTCCATTTTT-3’ (forward) and 5’-CTAGAAAAATTGAGACCCTGCTGGCCTCTCTTTGAAGGCCAGGGGTCTCCA-3’. HCT116 cells were transfected with pSN-TF and pSN-77 using Lipofectamine 2000 (Invitrogen), and clones screened for integration of the siRNA sequence by PCR (of genomic DNA) using the following primers: 5’-AATACGTGACGTAGAAAGTA-3’ (forward) and 5’-CACATGTGATATCCGTGCAG-3’ (reverse). TF gene downregulation in positive clones was then assessed by Northern blotting and TF activity assay.

**Matrigel Assay.**

HCT116, SI-2 and SI-3 cells were rendered mitotically incompetent by treatment for 2 hours with 10 µg/mL mitomycin C (Sigma), washed, and resuspended in Matrigel (BD Biosciences) at a concentration of 4x10\textsuperscript{6} cells/mL. A total of 0.2 mL of the mixture was injected subcutaneously in the flanks of SCID mice. As a negative control, cell-free Matrigel alone was injected. Plugs were excised after 11 days, placed in Carnoy’s fixative (60% methanol, 30% chloroform, 10% glacial acetic acid) for 4 h, then transferred to ethanol. Paraffin-embedded specimens were cut and stained with hematoxylin and eosin (H&E), or immunostained for von Willebrand Factor (vWF). The primary antibody was rabbit anti-human vWF (1:300, Dako), followed by goat anti-rabbit secondary antibody (1:1000, Zymed Laboratories), and colour was developed with DAB (Dako). Angiogenic response was quantified by measurement of the area occupied by vWF-positive endothelial networks per 20X field. A total of 5 fields were analyzed from 5 sections of
5 different Matrigel plugs per group. Image capture and analysis was performed using Northern Eclipse Software.

**Statistics.**

Data are presented as means of several independent measurements ± SD. Statistical analyses (t test) were performed with GraphPad InStat software (GraphPad Software, Inc., San Diego, CA). Linear regression analysis was performed with Origin 6.1 (OriginLab Co., Northampton, MA).
RESULTS

Impact of K-ras and p53 on TF expression by human colorectal cancer cells.

Clinical progression of colorectal cancer is paralleled by sequential alterations in K-ras and p53 genes, as well as by increasing TF immunoreactivity. Two independent human colorectal cancer (CRC) cell lines, DLD-1 and HCT116, both harbor one mutant (and one wild-type) K-ras allele and express elevated levels of TF. To determine whether mutation of the K-ras oncogene is directly linked to TF expression in CRC, we examined the consequences of selective disruption of the mutant K-ras allele by homologous recombination in previously characterized cellular variants. This procedure produced the DKs-8 and DKO-3 sublines (from DLD-1), as well as the HKh-2 subline (from HCT116), all of which consequently express only the wild-type K-ras allele and a diminished level of malignancy. DKO-1 cells were derived from DLD-1 after accidental disruption of the wild-type rather than the mutant K-ras allele (Figure 1).

Cell surface TF immunoreactivity was greatly diminished in cells without mutant K-ras (DKs-8, DKO-3 and HKh-2), relative to their respective isogenic, but mutant K-ras-expressing DLD-1 and HCT116 counterparts (Figure 2A and B). TF mRNA, total TF protein, and cell surface TF activity were all downregulated upon mutant K-ras disruption (Figure 2C-E). These results suggest that TF upregulation in CRC cells is directly linked to expression of the activated mutant K-ras oncogene.

During CRC progression, activation of K-ras is frequently followed by loss-of-function mutations in the p53 tumor suppressor gene. Therefore, we next investigated whether p53 status
impacts TF expression in cells representative of more advanced CRC. HCT116 cells harbor two wild-type $p53$ alleles, both of which have been disrupted in a previously generated variant (designated 379.2)$^{23}$. Thus, 379.2 cells recapitulate natural loss-of-function alterations (LOH) affecting this tumor suppressor in CRC. As with K-ras mutation, loss of $p53$ in 379.2 cells was associated with a marked further increase in cell surface TF activity, TF protein and mRNA relative to their parental $p53^{+/+}$ HCT116 counterparts (Figure 2B-E). It is noteworthy that the TF profile of $p53^{-/-}$ HCT116 (379.2) cells (which harbor mutant K-ras) reflects the cumulative impact of both transforming alterations (K-ras and $p53$), i.e. TF expression is progressively upregulated with these two sequential oncogenic events (HKh-2 < HCT116 < 379.2).

**Impact of oncogenes on circulating TF levels.**

To explore whether upregulation of TF in tumor cells translates into systemic release of this procoagulant, TF antigen concentration in the plasma of tumor-bearing mice was measured using a human-specific TF ELISA. Indeed, human (tumor-derived) TF was detected in the plasma of mice with HCT116 tumors, at a concentration corresponding with increasing tumor volume (Figure 3A). The plasma TF level in mice with extremely large tumors was somewhat reduced, possibly reflecting tumor necrosis and inefficient tumor perfusion. More importantly, the level of circulating human TF was significantly higher in mice bearing highly TF-positive 379.2 ($p53^{-/-}$) tumors, relative to mice with similarly sized HCT116 ($p53^{+/+}$) tumors; poorly tumorigenic HKh-2 cells were not included in this analysis (Figure 3B). Thus, the $p53$-dependent increase in TF expression in 379.2 cells translated into a higher level of TF in the circulation. Plasma of mice harbouring large murine fibrosarcomas engineered to express high levels of mouse TF (528ras+mTF) did not contain any human TF immunoreactivity detectable by this
ELISA, and neither did plasma of tumor-free animals (Figure 3B). Hence, essentially all circulating TF antigen detected in mice harbouring HCT116 and 379.2 tumors is attributable to tumor cell-derived TF.

Although this particular assay does not offer insight into TF procoagulant activity related to either tumor or host cells, such activity was detected in conditioned medium from HCT116 cells, as well as from its mutant K-ras and p53-disrupted sublines (Figure 3C). As with cell bound TF, the secretion of cell-free TF activity corresponded with the genetic status of the respective CRC cells (i.e. K-ras<sup>wt</sup>/p53<sup>wt</sup> < K-ras<sup>mut</sup>/p53<sup>wt</sup> < K-ras<sup>mut</sup>/p53<sup>mut</sup>). Surprisingly, all CRC cells expressed only minimal amounts of the recently described, soluble splice variant of TF (asHTF; data not shown)<sup>31</sup>, and instead most of the released cell-free TF activity could be isolated (pelleted) from the conditioned medium by ultracentrifugation (Figure 3C). As viable cells are known to actively shed plasma membrane-derived vesicles (also called microvesicles or microparticles) that may contain procoagulant activity<sup>32-35</sup>, this result is consistent with the ability of CRC cells to shed TF-containing microvesicles both into the media and the circulation. Indeed, such structures were observed under electron microscope in supernatants from CRC cell lines (data not shown). Again, the amount of cell-free vesicle-associated TF activity released by each cell line was proportional to their total TF production, which was dictated by their oncogenic status (Figure 3C). These data suggest that K-ras and p53 influence not only cell surface TF expression, but also the global amount of TF activity released from cancer cells into their surroundings and the circulation.
The link between TF expression and aggressiveness of cancer cells in vivo.

Low TF expressing, mutant K-ras negative HKh-2 cells are poorly tumorigenic in vivo (Figure 4A). Interestingly, HKh-2 tumors that eventually arise and assume more aggressive properties were found to display increased TF mRNA expression (50 days post cell injection), relative to their counterparts maintained in culture (Figure 4B, top). As analysis of early stage (day 7) HKh-2 tumors by TF immunostaining revealed the presence of extremely rare, but highly TF-positive cells among the TF-negative majority (Figure 4C-E), we examined whether this TF-positivity might be a signature of a cell subset with an overt growth advantage, which would subsequently contribute to the gradual increase in overall tumor aggressiveness in vivo. First, HKh-2 cells were stained with a TF antibody, and cell sorting was performed to collect the fractions with the 5% highest and lowest TF expression; these were designated ‘TF-positive’ (TF-POS) and ‘TF-negative’ (TF-NEG), respectively. Both cell subsets were expanded in vitro and analyzed for expression of TF mRNA and tumor forming ability in SCID mice. In this setting, the TF-NEG cell subset was poorly tumorigenic in SCID mice, while TF-POS cells grew more aggressively (Figure 4F), a finding that suggests close co-segregation between TF expression and malignancy within a single tumor cell line.

One obvious way by which genetically unstable HKh-2 cells (K-ras$^{del/\ast}$) could reacquire increased aggressiveness is by re-expression of a dominant genetic lesion, e.g. mutation in the remaining wild-type K-ras allele$^{36}$. Indeed, using a PCR-RFLP assay to detect mutations in K-ras, we found that HKh-2 cells expressed mostly wild-type K-ras allele, as expected. In contrast, HKh-2 cells isolated from tumors that eventually emerged in SCID mice contained a new K-ras mutation (Figure 4B, bottom). Moreover, analysis of TF-POS and TF-NEG cells collected from
two independent sorting experiments (including the one depicted in Figure 4F) revealed that even in culture, the more aggressive TF-POS subpopulation of HKh-2 cells (expressing high TF levels) is enriched in ‘revertants’ expressing mutant K-ras (Figure 4G). In contrast, low TF-expressing and poorly tumorigenic TF-NEG cells continue to express the wild-type K-ras allele. These results suggest that either TF is a consistent molecular ‘marker’ of greater malignancy (e.g. K-ras mutation), or a more direct effector of the malignant phenotype, at least in the context of mutant K-ras.

To reinforce the role of mutant ras in upregulation of TF expression, we examined the effect of expressing dominant inhibitory Ras-N17 mutant on TF promoter activity in Ras-driven cells. Even transient transfection of the Ras-N17 construct into HCT116 and DLD-1 CRC cells and A549 lung carcinoma cells diminished TF promoter activity relative to cells transfected with a control plasmid (Figure 4H). Expression of the dominant negative Ras-N17 mutant also decreased TF promoter activity in HKh-TUM2 cells (Figure 4H), which were isolated from HKh-2 tumors (50 days after cell injection) and found to be revertants harbouring de-novo K-ras mutation (see Figure 4B). Moreover, pharmacological inhibition of the MEK1/MAPK pathway (by PD98059) downstream of Ras resulted in similar inhibition of TF promoter activity in both HCT116 and 379.2 cells. Collectively, these results substantiate a causal role of the activated Ras pathway in upregulation of TF in CRC cells (both with and without defects in p53) and point to at least partial contribution of a transcriptional mechanism in this process (Figure 4B).
Regulation of TF expression in colorectal cancer cells downstream of mutant K-ras.

To further investigate some of the potential downstream events involved in TF upregulation by mutant K-ras, we treated HCT116 cells with a panel of pharmacological inhibitors. Again, selective inhibition of MEK1 (and MAPK pathway) with PD 98059 resulted in decreased TF protein levels (Figure 5A). Interestingly, blockade of PI3K activity also led to greatly diminished TF expression, to levels comparable to those of wild type K-ras-expressing Hkh-2 cells (compare Fig. 1 and Fig. 5). In contrast, treatment of HCT116 cells with a pan-ErbB inhibitor (CI-1033) only slightly decreased TF expression, while a PDGFR signaling inhibitor (AG1296) or vehicle (DMSO) had no effect. Similar results were observed following treatment of 379.2 cells with the same inhibitors (Figure 5B). These data suggest that the PI3K and MAPK pathways both contribute to upregulation of TF in CRC cells expressing mutant K-ras even when this effect is amplified by the simultaneous loss of p53.

Effect of TF gene silencing on HCT116 growth and angiogenesis in vivo.

To determine the extent (if any) to which TF expression may be required for K-ras driven tumorigenicity, we used a RNA interference (RNAi) approach to knock down TF expression in HCT116 cells. Previously validated small interfering RNA (siRNA) sequences were used to construct a TF siRNA expression plasmid suitable for generation of permanent cell lines, in order to achieve the long-term effects necessary for in vivo studies. Several TF siRNA-expressing HCT116 clones integrated the siRNA sequence (Figure 6A inset), and exhibited diminished TF activity and expression (Figure 6A and data not shown). Three of these clones (SI-2, SI-3, and SI-9) were selected for in vivo studies on the basis of their stable TF-suppressed phenotype.
While the *in vitro* growth properties of HCT116 cells were unaffected by downregulation of TF even in hypoxic or spheroid cultures (Figure 6B and data not shown), the growth of tumors established from TF-suppressed clones was markedly retarded relative to parental HCT116 cells (Figure 6C). This effect was highly specific as growth of HCT116 cells transfected with an expression plasmid encoding a siRNA sequence known to be ineffective in TF gene silencing were indistinguishable from parental HCT116 (data not shown). Since TF status only affected the behaviour of HCT116 cells *in vivo*, i.e. in a manner that was likely host-dependent, this could be indicative of the pro-angiogenic role of TF in the context of mutant K-*ras* driven tumorigenicity of these CRC cells. Hence, we assessed the global capacity of a fixed number of HCT116 cells or their TF-deficient variants to recruit host endothelial cells into Matrigel plugs *in vivo*. Cells were treated briefly with mitomycin C to prevent changes in cell number, resuspended in Matrigel, injected subcutaneously into mice, and plugs recovered 11 days later. Quantification of vWF-positive vascular structures by morphometry indicated a consistent, and statistically significant, 3-fold difference in the pro-angiogenic potential of HCT116 cells relative to their TF-downregulated SI-2 and SI-3 counterparts (Figure 6D-F). In SI-2 and SI-3 plugs, vWF-positive vasculature was found merely at the outer margins and rarely contained red blood cells (Figure 6E). Plugs consisting of Matrigel alone showed only minimal background invasion (Figure 6F). Because HCT116 cells ultimately depend on mutant K-*ras* for their tumorigenic and angiogenic properties, these results raise the possibility that TF is required for the expression of the angiogenic phenotype driven by this oncogene.
In keeping with this possibility, transcripts encoding two angiogenesis inhibitors (and oncogene targets), thrombospondin 1 (TSP-1) and 2 (TSP-2), were found to be expressed at much higher levels in TF downregulated cell lines than in their parental HCT116 counterparts (Figure 6G and H). Interestingly, this difference was only observed in vivo (and not between the respective cell lines in culture). This raises a possibility (but does not prove) that tumor-derived TF may interact with host-dependent ligands (e.g. fVIIa) to execute the ‘pro-angiogenic switch’ in tumors harbouring mutant K-ras. Expression of VEGF in HCT116 cells was minimally affected by their TF status (Figure 6I). Collectively, these results suggest that at least a portion of K-ras-dependent angiogenic activity expressed by colorectal cancer cells in vivo may be mediated through TF upregulation and its multiple downstream angiogenic targets exemplified by TSP-1 and -2 (Figure 6J).
DISCUSSION

The intriguing properties of TF, which acts as a procoagulant and proangiogenic cellular receptor in various pathological contexts, have recently been a subject of intense interest\textsuperscript{13}. Indeed, TF is consistently upregulated in human cancer cells\textsuperscript{13} but the causes and mechanisms of this change have remained unknown. In this regard our study led to several new findings.

First, our results suggest that TF is a target of at least two of the most common genetic alterations in human malignancy: inactivation of $p53$, and mutation of K-$ras$. Moreover, action of these respective transforming events appears to be cumulative in nature. Thus, in a unique and well characterized series of isogenic human colorectal cancer (CRC) cell lines engineered genetically to recapitulate two distinct steps in genetic tumor progression\textsuperscript{1; 22; 23}, activation of mutant K-$ras$ and subsequent inactivation of $p53$ upregulated TF expression and activity, in a cooperative manner. There is a remarkable similarity between this stepwise increase in TF levels and the reported pattern of gradual increase in TF immunoreactivity in clinical specimens of human colorectal cancer\textsuperscript{19-21}, as well as the increasing frequency of systemic coagulopathy in associated with advanced malignancy\textsuperscript{6; 37}. Both K-$ras$ and $p53$ likely act at the level of TF gene expression (rather than deencryption) as TF mRNA, total protein, immunoreactivity and procoagulant activity (both cell-associated and released in membrane vesicles) were affected in a parallel fashion by the genetic status of CRC cells. Taken together, our observations suggest the possibility that TF upregulation and hypercoagulability in cancer patients may have a hitherto unappreciated genetic cause.
The second major point presented in our study is that TF upregulation is not merely a correlate (‘marker’) or consequence of oncogenic alterations, but also an important effector of K-ras-dependent tumorigenesis and angiogenesis, at least in human CRC. This finding causally links two hitherto separately studied solitudes, namely the coagulation system and genetic tumor progression. In particular, tumor growth, angiogenesis and metastasis have long been attributed, at least in part, to both canonical and non-canonical TF activities, including generation of thrombin, deposition of fibrin, and activation of proangiogenic platelets. In some of these instances, elevated TF expression in cancer cells elicited such cellular responses as constitutive upregulation of VEGF, downregulation of TSP-2, and increased metastatic and angiogenic capacity.

Such TF-induced changes resemble those described previously in the context of mutant oncogenes (e.g. K-ras). Our present results indicate that this is not merely a coincidence, as we find TF expression to be required for full manifestation of the K-ras dependent angiogenic and aggressive tumor cell phenotype in vivo (but not in vitro). Thus, enforced TF downregulation in K-ras expressing colorectal cancer cells by RNA interference profoundly reduced global angiogenic capacity, led to increased levels of angiogenesis inhibitors (TSP-1 and TSP-2), and diminished growth of tumors in vivo, without affecting the transformed phenotype and mitogenic properties of the cancer cells themselves (in vitro). While TF does not seem to possess any overt cell-autonomous transforming or growth regulatory properties in this setting, it clearly possesses the ability to modify tumor cell behaviour in vivo, likely through interaction with host-derived entities. The nature of the latter remains unknown at present but the likely candidates include fVIIa, fXa and other putative TF ligands (e.g. plasminogen, TFPI). Regardless of the
molecular mechanism, the tumor-inhibitory effects of TF downregulation highlight the possible validity of targeting TF in the context of K-ras driven human tumors, such as pancreatic, colorectal and lung cancer. In this context, TF-dependent regulation of the angiogenic phenotype in cancer cells must be distinguished conceptually and mechanistically from the role TF appears to play in the context of angiogenic host endothelial cells. The latter was recently linked with signaling properties of the TF cytoplasmic domain.

At variance with earlier reports, we have not observed any significant link between the status of TF in HCT116 cells and expression of VEGF in vivo or in vitro. It is possible that VEGF in the context of HCT116 cells may be under TF-independent regulatory control (e.g. by mutant K-ras itself), a notion consistent with the pleiotropic and tumor/oncogene-specific nature of proangiogenic gene expression profiles characterized to date. This pleiotropism appears to extend to angiogenic mediators downstream of TF, as our findings suggest that TF is linked to the regulation of at least two different endogenous angiogenesis inhibitors (TSP-1 and TSP-2). A more complete survey of TF-dependent and TF-independent effectors of oncogene-driven tumor angiogenesis is warranted, using approaches of functional genomics and proteomics.

While many influences (including inflammation, hypoxia and other) could contribute to TF upregulation by cancer cells, our findings suggest that oncogenic events play an important and hitherto unappreciated role in this regard. It follows that oncogene-targeted therapies might be useful in reversing this increase. Unfortunately, no potent, specific and proven pharmacological modulators of K-ras and p53 status in the context of CRC are currently available. In contrast, recent clinical successes have been achieved with inhibitors of ErbB
receptor kinases. We have observed that in A431 epithelial carcinoma cells, the malignant properties of which are mainly driven by overexpression/activation of EGFR, both EGFR/ErbB1 and pan-ErbB inhibitors markedly diminished TF expression and reduced the levels of cancer cell-derived (human) TF in the circulation of tumor-bearing mice (unpublished data). It is worth considering whether TF levels in biopsy material or plasma of patients receiving certain oncogene-directed agents could provide a measure of the agent’s biological, and possibly also therapeutic efficacy.

Due to technical considerations (xenograft model), our experiments did not provide direct evidence of the contribution of tumor cell associated TF, and its oncogenic upregulation, to the overall hypercoagulability associated with cancer (i.e. with inclusion of host-dependent and inflammatory events). However, an observation that all-trans retinoic acid (ATRA) attenuates coagulopathy in patients with acute promyelocytic leukemia (APL) serves as a thought-provoking precedent, as ATRA acts essentially by blocking the oncogenic action of the PML/RARα gene product on the malignant phenotype of APL cells.

Collectively, TF expressed by cancer cells appears to act as both a regulatory target, and an important mediator of oncogene-driven tumor growth and neovascularization. Future studies will determine the extent to which targeting and monitoring TF expression may be useful, from a diagnostic, prognostic and therapeutic standpoint. A better understanding of the oncogenic defects driving malignant progression will likely provide important clues in this regard.
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FIGURE LEGENDS

Figure 1. Genetic changes associated with colorectal cancer (CRC) progression. The status of model CRC cell lines (HCT116 and DLD-1) with respect to changes in K-ras and/or p53 genes is indicated, in relation to the accepted model of CRC progression.

Figure 2. TF expression in cancer cells parallels genetic tumor progression – impact of K-ras and p53 status. Flow cytometric analysis of surface TF antigen levels present on (A) DLD-1 or (B) HCT116 CRC cells, and their respective sublines created by selective disruption of mutant K-ras or p53 by homologous recombination (+, wild-type allele; mut, mutant allele; del, disrupted allele). Analysis of TF mRNA and protein expression in these cells by (C) Northern and (D) Western blotting. (E) Cell surface TF activity of HCT116 sublines parallels TF antigen expression.

Figure 3. Oncogenic status of tumors influences circulating TF levels. (A) Circulating TF is detectable in the plasma of HCT116 tumor-bearing mice, and plasma TF concentration is proportional to tumor volume. Asterisked points were excluded from the linear regression. (B) Higher plasma TF concentrations are observed in mice with tumors originating from the p53−/− variant of HCT116 cells (379.2 cells), relative to those with HCT116 tumors of a similar size. The TF ELISA detects human (but not mouse) TF. Tumor volume (mean ± SD) for each group is indicated. (C) The cell-free TF activity produced by HCT116 and sublines is associated with pelleted membrane vesicles after ultracentrifugation.
**Figure 4. TF upregulation co-segregates with increased malignancy of CRC cells.** (A) Growth of HCT116 and variant sublines in SCID mice. (B) Elevated TF mRNA expression was observed in HKh-2 tumors 50 days after injection of cells *in vivo* (*top* and *middle*). PCR-RFLP assay for detection of K-ras codon 13 mutation (*bottom*). The HKh-2 cell line used to establish tumors displayed a faint mutant K-ras band, suggesting the presence of rare revertant cells that sustained codon 13 mutations in the remaining undisturbed (wild-type) K-ras allele. Such cells were enriched in HKh-2 tumors, as indicated by the bright mutant K-ras band. TF immunohistochemical staining of (C) HKh-2 tumor, (D) HCT116 tumor, and (E) 379.2 tumor. Only rare, single TF-positive cells (arrows in C) were observed in small early stage (day 7) HKh-2 tumors, while the greatest TF positivity was observed in 379.2 tumors, consistent with the levels of TF protein and mRNA expressed by these cells *in vitro*. (F) After sorting of cultured HKh-2 cells on the basis of TF positivity/negativity and injection into SCID mice, TF-positive (TF-POS) HKh-2 cells grew faster than the original HKh-2 cell population *in vivo*, while TF-negative (TF-NEG) HKh-2 cells were relatively non-tumorigenic. TF Northern blotting confirmed the purity of the sort (*inset*). Results from one of two independent sorting experiments are depicted. (G) The TF-POS cell subpopulation is enriched in revertant cells that express mutant K-ras, as indicated by the increased intensity of the mutant K-ras band (relative to the wild-type band). (H) Expression of dominant inhibitory Ras-N17 in mutant ras-expressing HCT116, HKh-TUM2, DLD-1, and A549 cells diminishes TF promoter activity relative to controls. (I) Marked attenuation of TF promoter activity by pharmacological inhibition of MEK1 (PD98059, 50 µM) downstream of Ras. This effect was observed in CRC cells harboring mutant K-ras (HCT116) and their derivatives with deleted p53 (379.2).
Figure 5. Downstream pathways regulating TF expression in CRC cells. (A) TF protein levels in mutant K-ras dependent HCT116 cells were diminished by selective inhibition of the PI3K and MAPK pathways using LY 294002 and PD 98059, respectively. Specific inhibition of EGFR and ErbB2 kinases with CI-1033, a pan-ErbB inhibitor, slightly decreased TF levels, while PDGFR inhibition (AG1296) had no effect. (B) Similar results were observed in 379.2 cells, which have altered p53 function in addition to mutant K-ras. Inhibitor concentrations used were as follows: AG1296 (20 µM), CI-1033 (10 µM), PD 98059 (50 µM), and LY 294002 (20 µM).

Figure 6. Effect of TF gene silencing on HCT116 tumor growth and angiogenesis. (A) TF siRNA-expressing clones (SI-2, SI-3, and to a lesser extent SI-9) demonstrate decreased cell surface TF activity consistent with TF gene knockdown. Presence of integrated TF siRNA sequence in the clones was confirmed by PCR (inset). (B) In vitro growth curves for HCT116 and the TF siRNA-expressing clones were found to be similar in monolayer culture. (C) In contrast, TF RNA interference results in diminished in vivo tumor growth of HCT116 tumors in SCID mice. (D) After 11 days in vivo, robust formation of vWF-positive vascular networks was observed in Matrigel plugs containing HCT116 cells. (E) Endothelial cell infiltration was noticeably reduced in plugs containing equivalent numbers of TF-deficient SI-3 cells. (F) The area occupied by vWF-positive vascular networks was significantly lower in Matrigel plugs containing TF siRNA-expressing cells (after 11 days in vivo), relative to plugs containing parental HCT116 cells (*P<0.0001; area was expressed as a percentage of the total area of a 20X field). No significant host cell invasion or vWF-positivity was detected in pellets consisting of Matrigel alone. Northern blotting of tumors derived from TF-downregulated SI-3 cells revealed
significantly increased expression of two angiogenesis inhibitors, (G) TSP-1 and (H) TSP-2, relative to their parental HCT116 counterparts, while expression of VEGF (I) was only slightly decreased in SI-3 tumors. Numbers indicate intensity of band relative to 28S rRNA loading control. (J) The working model that illustrates how TF may participate in mediating the K-ras dependent angiogenic phenotype, in part by deregulating expression of angiogenesis inhibitors TSP-1 and TSP-2.

References


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Figure 5
Oncogenic events regulate tissue factor expression in colorectal cancer cells: implications for tumor progression and angiogenesis

Joanne L Yu, Linda May, Vladimir Lhotak, Siranoush Shahrzad, Senji Shirasawa, Jeffrey I Weitz, Brenda L Coomber, Nigel Mackman and Janusz W Rak