Twist Is Required for Thrombin-Induced Tumor Angiogenesis and Growth

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Abstract

Twist, a master regulator of embryonic morphogenesis, induces functions that are also required for tumor invasion and metastasis. Because thrombin contributes to the malignant phenotype by up-regulating tumor metastasis, we examined its effect on Twist in five different tumor cell lines and two different endothelial cell lines. Thrombin up-regulated Twist mRNA and protein in all seven cell lines. Down-regulation of Twist in B16F10 tumor cell lines led to a ~3-fold decrease in tumor growth on a chorioallantoic membrane assay and ~2-fold decrease in syngeneic mice. Angiogenesis was decreased ~45% and 36%, respectively. The effect of Twist on angiogenesis was further examined and compared with the effect of thrombin. In studies using a Twist-inducible plasmid, several identical vascular growth factors and receptors were up-regulated ~2- to 3-fold in tumor cells as well as human umbilical vascular endothelial cells by both Twist as well as thrombin (vascular endothelial growth factor, KDR, Ang-2, matrix metalloproteinase 1, GRO-α, and CD31). Thrombin-induced endothelial cell chemotaxis and Matrigel endothelial cell tubule formation were similarly regulated by Twist. Thus, thrombin up-regulates Twist, which is required for thrombin-induced angiogenesis as measured by endothelial cell migration, Matrigel tubule formation, and tumor angiogenesis. [Cancer Res 2008;68(11):4296–302]

Introduction

The transcription factor Twist is a master regulator of embryonic morphogenesis (1–6). In various organisms, Twist is necessary for proper cell migration (1, 4, 5) and tissue reorganization during embryonic development (2–5), functions that are also required for tumor invasion and metastasis (7). It has been suggested that Twist contributes to spontaneous metastasis by promoting an epithelial-mesenchymal transition. This is a process in which epithelial cells lose polarity and cell-to-cell adhesion, which leads to remodeling of the cytoskeleton as well as cell migration. Twist has recently been reported to be responsible for metastasis in inducing a spontaneously metastatic mammary carcinoma cell line (8, 9). Ectopic expression of Twist inhibited E-cadherin–mediated cell-cell adhesion, while activating mesenchymal markers and cell motility. It also up-regulates microRNA-10b, which positively regulates cell migration, invasion, and metastasis (9). It has been speculated that Twist expression might be derepressed during tumor progression. Indeed, high levels of Twist expression correlate with invasive human breast lobular carcinoma, invasive gastric carcinoma, prostate cancer, and metastatic nasopharyngeal carcinoma—tumor types associated with loss of E-cadherin expression (8, 10–12). High levels of Twist also correlate with other cancers in which E-cadherin was not measured (11, 13, 14).

The association of deep vein thrombosis and cancer is well-documented (15). However, the role of thrombosis and thrombin in propagation of the promalignant phenotype has not been fully delineated or appreciated. This promalignant role of thrombin has been attributed to its stimulation of tumor adhesion (16–19), growth (20), metastasis (16, 17, 21–23), and angiogenesis (24–34). Because both Twist and thrombin share similar effects on tumor metastasis, we looked for a relationship between the two. We first examined the effect of thrombin on up-regulation of Twist and found it to be elevated in five different tumor cell lines as well as endothelial cells from two different sources. This prompted more extensive examination of its effect on the thrombin-activated promalignant phenotype. Thrombin up-regulation of Twist contributed to tumor growth, migration, and angiogenesis.

Although the role of thrombin in neoangiogenesis is well-documented, the precise roles of the various vascular growth factors and inhibitors have not been clearly defined. Vascular regulatory proteins and growth factors, particularly matrix metalloproteinases (MMP-1, MMP-2, and MMP-9), vascular endothelial growth factor (VEGF) and its KDR receptor, angiopoietin-1 (Ang-1) and Ang-2, and their Tie-2 receptor are required for angiogenesis. Thrombin-induced angiogenesis in a chorioallantoic membrane (CAM) is associated with up-regulation of the major VEGF (29) as well as Ang-2 (29). Thrombin also up-regulates VEGF in fibroblasts and tumor cells (24, 30) and the major VEGF receptor KDR in endothelial cells (28), and induces secretion of VEGF (31, 32) and Ang-1 (33) from platelets. Thrombin up-regulates Ang-2, MMP-1, and MMP-2 in endothelial cells (25, 34). However, the mechanisms responsible for thrombin-induced up-regulation of these genes have not been established.

In this report, we show that (a) thrombin up-regulates Twist mRNA and protein; (b) Twist enhances tumor cell growth and angiogenesis; (c) Twist enhances endothelial cell angiogenesis; (d) Twist up-regulates vascular growth factors and receptors; and (e) Twist regulates thrombin up-regulation of angiogenesis and vascular growth factors and receptors.

Materials and Methods

Reagents. All reagents were obtained from Sigma Co. unless indicated otherwise. Antibodies against VEGF, KDR, ANG-2, Tie-2, GRO-α, CD31, β-actin, and tubulin were purchased from Santa Cruz Biotechnology, Inc. Antibody to MMP-1 was purchased from Chemicon. Human recombinant GRO-α was purchased from Biosource International. Control IgG was purchased from Pierce. Growth factor–reduced Matrigel was purchased from Becton Dickinson.
Cell lines and culture conditions. Human breast carcinoma cell line MCF-7 and murine B16F10 melanoma and breast 4T1 cells were purchased from American Type Culture Collection. UMCL is an undifferentiated mouse cell line from our laboratory. All cell lines were maintained in DMEM (Sigma) supplemented with 10% fetal bovine serum (FBS; Life Technologies), 2 mmol/L L-glutamine, and penicillin-streptomycin. Human umbilical vascular endothelial cells (HUVEC) were obtained from Cambrex Bioscience and were maintained in EBM-2 (Cambrex Bioscience) supplemented with 2% FBS and growth factors (singlequots) according to the instructions of the manufacturer. Human brain microvascular endothelial cells (HBMEC) were provided by Dr. Jorge Ghiso (New York University Medical Center, New York, NY). All cells were grown at 37 °C in 5% CO2. Tumor cells were starved for 17 h and HUVECs for 4 h in the absence of FBS before incubation with agonists.

CAM tumor/angiogenesis assay. The CAM was prepared as previously described (29). Briefly, 10-day-old chick embryos were used. The CAM was separated from the shell membrane. B16F10 cells were added to the CAM and examined at day 7 for tumor weight and angiogenesis. The angiogenesis index was quantified from frozen sections with immunohistochemical staining of CD31. The number of branching blood vessels was counted in five random fields of each CAM and the mean obtained. Each of the four experimental categories was performed on three CAMs. Results are mean ± SE of the angiogenic index.

Munir tumor angiogenesis assay. Tumor nodules were fixed, embedded in paraffin, and stained immunohistochemically for Von Willebrand Factor (VWF) as well as CD105 as described (35). The entire tissue section was examined, and areas with the highest intensity of staining (i.e., hotspots; ref. 36) were identified and further examined under high power magnification (×400) for VWF. The number and intensity of staining of small vessels in the hotspots were scored in a “blinded” fashion. An angiogenesis index was obtained by multiplying the number of vessels by the intensity of staining (scored 1–3).

Endothelial cell cord formation. Matrigel (100 μL) was added to a 96-well plate and left to polymerize for 1 h at 37 °C. HUVECs were trypsinized and resuspended in EBM-2, 2% FBS with or without thrombin, hirudin, or Twist knockdown (KD) cells. One hundred microliters were added to the top of the Matrigel and incubated for 24 h at 37 °C. Tumor cells were starved for 17 h and HUVECs for 4 h in the presence or absence of FBS.

Endothelial cell culture in vitro. HUVECs were grown to near confluence, starved for 2 h in the presence of FBS, and then incubated for 48 h in the presence or absence of thrombin.

Chemotaxis assay. Transwell plates (obtained from Costar 3422, Corning, Inc.) were used to measure tumor chemotaxis. Cells were grown to 75% confluence in 10% bovine serum albumin (BSA)-DMEM culture medium for 24 h, followed by 0.1% BSA-DMEM for an additional 4 h. Cells were then trypsinized, washed with PBS, resuspended in 0.1% BSA-DMEM, and 200 μL (5 × 104 cells) were added to the upper chamber. The lower chamber contained 0.1 unit/ml thrombin or PBS in 600 μL of 0.1% BSA-DMEM. Plates were incubated at 37 °C, 5% CO2 for 3 h. Inserts were removed, washed with PBS, and then stained with crystal violet for 10 min. Cells and solutions had been removed from the inside of the insert. Excess stain was removed from the bottom of the insert by swabbing with a cotton-tipped applicator and then allowed to dry. Destaining was performed in 10% acetic acid for 10 min. The solution was then transferred to a 96-well plate, and absorbance was read at 595 nm. Relative migration refers to the absorbance reading obtained from the stained cells. Similar results were obtained with microscopic enumeration of stained cells.

Tet-on plasmid constructs. Both Twist and GRO-α Tet-on cell lines were prepared in the same way, except for their PCR primers. The Twist primers were selected to generate a 970-base fragment, which was eluted from a 10% agarose gel electrophoresed to separate the band. Forward primer 5′-ccctgctgccacccc-3′ and reverse primer 5′-aacaacagaggtcttacagc-3′ were used. The fragment was then ligated into a PCR cloning vector (pGEM T-Easy Vector) by prior digestion with BamHI/NotI and then transfected into a competent bacterial cell (DH-5α) grown in Luria-Bertani medium. The plasmid was then extracted, digested, and electrophoresed to obtain a Twist DNA fragment and then ligated into a PTE-tight vector to generate the PTE-twist plasmid. A pTet-on cassette vector containing the Neo resistance gene (Clontech Laboratories) was then used to develop a stable B16F10 cell line. The PTE-Twist plasmid was cotransfected with a linear Hygromycin marker into B16F10 cells, which were selected with Hygromycin and G-418. Doxycycline was used to turn on transcription.

Knockdown of Twist in B16F10 cells and HUVEC by shRNA. B16F10 or HUVEC shRNA was introduced into the SIRNA-RetroQ retrovirus (BD Biosciences) at the BamHI and EcoRI ligation sites according to the directions of the manufacturer. shRNA oligonucleotides were derived from the murine and human Twist sequence and synthesized after derivation from the computer program supplied by BD Biosciences. Successful inhibitory shRNAs were discovered.

The following sequences were used to knock down mouse and human Twist:

Mouse strand sequence: 5′-GATCCAGCTGACAGATCAGCTCTTTTTTTTTGG-3′
Plus complementary strand: 5′-AAATTCAAAAAAGCTGACAGATTCAAGAGGCTTCTTGAGTTCTTCTTCTTTTTTT-3′

Mouse scrambled sequence as control: 5′-GATCCGAGTTAGCCTAAACCAAGATGACAGATCAGCTCTTTTTTTTTGG-3′
Plus complementary strand: 5′-AAATTCAAAAAAGCTGACAGATTCAAGAGGCTTCTTGAGTTCTTCTTTCTTTTTTTTT-3′

Human strand sequence: 5′-GATCCGAGTCAAGCTGAGCAAGATTCAAGAGGCTTCTTGAGTTCTTCTTTTTTTTTGG-3′
Plus complementary strand: 5′-AAATTCAAAAAAGCTGACAGATTCAAGAGGCTTCTTGAGTTCTTCTTTCTTTTTTTTTTTTT-3′

The two paired oligonucleotides were annealed to form double strands. Verification of the inserted sequence was obtained by automated DNA sequencing from the Core laboratory at New York University Medical Center. The plasmids were packaged into Phoenix Amphi cells (BD Biosciences) by standard calcium phosphate transfection. Virus supernatants were collected at 48 h posttransfection, centrifuged to remove non-adherent cellular and cellular debris, and frozen in small aliquots at −80 °C. B16F10 cells were seeded at 20,000 cells per well in 24-well plates. The following day, the culture medium was aspirated and replaced with retroviral supernatant diluted 1:2 DMEM in to a final volume of 1 mL in growth medium (DMEM) plus 10% FBS and penicillin-streptomycin. Polybrene was added to a final concentration of 4 μg/mL. Twenty-four hours after infection, the cells were collected by trypsinization and reseeded in 6-well dishes in selective medium + 1 μg/mL puromycin. Single-cell colonies were picked and reseeded with selective medium. Cell viability was verified by propidium iodide stain exclusion measured by Flow Cytometry, ~90% viable.

Traditional reverse transcription-PCR (RT-PCR) and real-time RT-PCR were done to validate KD of Twist mRNA as well as to measure the effect of thrombin on these cells. iCycle iQ real-time PCR (iScript one-step RT-PCR) with SYBR green was purchased from Bio-Rad and performed according to the instructions of the manufacturer.

Immunoprecipitation and Western blotting. HUVECs were starved for 4 h. Medium was then removed and 5% BSA plus agonist added for experimental studies. Tumor cells were starved for 17 h and agonist added for an additional 24 h. Total cell extracts were prepared after removal of medium and lysing of cells by lysis buffer containing 1% Triton X-100, 150 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L EGTA, 2.5 mmol/L sodium PPI, 1 mm β-glycerol phosphate, 50 mmol/L Tris-HCl (pH 7.5), 10% glycerol, 1 mmol/L NaVO3, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 μg/mL leupeptin. After centrifugation, the supernatants
(1 mg/mL) were incubated with 3 μL of antibody (200 μg/mL) overnight at 4°C, followed by the addition of 30 μL protein A/G beads (0.5 mL/mL; Santa Cruz Biotechnology), and further incubated at 4°C for 2 h followed by centrifugation. The immune complexes (beads) were washed thrice with lysis buffer and then suspended in 50 μL of SDS-PAGE loading buffer. The washed, suspended beads were boiled at 95°C for 5 min, chilled at 4°C, and centrifuged. Thirty microliters of supernatant were then run on 10% SDS-PAGE under reducing conditions and transferred to a nitrocellulose membrane. Membranes were incubated with the appropriate antibody (1 μg/mL) for 1 h at room temperature, washed, and incubated with horseradish peroxidase–conjugated secondary antibody for another hour and developed by enhanced chemiluminescence (Amersham Biosciences).

Results

Up-regulation of Twist mRNA and protein by thrombin. There is now abundant evidence that both thrombin (15) and Twist enhance the malignant phenotype in immortal cell lines as well as clinically with respect to prognosis and invasiveness. Because both thrombin and Twist enhance many of the same promalignant functions on cells, we proposed that thrombin might be acting through the up-regulation of Twist. We therefore determined whether thrombin up-regulates Twist in various tumor and primary cell lines. Figure 1 shows thrombin-induced up-regulation of Twist mRNA for HUVEC, human prostate DU145, Breast MCF7 and murine melanoma B16F10, and undifferentiated UMCL. The thrombin PAR-1 receptor–activating peptide, SFLLRN, also up-regulates B16F10 cells in a similar manner as thrombin, indicating that activation was through PAR-1. Similar results were noted for protein by immunoblot. A similar relationship between thrombin and Twist was noted for protein by immunoblot. Similar results were noted for protein by immunoblot. Similar results were noted for protein by immunoblot.

Twist enhances tumor cell growth. In a preliminary experiment, Twist mRNA was knocked down with shRNA in B16F10 cells and then applied to a CAM to determine whether Twist had any effect on B16F10 tumor growth in this xenograft experimental system. Figure 2A shows successful KD of Twist in B16F10 cells in clones TB6, TB8, and TAB. Figure 2B shows a ~3-fold decrease in tumor weight of TAB KD cells compared with empty vector (EV), scrambled vector (SV), or wild-type (WT) in two representative tumors from each group of seven tumors. WT, EV, or SV versus KD, P < 0.03.

A similar more extensive confirmatory study was performed in a more relevant pathophysiological system in which Twist KD B16F10 cells were injected into syngeneic C57BL/6j mice. Figure 2C shows a 2-fold decrease in tumor volume at 8 to 17 days (P = 0.02), as well as a 2.3-fold decrease in weight on day 17 (P < 0.03). Impaired thrombin-induced chemotaxis of Twist TAB KD B16F10 cells was also noted (−70%; P = 0.02; n = 4), which is consistent with the requirement of Twist for metastasis (8), and similarly reported for GRO-α (35).

Twist is required for B16F10 tumor cell–induced angiogenesis in the CAM and mouse tumor assays. It has been recognized that tumors can contribute to angiogenesis and "self-nourishment" by secretion of vascular growth factors (24, 35), and that thrombin contributes to angiogenesis (24–35, 37). We therefore focused our attention on the effect of Twist and thrombin on angiogenesis because thrombin up-regulates Twist as well as angiogenesis. In a preliminary xenograph experiment, Fig. 3A examines angiogenesis in Twist KD B16F10 cells applied to the CAM. Note the ~45% decrease in angiogenesis after 72 hours for KD versus WT, EV, or SV (P < 0.03; n = 3). Similar results were obtained with a more physiologically relevant experiment, using B16F10 Twist KD cells in syngeneic mice (Fig. 3B). Note the 36% decrease of angiogenesis in KD versus EV cells at 17 days. Real-time PCR of VWF (a surrogate marker for thrombin-induced chemotaxis of Twist TAB KD B16F10 cells was also noted (−70%; P = 0.02; n = 4), which is consistent with the requirement of Twist for metastasis (8), and similarly reported for GRO-α (35).

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![Up-regulation of Twist mRNA and protein by thrombin immunoblot](image)
Twist up-regulates angiogenesis growth factor and receptor mRNA and protein in B16F10 cells. The above data suggested that B16F10 cells might contain inducible vascular growth factors and receptors, known to be present in endothelial cells, which can contribute to tumor-induced angiogenesis. Therefore, a Twist-inducible plasmid was introduced into B16F10 cells, and the up-regulation of various angiogenesis growth factors and receptors was sought and analyzed by immunoblot. Figure 4 shows up-regulation of mRNA and a 2.8 to 3.5 increase in protein for various angiogenesis growth factors and receptors, which include VEGF and its receptor, KDR, Ang-2 and its receptor Tie 2, as well as GRO-α by Twist. Twist induced up-regulation of GRO-α is of particular interest because GRO-α is an angiogenesis/growth factor chemokine shown to be required for thrombin-induced up-regulation of angiogenesis and tumor growth [35].

Requirement of Twist for thrombin-induced up-regulation of angiogenesis growth factor and receptor mRNA and protein in Twist KD B16F10 cells. Because thrombin has been shown to up-regulate many angiogenesis growth factors through the up-regulation of GRO-α, we designed an experiment to test the effect of thrombin on Twist KD B16F10 cells to determine whether thrombin-induced tumor angiogenesis also required Twist in B16F10 cells. Figure 5A shows that Twist is required for thrombin-induced up-regulation of mRNA (~2-fold) for vascular growth factors and receptors: Ang-2, VEGF, KDR, MMP-1, and GRO-α. KD cells were not rescued by thrombin (P < 0.02; KD + thrombin versus EV + thrombin for all factors; n = 5). Similar results were noted for VEGF protein (Fig. 5B).

Twist is required for thrombin-induced angiogenesis with HUVEC. We then studied a more directly relevant angiogenesis primary endothelial cell line (HUVEC) to determine whether Twist plays a similar role in endothelial cells as it does with B16F10 tumor cells.

Because the initial requirements of endothelial cells for neo-angiogenesis entails cell migration and tube formation, we examined the effect of Twist KD in HUVEC in the presence and absence of thrombin. Figure 6A shows successful KD of Twist in HUVEC. Figure 6B is a quantitative analysis demonstrating enhanced chemotaxis with thrombin and ~50% impairment of chemotaxis with Twist KD, indicating that Twist is required for chemotaxis in the absence of thrombin. Thus, the effect of thrombin is in addition to an independent effect of Twist requirement (n = 3). Note that Twist KD was not rescued by thrombin. We next examined Matrigel tube formation in the presence and absence of thrombin. Figure 6C shows a ~5.5-fold impaired vascular tube formation in Twist KD HUVEC compared...
with WT and SV, which were not rescued by thrombin, verifying an independent requirement of Twist for tubule formation in addition to the enhanced thrombin effect. Similar Twist KD results were also noted with a HBMEC line (data not shown).

**Twist is required for thrombin-induced up-regulation of HUVEC vascular growth factors and receptors.** The effect of thrombin on HUVEC was next evaluated in Twist KD HUVEC. Again, the thrombin-induced up-regulation of angiogenesis growth factor and receptor proteins requires Twist (Fig. 6D). Thus, Twist up-regulates the same vascular growth factors and receptors measured in both HUVEC and B16F10 cells, suggesting that this is a general biological effect. Similar up-regulation has recently been reported for GRO-α (VEGF, KDR, Ang-2, MMP-1, CD31; ref. 35).

**Discussion**

These data clearly show a new role for thrombin, the up-regulation of Twist, and a new role for Twist, the up-regulation of angiogenesis. Thrombin up-regulation of Twist mRNA and protein was shown in seven of seven cell lines tested (tumor cells as well as endothelial cells), suggesting that this may be a generalized biological response.

The role of Twist in the up-regulation of angiogenesis and tumor growth was shown with tumor cells as well as endothelial cells and is supported by the following observations noted after the KD of Twist: (a) tumor cell growth on the chick CAM was inhibited ~3-fold and tumor cell growth in syngeneic mice inhibited ~2-fold; (b) tumor cell–associated angiogenesis was similarly...
inhibited; (c) thrombin-induced chemotaxis is impaired; and (d) endothelial cell tubule formation is inhibited. Up-regulation of Twist leads to the up-regulation of mRNA and protein of several vascular growth factors and receptors (VEGF, KDR, ANG-2, Tie 2, MMP-1, and CD31). These observations are further supported by a recent report on increased tumor growth and up-regulation of angiogenesis (detected by magnetic resonance imaging) in MCF-7 xenografts transfected with Twist and injected into severe combined immunodeficient mice (37). They are inconsistent with observations reported by others with 4T1 cells in which tumor growth was not affected in vitro or in vivo (8), suggesting that the effect of Twist on growth could vary with different tumors.

Of particular interest is our recent report on induction of angiogenesis with the chemokine GRO-α in tumor tissue and HUVEC (35). GRO-α, like Twist, also up-regulates angiogenesis in the CAM assay as well as in a syngeneic mouse model, is also up-regulated by thrombin, and is also required for thrombin-induced up-regulation of the same vascular growth factors described for Twist. Of further interest in a preliminary observation is that GRO-α can up-regulate Twist.

We conclude that at least one of the facets of thrombin-induced up-regulation of the malignant phenotype is through the up-regulation of Twist. Twist has been reported to have a strong association with human tumor progression (breast,
gastric, prostate, and nasopharyngeal carcinomas; refs. 8, 10–14. The tumor-related mechanisms of Twist have been reported to stimulate tumor invasion, and metastasis, purportedly via inhibition of E-cadherin– or microRNA-10b–mediated cell-cell adhesion, migration, and invasion. Our present report on Twist up-regulation of angiogenesis contributes to our understanding of the role of thrombin in stimulating tumor-associated angiogenesis as well as other facets of the malignant phenotype.

References
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