Endostatin Binds Tropomyosin

A POTENTIAL MODULATOR OF THE ANTITUMOR ACTIVITY OF ENDOSTATIN*

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The mechanism of action of Endostatin, an endogenous inhibitor of angiogenesis and tumor growth, remains unknown. We utilized phage-display technology to identify polypeptides that mimic the binding domains of proteins with which Endostatin interacts. A conformed peptide (E37) was identified that shares an epitope with human tropomyosin implicating tropomyosin as an Endostatin-binding protein. We show that recombinant human Endostatin binds tropomyosin in vitro and to tropomyosin-associated microfilaments in a variety of endothelial cell types. The most compelling evidence that tropomyosin modulates the activity of Endostatin was demonstrated when E37 blocked greater than 84% of the tumor-growth inhibitory activity of Endostatin in the B16-BL6 metastatic melanoma model. We conclude that the E37 peptide mimics the Endostatin-binding epitope of tropomyosin and blocks the antitumor activity of Endostatin by competing for Endostatin binding. We postulate that the Endostatin interaction with tropomyosin results in disruption of microfilament integrity leading to inhibition of cell motility, induction of apoptosis, and ultimately inhibition of tumor growth.

Angiogenesis, the formation of new blood vessels from existing capillaries, is required for tumors to expand beyond 1–2 mm² in size and is the principal determinant for tumor growth (1, 2). Consequently, antiangiogenic molecules offer promise as novel therapeutic modalities for the treatment of cancer. Endostatin, a 20-kDa cleavage fragment of collagen XVIII first identified in the conditioned medium of hemangiendothelioma cells (3), is a potent inhibitor of angiogenesis and growth in murine models, and recombinant human (rh) Endostatin is currently being evaluated in the clinic (4, 6). Both recombinant murine (rm) and rhEndostatin have been successfully produced and shown to inhibit endothelial cell proliferation and migration in vitro, as well as tumor growth in vivo tumor models (3, 5, 6). In fact, the first demonstration of tumor dormancy was achieved with systemic treatment of rmEndostatin (7).

It is increasingly recognized that alterations in the actin cytoskeleton play a crucial role regulating the proliferation and migration of endothelial cells (8, 9). Tropomyosins are a large family of proteins; at least 20 different isoforms exist that are generated by alternative splicing of a multigene family (Refs. 10, 11 and references therein). They bind the a-helical groove of actin filaments to stabilize actin in the polymerized state directly influencing the integrity of microfilaments and thus play a role regulating reorganization of the actin cytoskeleton. Tropomyosins have been identified in organisms as diverse as yeast and man and are core components of the cell cytoskeleton. Many vertebrate non-muscle cells express between five and eight isoforms of tropomyosin in a tissue-specific manner, leading to speculation that tropomyosin isoforms may have evolved to perform specific functions in the microfilaments of non-muscle cells. Indeed, mutational analysis indicates that tropomyosin isoforms have distinct functions and that they play important roles in a variety of cellular functions, including contraction, cytokinesis, intracellular transport, secretion, motility, morphogenesis, and cell transformation (Ref. 11 and references therein).

The mechanism of action of the antiangiogenic activity of Endostatin is unknown. To elucidate the mode of action of Endostatin, we screened a phage-display library to identify the binding domains of proteins with which Endostatin potentially interacts. Here we present evidence that human tropomyosin isoform 3 (hTM3) shares an epitope with an Endostatin-binding peptide, demonstrate an interaction between rhEndostatin and hTM3 in vitro, and show that rhEndostatin protein binds tropomyosin-containing microfilaments of endothelial cells. We further show that the peptide mimotope of the hTM3 Endostatin-binding site blocks the antitumor activity of rhEndostatin in vivo and suggest that the antitumor activity of Endostatin results from an interaction with tropomyosin-containing microfilaments that leads to inhibition of microfilament function and an induction of apoptosis.

MATERIALS AND METHODS

Screening of the Phage-display and cDNA Libraries—The Ph.D.-C7C, a disulfide-constrained 7-mer phage-display library (New England BioLabs, Beverly, MA), was screened as recommended by the manufacturer. Following a third round of amplification, individual phage were isolated, and the peptide sequences were deduced by DNA sequencing.

Peptide Synthesis and Generation of Anti-peptide Serap—The E37 (CTHWHHKRCGGGS) and control (CSNSDKPKCGGGS) peptides were synthesized, cyclized at high dilution, and purified to at least 95% purity by high-performance liquid chromatography (Infinity Biotech Research and Resource, Aston, PA). The E37 peptide was coupled to hemocyanin from keyhole limpets (Spectra) in the presence of glutaraldehyde and used to immunize New Zealand White rabbits.

cDNA Library Screening—The Lambda ZAP II (Stratagene, La Jolla, CA) bFGF-stimulated HUVEC cDNA library was constructed by directional cloning of oligo (dT)-primed cDNA into EcoRI and XhoI cloning sites. 1.4 × 10⁶ recombinant phage were screened for immunoreactivity to E37 peptide antisera.

Native and Recombinant Proteins—Plasmid p21-1 was excised as a BBlueScript phagemid (as recommended by Stratagene) from a recombinant phage isolated from the HUVEC cDNA library based on its recognition by E37 antisera. DNA sequencing of the cDNA insert of
p21-1 showed it contained the complete hTM3 coding sequence fused in frame downstream of the Lac Z gene. Escherichia coli DH5α cells were transformed with p21-1 and β-lactamase plasmids and crude bacterial lysates were prepared (12). Human cardiac tropomyosin and muscle tropomyosins were purchased from Trichem Resources (West Chester, PA). A chimeric cDNA was generated using overlapping cDNA fragments obtained by reverse transcriptase-polymerase chain reaction (PCR) from total RNA isolated from rat skeletal muscle and human umbilical vein endothelial cell (HUVEC) cDNA (Sigma, St. Louis, MO). PCR products were cloned into pBluescript plasmids and screened by colony hybridization using a 32P-labeled probe containing the E37 peptide sequence.

The cDNA encoding hTM3 cDNA was amplified by PCR using forward primer 596 (5′-ATGCCATATGGCCAGCCATCAAG-3′) and reverse primer 597 (5′-ATGCAACTTTCCATGTTGTTAATCCAG-3′) and cloned into pET21a(+) (Novagen, Madison, WI). BL21 (DE3) cells were transformed with the pET plasmid and grown in Luria-Bertani (LB) broth containing 50 μg/ml carbenicillin (Novagen, Madison, WI). The cells were induced with 5 mM isopropyl-β-D-thiogalactopyranoside, harvested by centrifugation, resuspended in 1× Tris-HCl pH 8, 1 mM NaCl, and heated 45 min at 90 °C. The lysate was cooled to room temperature, centrifuged, and the supernatant was loaded onto a SulfoProlyl-Sepharose Fast Flow column (SP-PF, Amersham Pharmacia Biotech), which had been equilibrated in a 50 mM phosphate buffer, pH 7.5. The proteins were eluted with a linear NaCl gradient, the recombinant (r) hTM3 fractions were acidified to a pH of 2 with trifluoroacetic acid, loaded onto a reverse phase C4 column (Vydac Inc., Hesperia, CA) and eluted with an acetonitrile/trifluoroacetic acid gradient. The fractions containing hTM3 were concentrated and dialyzed against PBS. rhAngiostatin K1–38 and rhEndostatin (6) were produced in P. pastoris and purified to homogeneity.

ELISA—Plates were coated with 5 μg/ml of bovine serum albumin or rhEndostatin and blocked. Dilutions of 21-1 or control lysate were added and incubated for 1 h at 37 °C, followed by incubation with 1:500 diluted anti-tropomyosin TM311 ascites fluid (Sigma). Following incubation in 1:5000 diluted anti-mouse IgG (H+L) conjugated to alkaline phosphatase (Promega, Madison, WI) wells were developed using Blue Phos Phosphate Substrate (Kirkegaard and Perry, Gaithersburg, MD) and the A405 was measured.

Surface Plasmon Resonance (BIAcore)—Interactions between purified soluble rhEndostatin and rhTM3 were evaluated by surface plasmon resonance using the BIAcore 3000 (BIAcore, Piscataway, NJ). The purified rhTM3 was immobilized on the flow cell of a CM-5 BIAcore biosensor chip. The running buffer was 0.1 M HEPES, pH 7.4, 0.15 mM NaCl, and 0.005% polysorbate 20 (v/v). The competition experiments involving soluble rhTM3 were performed using PBS, pH 7.4, as running buffer. All measurements were performed at 25 °C. To correct for differences in bulk refraction all protein preparations were passed over an acidic Prep-Agilent column to remove contaminating proteins. Effects due to bulk shift were subtracted from experimental data.

Fluorescent Staining of Endothelial Cells—To detect the binding of Alexa-labeled rhEndostatin 1×10⁴ HUVEC, HMVEC, and HAEPCs were plated on 1.5% gelatin-coated Nalge Nun Lab Tek II chamber slides (Naperville, IL) and fixed in 10% neutral buffered formalin followed by a methanol wash. The chamber slides were incubated in PBS/0.1% calf serum containing 1:200 Alexa-labeled rhEndostatin solution for 1 h at room temperature. The slides were washed in PBS, coverslips mounted using fluorescent mounting media (Kirkegaard and Perry Laboratories), and cells were photographed under Alexa 488 excitation wavelength. Co-localization studies were performed as above except cells were incubated in PBS/0.1% calf serum containing 1:200 mouse anti-tropomyosin TM311 (Sigma) and 40 μg/ml of Alexa 488-labeled rhEndostatin for 1 h at room temperature. The slides were washed in PBS, coverslips mounted using fluorescent mounting media (Kirkegaard and Perry Laboratories), and cells were photographed under Alexa 488 excitation wavelength.

RESULTS

An Endostatin-binding Peptide Shares an Epitope with hTM3—A cyclic (disulfide bond-constrained) random 7-mer peptide phage-display library was screened to identify peptides that interacted with rhEndostatin (6). The cyclic, disulfide-bonded peptide, E37 (CTHWHHKCGGGS) was found to bind rhEndostatin. A BLAST search of the National Center for Biotechnology Information database failed to identify proteins with any significant homology to the primary sequence of the E37 peptide. To identify proteins that share common epitope(s) with the E37 peptide, rabbit antisera was raised against KLH-coupled E37 peptide and used to probe an FGF-2 stimulated human umbilical vein endothelial cell (HUVEC) cDNA expression library. DNA sequencing of 9 clones recognized by E37 antisera identified seven as being unique cDNAs of different lengths encoding non-muscle human tropomyosin isoforms 3 (hTM3), suggesting that E37 and hTM3 share a common epitope.

A comparison of the primary sequence of hTM3 with that of the E37 peptide failed to reveal significant homology, suggesting that the E37 peptide sequence represents a conformational mimotope of the Endostatin-binding domain of tropomyosin. To confirm that the E37 peptide and human tropomyosin share an epitope, immunoblots of lysate from rhTM3 expressing E. coli (21-1) were probed with either TM311 anti-tropomyosin monoclonal antibody (Sigma) or anti-E37 polyclonal sera (Fig. 1). The anti-E37 sera and TM311 anti-tropomyosin antibody specifically recognized common bands of ~49 and 43 kDa in the rhTM3 expressing λ21-1 lysate whereas neither band was detected in immunoblots of control lysate (Fig. 1). Furthermore, when a lysate of HUVECs was probed with anti-E37 sera or TM311 anti-tropomyosin antibody, a common band of ~38 kDa was identified that co-migrated with E. coli expressed rhTM3 (data not shown). These findings demonstrate that the E37 peptide and hTM3 share a common epitope. The anti-E37 sera however failed to recognize tropomyosins purified from human cardiac tissue, rabbit muscle, or chicken muscle (Fig. 1B).

The amino acid sequences of rabbit muscle tropomyosin and human cardiac tropomyosin each share 86% identity with hTM3, diverging in two differentially spliced exons, exon 6 (amino acids 189–212) and exon 9 (amino acids 258–284). The inability of the anti-E37 sera to recognize either rabbit muscle or human cardiac tropomyosins suggests that the putative Endostatin-binding domain of hTM3 resides within either exon 6 or 9 of hTM3. Because the E37 peptide was identified based on its ability to bind rhEndostatin, the presence of the E37 mimotope may define the specific isoforms of tropomyosin that bind Endostatin.

rhEndostatin and rhTM3 Interact in Vitro—We characterized the interaction between rhEndostatin and tropomyosin by ELISA and showed that rhEndostatin binds rhTM3-expressing bacterial lysate (λ21-1) (Fig. 2). This result provides evidence of a binding interaction between rhEndostatin and rhTM3.

Surface Plasmon Resonance, with the BIAcore 3000, was used to assess the kinetics of rhEndostatin/rhTM3 binding. 3200 resonance units (RU) of purified rhTM3 were immobilized on the surface of a sensor chip via amine coupling and rhEndostatin injected over the chip as analyte. The rhEndostatin showed a dose-dependent increase in RU, demonstrating the real-time binding of rhEndostatin to immobilized rhTM3 (Fig. 3A). The
dissociation constant ($K_D$) for the rhEndostatin/rhTM3 interaction was calculated to be $100 \mu M$ using a steady-state model. When, as a negative control, similar concentrations of rhAngiostatin K1–3 were passed over the rhTM3 chip, no evidence of specific binding to rhTM3 was observed (Fig. 3B), demonstrating the specificity of the rhEndostatin/rhTM3 interaction. To further evaluate the specificity of rhEndostatin/rhTM3 binding, competition experiments using soluble rhTM3 were performed. Soluble rhTM3 competed for the binding of rhEndostatin to a flow cell containing 2000 RU of immobilized rhTM3 in a dose-dependent manner (Fig. 3C), further demonstrating the specificity of the rhEndostatin/rhTM3 interaction.

Endostatin Binding and Tropomyosin Co-localize to Actin Microfilaments—Incubation of formalin-fixed HUVECs, human microvascular endothelial cells (HMVECs), and human aortic endothelial cells (HAECs) with biologically active Alexa Fluor™ 488-labeled rhEndostatin showed that, given access to the cytosolic compartment, rhEndostatin displays a pattern of binding identical to that observed in cells immunostained for tropomyosin or filamentous actin (Fig. 4, A–C). Fluorescently labeled rhEndostatin and anti-tropomyosin antibodies co-localized to HUVEC microfilaments (Fig. 4, D–E). Similar results were obtained using anti-actin antibodies (data not shown). Thus, in fixed cells, Endostatin-binding, tropomyosin, and actin co-localize to microfilament bundles of the cell cytoskeleton. We postulate that Endostatin, which is specifically internalized by endothelial cells (13), binds tropomyosin to inhibit microfilament function, and as a consequence, tumor angiogenesis.

To determine whether the binding of Alexa-labeled rhEndostatin to the HUVEC cytoskeleton was the result of an interaction with tropomyosin competition experiments were performed. A 50-fold molar excess of the E37 peptide, but not the control peptide inhibited the binding of rhEndostatin to HUVEC microfilaments (Fig. 5, A–C), thus demonstrating the role tropomyosin plays in Endostatin binding to the HUVEC cytoskeleton. This observation further confirms the role of the E37 mimotope in the interaction between Endostatin and hTM3.

E37 Peptide Blocks the Antimetastatic Activity of rhEndostatin in Vivo—To further evaluate the biological significance of the interaction between E37 and Endostatin, we assessed the effect of the E37 peptide on the antitumor activity of rhEndostatin. rhEndostatin has been shown previously to inhibit the growth of pulmonary metastases in the murine B16-BL6 experimental metastasis model in a dose-dependent manner (6). Daily administration of 1.5 nmol of rhEndostatin (1.5 mg/
kg/day) inhibited the growth of experimental B16-BL6 lung metastases by greater than 70% when compared with buffer-treated control mice (Fig. 6). The number of metastatic lesions on the lungs of the mice treated with up to 375 nmol/day of E37 peptide in the absence of rhEndostatin was not statistically different from that of the control group, demonstrating that the E37 peptide alone has neither tumor promoting or inhibitory activity in this assay. Co-administration of rhEndostatin with E37 peptide dramatically blocked the tumor growth inhibitory activity of the rhEndostatin (Fig. 6). Co-injection of 1.5 nmol of rhEndostatin at 5 μl/minute for 240 s in the presence of 0, 6, 15, 30, and 45 μM soluble rhTM3 over a flow cell containing 2000 RU of rhTM3. Samples were run in duplicate, the reference cell values subtracted, and the average of each of the 2 curves plotted. The spikes in RU seen at the beginning and end of each injection are due to the lag in flow caused by the fact that the rhTM3 and reference cells are non-adjacent.

Antitumor Activity of Endostatin Is Modulated by Tropomyosin

Fig. 3. Kinetics of the interaction of soluble rhEndostatin with immobilized rhTM3. A, 0, 10, 20, 40, 80, and 160 μM rhEndostatin were injected at 5 μl/minute for 960 s through a biosensor flow cell that had been activated and blocked as a control for bulk refractive index and subsequently through a flow cell to which 3200 RU of rhTM3 had been immobilized. The binding kinetics were recorded, and the differences between the two curves plotted for each rhEndostatin concentration. B, 0, 11.5, 23, and 46 μM rhAngiostatin were injected at 5 μl/minute for 900 s over a flow cell containing 3200 RU of rhTM3 as above. Samples were run in duplicate, the data corrected for bulk shift and the average of the 2 curves plotted. C, injection of 5 μM rhEndostatin at 5 μl/minute for 240 s in the presence of 0, 6, 15, 30, and 45 μM soluble rhTM3 over a flow cell containing 2000 RU of rhTM3. Samples were run in duplicate, the reference cell values subtracted, and the average of each of the 2 curves plotted. The spikes in RU seen at the beginning and end of each injection are due to the lag in flow caused by the fact that the rhTM3 and reference cells are non-adjacent.
An alternative explanation for the inhibitory activity of E37 is that co-administration of peptide with rhEndostatin results in the formation of an insoluble precipitate at the site of injection, leading to reduced bioavailability of rhEndostatin. To test this hypothesis, we measured sera concentrations of rhEndostatin over time following subcutaneous (s.c.) administration of 1.5 nmol of rhEndostatin alone or in combination with either 375 nmol (250-fold molar excess) of the E37 or control peptide. rhEndostatin co-administered with a 250-fold molar excess of E37 peptide had an area under the curve last (AUC last) equal to 85% of that observed when rhEndostatin was administered alone or when rhEndostatin was dosed with 375 nmol of control peptide (Table I). Assuming 100% bioavailability of rhEndostatin following its administration alone or with control peptide, then the bioavailable dose of rhEndostatin resulting from the co-administration of rhEndostatin and E37 peptide would correspond to 1.3 nmol (1.25 mg/kg). Inference from the B16-BL6 experimental metastasis model where a dose titration of rhEndostatin was used to inhibit metastatic growth (6) suggested that a dose of 1.3 nmol would inhibit lung metastases by 77%. In this study, 1.5 nmol of rhEndostatin co-administered with a 250-fold molar excess of the E37 peptide resulted in an 11% inhibition of lung metastasis (Fig. 6). Thus, despite having a lower AUC last, reduction of the activity of rhEndostatin in the B16-BL6 metastasis model by the E37 peptide cannot be attributed to decreased levels of circulating rhEndostatin. We thus conclude that the loss of tumor growth inhibition was caused by inactivation of rhEndostatin that results from the specific interaction of E37 with rhEndostatin.

DISCUSSION

We identified hTM3 as an Endostatin-binding protein. Using an epitope-specific antibody (Fig. 1B) we further demonstrated that the Endostatin-binding epitope of hTM3 is not present in all tropomyosins and consequently that Endostatin-binding is presumably not a characteristic of all tropomyosin isoforms. This finding has broad significance. Endostatin inhibits the growth of tumors by inhibiting endothelial cell function (3, 5, 6). Systemic rhEndostatin therapy was shown to induce a 50%
reduction of intratumoral blood flow whereas other non-tumor affected organs in the same animal were unaffected. Furthermore, other events that require angiogenesis such as wound healing remained unaffected by rhEndostatin (15, 16). These observations suggest that the antiangiogenic effects of Endostatin are tightly controlled. Our finding that Endostatin may only affect specific isoforms of tropomyosin provides an explanation for how this tight control could be achieved and maintained. It is possible that specific isoforms of tropomyosin are expressed and regulated in endothelial cells during tumor angiogenesis. We speculate that these specific isoforms bind Endostatin preferentially leading to potent inhibition of tumor growth. We also postulate that expression of such isoforms of tropomyosin that interact with Endostatin with different affinities and dissociation constants provides the natural homeostatic balance of endothelial cell growth under normal physiological conditions.

The binding of rhEndostatin to immobilized hTM3 displays both rapid rates of association and dissociation and a $K_D$ of $10^{-10} \text{M}$ (Fig. 3A). Similar binding kinetics have been observed between T cell receptors and their cognate ligands on the surfaces of antigen-presenting cells and between the glycosylation-dependent cell-adhesion molecule-1 (GlyCAM-1) and $\alpha$-selectin (17–19). Kinetic analysis of these interactions shows they have relatively low affinities that are also the result of rapid dissociation rates. Such kinetics are suggestive of highly dynamic interactions. It is possible that Endostatin may have higher affinity for an as yet undefined isoform of tropomyosin or that a third, as yet unidentified component is involved in stabilizing the Endostatin/hTM3 interaction in vivo, and that in the presence of this molecule(s) the $K_D$ of binding would be significantly lower.

Whereas pharmacokinetic analysis indicates that parenteral administration of rhEndostatin results in circulating levels in the ng range (6), local concentrations in the tumor microenvironment may be significantly higher. Planar imaging of mammary tumor-bearing rats following i.v. injection of technetium ($^{99m}$Tc) labeled rhEndostatin (100 $\mu$Ci/rat) showed that the tumor could be visualized from 0.5–4 h post-injection. This observation suggests that Endostatin targets the tumor microenvironment and indicates significantly higher Endostatin concentrations at the tumor bed in comparison to circulating levels. These elevated local concentrations may favor the interaction of Endostatin with hTM3 in the tumor vasculature.

Whereas normally located intracellularly, tropomyosin has been localized to the surface of colon epithelial cells in ulcerative colitis (20) but surface expression has not been reported for endothelial cells. This does not eliminate the possibility that tumor endothelial cells more closely resemble damaged colonic epithelium in this regard. Dixeius et al. (13) recently showed that endothelial cells, but not fibroblasts internalized Endostatin by an endocytic pathway. This observation together with well established differences that exist between the endothelial cells of normal and tumor tissues, may explain the anti-endothelial cell activity of Endostatin in vitro and the tumor specificity in vivo. However, the precise mechanism(s) by which endocytosed Endostatin gains access to the cytosolic compartment remains to be elucidated.

For the first time we present evidence that links a mechanism of action to the in vivo antitumor activity of Endostatin. Co-injection of the E37 peptide with rhEndostatin resulted in a dose-dependent inhibition of the antitumor activity of rhEndostatin in the B16-Bl6 experimental metastasis assay (Fig. 6). We postulate that the E37 peptide mimics the Endostatin protein-binding epitope of hTM3 to compete for Endostatin

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binding and consequently inhibits the antitumor activity of Endostatin.

We believe that the antiangiogenic effect of Endostatin is mediated via an interaction with tropomyosin-containing microfilaments. Rearrangement of the actin cytoskeleton has been reported to be characteristic of, and sufficient to induce endothelial cell apoptosis (21). Taken together, the ability of rhEndostatin to induce endothelial cell apoptosis (3, 22–24) and our observations that rhEndostatin binds tropomyosin-containing microfilaments, suggests that Endostatin disrupts microfilament function to initiate apoptosis. Further observations consistent with Endostatin exerting an effect upon the actin cytoskeleton come from an endothelial cell monolayer-wound assay (6, 25). In this assay, cell migration at the wound edge has been correlated with increased incorporation of monomeric actin into filaments and a reorganization of the actin cytoskeleton (26). Endostatin binding to microfilaments may inhibit the cytoskeletal reorganization required for cell migration, leading to an inhibition of migration.

Whereas the mechanism by which the Endostatin and hTM3 interaction may induce endothelial cell apoptosis is poorly defined, tropomyosin and the cytoskeleton have critical roles in cell survival. Ingber and co-workers (27–30) have demonstrated that actin-based microfilaments play a crucial role in cell shape-regulated determination of cell fate and that whereas growth factors and integrin signaling are required for endothelial cell growth, they are not sufficient (31). Progression through the cell cycle is inhibited following disruption of the cytoskeleton or by release of cytoplasmic tension, implicating the actin-based cytoskeleton and its ability to generate tension against integrin-ECM contacts in the regulation of endothelial cell cycle progression and cell fate (29, 32). Thus, we surmise that Endostatin, which has been reported to regulate cell cycle progression in endothelial cells (13, 23), may bind cytoskeletal tropomyosin leading to a release of cytoskeletal tension and subsequent induction of cell apoptosis.

Our data indicate that Endostatin exerts its antitumor and antiangiogenic effects as a result of its interaction with tropomyosin isoforms containing the E37 mimotope. We suggest that this interaction leads to disruption of the actin cytoskeleton, that blocks cell migration and contributes to the induction of apoptosis. Interestingly troponin I, better known for its role in muscle contraction where it forms a regulatory complex with troponin T, troponin C, and tropomyosin, has been identified as a cartilage-derived inhibitor of tumor angiogenesis and metastasis (33). Troponin I is the actin-binding component of the tropomyosin complex, and thus may inhibit endothelial cells via an interaction with microfilaments that disrupts cytoskeletal function. Thus, troponin I and Endostatin may represent a family of naturally occurring antiangiogenic proteins that block microfilament function.

We recognize that the antiangiogenic activity of Endostatin is complex and potentially involves multiple mechanistic pathways, the convergence of which results in the inhibition of endothelial cell migration, proliferation, differentiation, and an induction of apoptosis. Our study has identified tropomyosin as an Endostatin-binding protein, thus providing a mechanism that may explain the antitumor activity of Endostatin. Endothelial cells rapidly internalize Endostatin (13), and we showed here that rhEndostatin and tropomyosin co-localize to the microfilaments of formalin-fixed human endothelial cells (Fig. 4). The biological relevance of the tropomyosin/rhEndostatin interaction to the antiangiogenic activity of Endostatin was demonstrated by blocking antitumor activity of rhEndostatin with a peptide mimotope of tropomyosin. We postulate that the antiangiogenic activity of Endostatin may result, at least in part, from internalization of Endostatin by endothelial cells and its subsequent interaction with tropomyosin; leading to a disruption of microfilament integrity, inhibition of cell motility and induction of apoptosis, and ultimately tumor growth inhibition.

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