Targeting Tumor Angiogenesis with Adenovirus-Delivered Anti-Tie-2 Intrabody

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Abstract

Inhibition of tumor angiogenesis is a promising approach for cancer therapy. As an endothelial cell–specific receptor kinase expressed almost exclusively on the surface of vascular endothelium, Tie-2 has an important role in tumor angiogenesis. To explore the therapeutic potential of blocking Tie-2 receptor–interaction pathway, an adenoviral vector was used to deliver a recombinant single-chain antibody fragment rabbit intrabody (pAd-2S03) capable of inhibition of both mouse and human Tie-2 surface expression. pAd-2S03 was given to mice with well-established primary tumors, either a human Kaposi's sarcoma (SLK) or a human colon carcinoma (SW1222). The intrabody significantly inhibited growth of both tumors (75% and 63%, respectively) when compared with pAd-GFP control-treated tumors (P < 0.01). Histopathologic analysis of cryosections taken from mice treated with pAd-2S03 revealed a marked decrease in vessel density, which was reduced by >87% in both tumor models when compared with control-treated tumors (P < 0.01). In contrast, human Tie-2–monospecific pAd-1S05 intrabody did not affect the growth of tumors, indicating that the antitumor effect of pAd-2S03 was due to the inhibition of tumor angiogenesis in these murine models. Our results show that the Tie-2 receptor pathway is essential for both SLK sarcoma and SW1222 colon carcinoma xenograft growth. The present study shows the potential utility of antiangiogenic agents that target the endothelium–specific receptor Tie-2 for down-regulation or genetic deletion. (Cancer Res 2005; 65(3): 972-81)

Introduction

Collective experimental and clinical studies show that, in order for a solid tumor to grow to a clinically relevant size, an adequate blood supply is required (1). Vascular endothelial growth factor (VEGF) receptors (VEGFR) and their ligand are currently considered the best candidates for treatment of human cancers via angiogenesis inhibition (2). Inhibition of angiogenesis and the resultant tumor growth has been achieved by using specific agents that either interrupt the VEGF/VEGFR interaction or block the VEGFR2 signal transduction pathway (3). However, several studies have shown that not all types of tumors respond to interruption of the VEGF/VEGFR pathway. This suggests that alternative pathways for vascular growth exist and can drive tumor angiogenesis (4, 5). Two structurally related tyrosine kinases with immunoglobulin and epidermal growth factor motifs (Tie-1 and Tie-2) constitute a second group of receptor tyrosine kinases found mainly in endothelial cells and their progenitors (6). Whereas signaling ligands for Tie-1 are not known, angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) are ligands of the Tie-2 receptor (7, 8). In contrast to the VEGFR system, Tie receptors are not required for vasculogenesis. Correct regulation of Tie-2 is required for normal vascular development perhaps via the regulation of vascular remodeling and endothelial cell interactions with supporting pericytes and smooth muscle cells (8–10). Tie-2 is unique among receptor tyrosine kinases in that its ligands, Ang1 and Ang2, apparently have opposing actions on Tie-2 signaling and function (11). Ang1, the activating ligand, blocks increases in vascular permeability induced by inflammatory agents and VEGF (12). This suggests a role for Ang1 in vessel maturation and stabilization. In contrast, Ang2 is required for VEGF-induced corneal angiogenesis (13), suggesting that Ang2 blocks the actions of Ang1 and promotes vascular destabilization and facilitates angiogenesis. This is supported by the fact that Tie-2−/− mice lack proper hierarchical organization of the vasculature and die during embryogenesis (14). Although Tie-2 expression is down-regulated postnatally, it does persist in quiescent adult endothelial cell, and the presence of tyrosyl phosphorylation in the normal vasculature suggests an active role in the maintenance of blood vessels (15). Furthermore, Tie-2 is up-regulated in capillaries during neovascularization processes produced in skin wounds and tumors (16, 17). Several reports have shown that interference with the Tie-2 receptor pathway results in the inhibition of tumor angiogenesis and growth. Such inhibition has been achieved by using specific blocking agents that include soluble dominant-negative receptors (18–21), antisense oligonucleotides (22), RNA aptamers and RNA interference (23, 24), and short synthetic peptides (25). There have been no published reports based on the use of anti-Tie-2 intrabody to inhibit tumor growth and angiogenesis. The potential of intrabodies (i.e., antibodies for intracellular applications) has been exploited in several laboratories (26). The aim has been to neutralize the function of intracellular and extracellular proteins. One widely successful strategy has been the misdirected localization of cell membrane proteins to the endoplasmic reticulum (ER) based on a KDEL tetrapeptide motif (27, 28). Intrabodies present a potent alternative to methods of gene inactivation that target at the level of DNA or mRNA, such as antisense oligonucleotides (29), zinc finger proteins (30), targeted gene disruption, or RNA interference (31). Operating at the post-translational level, intrabodies can be directed to relevant subcellular compartments and precise epitopes on target proteins.
proteins, potentially blocking only one of several functions of an expressed protein.

The goal of our study was to generate an ER-targeted intrabody against Tie-2 to investigate whether a phenotypic knockout of Tie-2 could inhibit tumor cell growth in vivo. To accomplish this, an ER-targeted single-chain antibody fragment (scFv) was generated and a replication-deficient recombinant adeno virus was used for its local delivery in two mouse models of primary tumor growth.

**Materials and Methods**

**Cell Lines.** Human umbilical vein endothelial cells (HUVECs) were purchased from BioWhittaker (Walkersville, MD) and MAECs were produced by W.B. Stalack (Burnham Institute, La Jolla, CA) and maintained in DMEM supplemented with 4 mmol/L L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 1 mmol/L sodium pyruvate, 10% FCS, and antibiotics. Human Kaposi’s sarcoma cell line (SLK) was provided by R. Pasqualini (University of Texas M.D. Anderson Cancer Center, Houston, TX) with permission from S. Levintov-Kriss (Tel Aviv, Israel). Human colon cancer cell line (SW1222) was provided by L.J. Old (Dudwig Institute for Cancer Research, New York, NY). All human cell lines were maintained in RPMI 1640 containing 10% FCS and antibiotics.

**Antibodies and Other Proteins.** The lyophilized recombinant human Tie-2/Fc chimera (330 kDa) and mouse Tie-2/Fc chimera (270 kDa), which contain the extracellular domain of human or murine Tie-2 receptors fused to human IgG Fc via a polypeptide linker, were purchased from R&D Systems (Minneapolis, MN). Biotinylated goat anti-human Tie-2 polyclonal antibodies and biotinylated normal goat IgG were also purchased from R&D Systems. Mouse anti-human/mouse Tie-2 monoclonal antibody (mAb), rat anti-mouse CD31 (platelet/endothelial cell adhesion molecule 1) mAb, APC-conjugated streptavidin, and biotinylated antibody anti-rat IgG polyclonal antibodies were purchased from Pharmingen (San Diego, CA). FITC-conjugated donkey anti-mouse and human IgG polyclonal antibodies, Cy5-conjugated donkey anti-rat IgG polyclonal antibodies, horseradish peroxidase–conjugated goat anti-mouse IgG polyclonal antibodies, and rhodamine red-X–conjugated streptavidin were from Jackson ImmunoResearch Laboratories (West Grove, PA). High-affinity rat anti-hemagglutinin (HA) mAb 3F10 was from Roche Diagnostics (Indianapolis, IN). Mouse anti-HA mAb was from Covance Research Products (Denver, PA). Horseradish peroxidase–conjugated goat anti-human α light-chain polyclonal antibodies and mouse IgG1 mAb were from Southern Biotechnology Associates (Birmingham, AL). Protein A tagged with 10-nm gold was provided by J. Slot (Department of Cell Biology, University of Utrecht, Utrecht, the Netherlands).

Chimeric rabbit/human F(ab) 1S05 and 2S03 against the extracellular domain of Tie-2 were generated as described previously (32).

**Analysis of Tie-2 Binding in ELISA.** Costar 96-well ELISA plates (Corning, Acton, MA) were coated with 100 ng antigen (human or mouse Tie-2 or bovine serum albumin) in 25 μL PBS and incubated overnight at 4°C. After blocking with 150 μL TBS/3% bovine serum albumin for 1 hour at 37°C, 50 μL of 2 μg/mL F(ab) 1S05, 2S03, or control mAb solution was added to each well, and the plates were incubated for 2 hours at 37°C. Washing and detection were done essentially as described (33) using horseradish peroxidase–conjugated goat anti-human α and anti-mouse IgG antibody (diluted 1:2,500 in TBS/1% bovine serum albumin).

**Conversion of a Tie-2-Specific F(ab) 2S03 into a scFv.** Specific oligonucleotide primers were used to amplify VH and VL gene segments from purified phagemid DNA of F(ab) 2S03 (34). VH was amplified with extomseq (5’-GCGGAGGAGGTCACGGTCGAGAAGACGCTACGCGATTG-CATGT-3’) and BJ/JO-BL; VL was amplified with RSCVH3 and HSGC1234-B. Overlap extension PCR was done using primers ext and RSC-B. The resulting overlap-PCR product encoded a scFv in which the COOH-terminal V\_L region was linked to the NH\_2-terminal V\_H region through a peptide linker (SSGGGGSGGGGSGSSBSSS). Control constructs 1S05 and JC7U (against integrin α\_5β\_3) were described previously (35). The scFv encoding sequences were cloned into phagemid vector pComb3X using asymmetrical SfiI sites, and binding activity of the expressed scFv was confirmed by ELISA.

**Construction of pAd-2S03 Adenoviral Vector and Production of Recombinant Virus.** 2S03 scFv coding regions were initially assembled in pBabePuro essentially as described (36). In these constructs, the scFv coding regions were flanked by a human light-chain leader sequence at the 5’ end and a sequence encoding the HA-tag (YPYDVPDYA) and the ER retention signal (KDEL) at the 3’ end. The scFv coding regions were excised by digestion with BamH\_1 and SalI and ligated into pAd-TrackCMV (37). Control adenovirus vectors, pAd-1S05 and pAd-JC7U, encoding 1S05 and JC7U scFvs and empty adenovirus vector, pAd-GFP, were generated as described previously (35). The generation of recombinant adenoviruses was done essentially as described (37). High-titer viral stocks were produced and purified by CsCl banding. Final yields were between 5.3 x 10\_11 and 1.5 x 10\_12 particles/mL. All virus preparations were green fluorescent protein (GFP) corrected (38).

**Flow Cytometry.** Analysis of Tie-2 binding on HUVECs and MAECs was done as described previously (32). Briefly, 100 μL of F(ab) 1S05, 2S03, or control mAb solution at 5 μg/mL in fluorescence-activated cell sorting (FACS) buffer were added to the 10\_6 cells and incubated for 40 minutes at room temperature. Cells were washed once with 200 μL FACS buffer and incubated for 40 minutes at room temperature with 100 μL FITC-conjugated donkey anti-human or anti-mouse IgG antibody diluted to 1:100 in FACS buffer. Cells were washed twice, resuspended in 200 μL FACS buffer, and transferred to FACS tube for analysis in a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

For Tie-2 detection on adenovirus-infected cells, 1.5 x 10\_6 HUVECs were infected with 10 multiplicities of infection (MOI) of adenovirus (~80% of the cells were infected) encoding Tie-2-specific intrabody (pAd-1S05 or pAd-2S03) or with control adenovirus (pAd-JC7U) and binding activity of the expressed scFv was confirmed by ELISA.

**Analysis of Tie-2/Intrabody Colocalization by Immunofluorescence.** For analysis of Tie-2, 2.5 x 10\_6 HUVECs were seeded on collagen-coated LabTek 8-chambered slide (Nalge Nunc International, Naperville, IL) and infected with 50 MOI of adenovirus encoding Tie-2-specific intrabody (pAd-1S05 or pAd-2S03) or with control adenovirus (pAd-JC7U). Forty-eight hours postinfection, HUVECs were fixed and double stained with biotinylated goat anti-human anti-Tie-2 polyclonal antibody and rat anti-HA mAb as described previously (35). Three-color (GFP, rhodamine red-X, and Cy5) three-dimensional data sets were collected with a DeltaVision system (Applied Precision, Issaquah, WA), and images were deconvolved (based on the Argard-Sadat inverse matrix algorithm) and analyzed with softWoRx version 2.5.

**Ultrastuctural Characterization.** For transmission electron microscopy (TEM), adenovirus-infected cells were cultured in 35-mm dishes to 90% confluence and fixed in 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer. After a brief rinse, cells were postfixed in 1% osmium tetroxide, rinsed, incubated in 0.5% tannic acid followed by 1% sodium sulfate, and subsequently dehydrated in graded ethanol series. Cells were embedded in LX112 resin (Ladd Research Industries, Williston, VT), each disc of embedded cells was cut into small pieces and mounted on blank resin blocks, and ultrathin sections were cut en face. Sections were mounted on parlodion-coated copper slot grids, stained with uranyl acetate and lead citrate, and viewed in a Philips CM100 TEM (FEI, Hillsbrough, OR) at 80 kV. Images were documented on Kodak SO-163 film for later analysis.

Immunoelectron microscopy on ultrathin cryosect sections was done as described (39) with minor variations. Cells were cultured as described above, fixed in 4% paraformaldehyde-0.025% glutaraldehyde in 0.1 mol/L
phosphate buffer, and embedded in gelatin. Cryosections (~100 nm) were cut and mounted on nickel mesh grids, and each grid was processed on individual droplets of the solutions [50 mmol/L glycine, 10% FCS in PBS, and primary mouse anti-HA mAb diluted in 5% FCS (controls grids were incubated in an irrelevant mouse IgG1 mAb)] at room temperature, washed in 0.2% FCS, and incubated in protein A tagged with 10-nm gold. Grids were then washed in PBS, fixed in 1% glutaraldehyde in PBS, washed in ddH₂O, and contrasted in uranyl oxalate (pH 7). Each individual grid was then picked up in ice-cold uranyl acetate/methyl cellulose mixture (pH 4). Once dry, the grids were examined on the CM100 microscope and documented as described above.

In vitro Cell Proliferation Assay. A total of 1 × 10⁴ (SLK), 2 × 10³ (HUVEC), 2.5 × 10² (SW1222), or 5 × 10¹ (MAEC) cells per well in a 96-well tissue culture plate (type I collagen-coated 96-well plate for HUVEC) were infected with various amount of different adenoviruses ranging from 0.5 to 500 MOI, and plate was incubated at 37 °C for 64 hours (72 hours for HUVEC) in a humidified CO₂ incubator. [³H]Thymidine (ICN Radiochemicals, Irvine, CA) was added to 0.5 μCi/well (1 Ci = 37 GBq) during the last 16 hours of incubation (24 hours for HUVEC). The cells were frozen at −80°C overnight and subsequently processed on a multichannel automated cell harvester (Cambridge Technology, Cambridge, MA), and the [³H]thymidine incorporated into DNA was determined by a in liquid scintillation β counter (Beckman Coulter, Fullerton, CA). The background was defined by running the same assay in the absence of adenovirus. The inhibition in experiment E was calculated according to the formula: (background − E) / background × 100%. All experiments were done in triplicate.

In vivo Tumor Growth. Confluent cultured tumor cells (SLK and SW1222) were harvested by incubation with 5 mL trypsin solution (0.25%). Viable cells were counted by trypan blue exclusion. Fractions containing >95% of viable cells were used in this study. SLK cells were suspended in PBS, and 0.2 mL (3 × 10⁸ cells per mouse) of the suspension was inoculated s.c. into right flanks of 20 nude mice on day 0. Tumors were allowed to establish for 6 days. On day 7, four groups of five mice were formed, and each mouse received 1.5 × 10⁸ plaque-forming units per mouse (50 MOI per initial tumor load) of adenovirus-encoded intrabody (pAd-2S03, pAd-IS05, or pAd-JC7U) or control adenovirus (pAd-GFP) administrated peritumorally in 70 μL PBS on days 7, 14, 21, and 28. The resulting tumor was measured over the skin in two dimensions using a slide caliper, and the tumor volume was calculated according to the formula: 0.5(width)² × length. All animals were sacrificed after 45 days, and the tumors were completely dissected and then weighed. The same experiment was done with SW1222 tumor cells (2 × 10⁶ cells per mouse), except three injections of 10⁸ plaque-forming units per mouse were administrated on days 7, 14, and 21, and the animals were sacrificed on day 24. Results are reported as means ± SD of each group.

Immunohistochemistry and Microvessel Density Determination. Immunohistochemical localization was done with rat anti-mouse CD31 using a Vectastain Elite Peroxidase kit (Vector Laboratories, Inc., Burlingame, CA). Tumors represented each group of treated animals were freshly frozen in OCT solution and stored at −80 °C. Cryostat sections (6 μm) were cut, fixed in 3.7% paraformaldehyde, and blocked with 0.3% H₂O₂, 5% goat serum, and avidin-biotin blocking reagents (Vector Laboratories). With PBS washes between all steps, mouse endothelial cells were detected with rat anti-mouse CD31 (platelet/endothelial cell adhesion molecule 1) mAb at 0.5 μg/mL (incubation overnight at 4°C) followed by mouse-adsorbed biotinylated goat anti-rat IgG polyclonal antibodies at 5 μg/mL (incubation for 1 hour at room temperature) and visualized using Avidin-Biotin-Peroxidase Complex kit from Vector Laboratories. Peroxidase activity was revealed by the 3,3'-diaminobenzidine (Vector Laboratories) cytochemical reaction. Sections were then weakly counterstained with hematoxylin (Vector Laboratories) and mounted with VectaMount (Vector Laboratories). The microvessel density was determined by digitally imaging five fields of the tumor stroma that showed the highest vascularity [hotspots; at ×100 magnification with a CCD1317 camera (Princeton Instruments, Inc., Trenton, NJ) mounted on an Axiosvert 100 microscope (Carl Zeiss, Inc., Thornwood, NY)] and processing them with Abohe Photoshop 7.0 software (Adobe Systems, Mountain View, CA) as follows: (CD31-stained pixels per field) / (total pixels per field) × 100%. The final count per group represents the average (mean ± SD) of five fields.

Statistics. Tumor volume, tumor weight, and percentage of CD31-positive cells were compared by using two-tailed Student's t test. Differences were considered statistically significant at P < 0.05.

Results

Characterization of Rabbit Antibody Fragments and Binding to Tie-2. F(ab) IS05 and 2S03 were subcloned and overexpressed in Escherichia coli with subsequent purification to yield near-homogenous protein solutions (data not shown). ELISA was used to check for cross-reactivity of rabbit F(ab) IS05 and 2S03 with human and mouse Tie-2. F(ab) IS05 bound only to human Tie-2 and did not reveal any cross-reactivity with mouse Tie-2. In contrast to F(ab) IS05, F(ab) 2S03 was found to bind both human and...
and mouse Tie-2 (Fig. 14). Flow cytometry analysis further revealed that F(ab)2 2503 bound to both HUVECs, which express native human Tie-2 on their surface, and to MAECs, which express a very low level of mouse Tie-2 (Fig. 1B). In contrast, F(ab)1 1505 was able to bind HUVECs but not to MAECs (Fig. 1B), thus confirming previous ELISA results demonstrating cross-reactivity within the Tie-2. It should be emphasized that this anti-Tie-2 cross-reacting rabbit F(ab)2 2503 was generated and selected using human Tie-2 for both immunization and panning (32). Despite using human Tie-2 for immunization, rabbit F(ab)2 2503 binds to the mouse antigen as well. The affinity of F(ab)2 2503 for the human or mouse Tie-2 was determined by surface plasmon resonance. The rabbit F(ab)2 2503 was found to bind human and mouse Tie-2 with a monovalent affinity of 6.8 and 9.6 nmol/L (Supplementary Table S1). The $K_d$ for F(ab)1 1505 for human Tie-2 was described previously and equal to 15 nmol/L (32). The relative ease with which cross-reacting antibodies can be selected points at the potential of the rabbit antibody repertoire as a source for therapeutic antibodies.

**Tie-2 Surface Depletion with Intrabodies.** F(ab)2 2503 was converted into scFv in which the $V_h$ and $V_l$ fragments were covalently linked with a peptide linker consisting of 18 amino acids. Antigen binding was confirmed by ELISA (data not shown). Next, the 2503 scFv against Tie-2 was cloned into a modified adenovirus shuttle vector, pAd-TrackCMV.

HUVECs were infected with 10 MOI of adenovirus encoding Tie-2-specific intrabody (pAd-1505 or pAd-2503) or, as a control, with an intrabody against integrin $\alpha_v\beta_3$ (pAd-JC7U). Surface Tie-2 expression was monitored for 7 days by flow cytometry as described in MATERIALS AND METHODS (Fig. 2A). More than 90% of the infected HUVECs showed GFP expression, suggesting that virtually all infected cells expressed the vector-delivered intrabody (data not shown). At 24 hours postinfection, the surface expression of human Tie-2 on HUVECs infected with the 1505 intrabody was blocked specifically up to 62% (Fig. 2A). On day 2 after infection, Tie-2 expression was reduced by 95%. Seven days after infection, surface expression remained efficiently blocked up to 92%. In comparison, the surface expression of human Tie-2 on HUVECs infected with the 2503 intrabody was only blocked with 25% efficiency on day 1. However, on day 3, Tie-2 surface depletion reached a maximum of 82% and remained >65% inhibition through day 7 postinfection (Fig. 2). No inhibition of Tie-2 expression was observed for the cells infected with JC7U intrabody (Fig. 2). These results underscore the ability of ER-targeted intrabodies 1505 and 2503 to down-regulate the HUVEC surface receptor Tie-2.

**Colocalization of Intrabody and Tie-2 Protein in the ER.** To investigate whether the intrabody constructs were expressed in the ER and able to trap the targeted proteins in the same compartment, HUVECs were infected with the adenovirus-encoding intrabodies (pAd-1505, pAd-2503, and pAd-JC7U; 50 MOI). As a control, HUVECs were infected with a vector alone (pAd-GFP). Representative data from immunogold electron microscopy, which is the technique of choice for precise subcellular localization, revealed that on day 3 after infection most of the 10-nm gold particles were present in the ERs of 1505, 2503, and JC7U pAd-infected HUVECs (Fig. 3B). No signal was detected in the nucleus (data not shown). On the other hand, the control infection with the vector alone (pAd-GFP) did not reveal any ER staining.

**Intrabodies Effect on Tumor Cell Proliferation.** We next sought to evaluate general cytotoxicity from adenoviral infection and the cytototoxicity related to scFv expression in ER. For this analysis, HUVEC, MAEC, SW1222, and SLK cells were treated in vitro with adenovirus encoding the ER-directed anti-Tie-2 scFv (pAd-1505 and pAd-2503), anti-\(\alpha_v\beta_3\) scFv (pAd-JC7U), or an adenovirus encoding the GFP reporter gene (pAd-GFP). Adenoviral vector-infected cells were examined for viability using the proliferation assay described in MATERIALS AND METHODS. Using this assay, it was observed that scFv accumulation in the ER results in an elevated inhibition of proliferation in all the cell lines tested when compared with control pAd-GFP (Fig. 4). The most sensitive cell line was HUVEC, where a MOI of 5 resulted in a $>20\%$
inhibition of proliferation. Colon carcinoma cell line SW1222 was less affected by intrabody accumulation in the ER, with 32% to 58% inhibition observed at a MOI of 50 (Fig. 4A). However, a MOI of 50 did only slightly inhibit the proliferation of MAEC and human tumor SLK cells (<20% and <10%, respectively). Because low cytotoxicity of intrabodies was usually observed at MOI 50, we selected this concentration for antiangiogenic studies in vivo.

To further investigate the nature of pAd-mediated cytotoxicity, HUVEC treated with 50 MOI of pAd-1S05, 2S03, JC7U, and GFP in vitro were analyzed by TEM. Image analysis of high-magnification TEM micrographs (five for each cell culture conditions) allowed us to identify a pronounced swelling of ERs in 10% to 15% of the cells treated with pAd-1S05, 2S03, and JC7U (Fig. 4B, arrows). The ER in GFP-treated cells as well as the nuclear and mitochondrial morphology in all cells remained unaffected (Fig. 4B). This observation suggests that the elevated cytotoxicity of adenoviral vectors encoding intrabodies could be attributed to extensive scFv accumulations in the ER.

**Figure 3. Localization of adenovirus-delivered intrabodies.** A, maximal projections of three-dimensional data sets show the intrabody and Tie-2 colocalization in HUVEC. Cells were infected with a MOI of 50 with the intrabodies against Tie-2, 1S05 (d-f), and 2S03 (g-i) and a control intrabody against integrin αvβ3, JC7U (a-c). Left, distribution of Tie-2 in red; middle, intrabodies in blue; right, overlapping signal between Tie-2 (red) and intrabody (blue) in pink and cell nuclei in green. Tie-2/intrabody colocalization was observed in cells expressing 1S05 and 2S03 (f and i, pink). Note the complete overlap in signal. By contrast, a homogenous distribution of Tie-2 in control intrabody-infected HUVECs and a minimal overlap in signal were observed (c). For three-dimensional images of c, f, and i, see Supplementary Movies S1-S3. B, immunolocalization of intrabodies in HUVEC by electron microscopy. Cells were cultured in 35-mm dishes, fixed, and processed for immunoelectron microscopy as described in MATERIALS AND METHODS. Intrabodies were detected with anti-HA mAb and 10-nm colloidal gold-conjugated protein A. Localization of intrabodies can be seen in ER of HUVEC infected with scFv-containing adenovirus (JC7U, 1S05, and 2S03) and not in HUVEC infected with empty vector (GFP). Bar, 200 nm.

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verifying the quality of DNA in these samples. The presence of scFv and/or GFP genes was restricted to the tumor tissue as shown by gel electrophoresis (Supplementary Fig. S1). All tested mouse organs failed to show the presence of scFv/GFP sequences at the limits of detection.

Toxicity Study with Adenoviral Vector Encoding scFv Intrabody. A toxicity study was carried out with the adenoviral vector to determine any potential toxicity associated with vector delivery of intrabody genes. For this study, SLK tumor-bearing nude mice, ages 8 weeks, were injected peritumorally four times with \(1.5 \times 10^8\) plaque-forming units per mouse of pAd-2S03, pAd-1S05, pAd-JC7U, or pAd-GFP on days 7, 14, 21, and 28. On day 34, animals were sacrificed and underwent autopsy. On pathologic examination, none of the animals showed any detectable pathologic changes, and all organs were appropriately placed without evidence of abscess formation, necrosis zones, or infarction. In addition, the major organs were retained for histologic examination and found to be within normal limits with no pathologic changes in the vasculature of these organs (Supplementary Fig. S2).

Antiangiogenic Activity of 2S03 Intrabody In vivo. The ability of F(ab) 2S03 to recognize mouse endothelial cell receptor Tie-2 enabled us to use this adenovirus-encoded scFv intrabody for an experimental antiangiogenic therapy in xenograft tumor models. To determine whether administration of pAd-2S03 could inhibit the growth rate of well-established primary tumors, two human tumor cell lines, SLK Kaposi sarcoma and SW1222 colon carcinoma, were used. SLK, an \(\alpha_v\beta_3\) integrin-positive tumor cell line, is of interest because of its endothelial origin. In this analysis, tumor cells \(3 \times 10^6\) in 100 \(\mu\)L PBS) were implanted s.c. into the right flank of female nude mice on day 0 followed by the peritumoral administrations of the control adenovirus pAd-GFP or the adenovirus encoding the anti-Tie-2 scFv pAd-1S05 and pAd-2S03 as well as the anti-\(\alpha_v\beta_3\) scFv pAd-JC7U adenovirus. Four injections of \(1.5 \times 10^8\) plaque-forming units each were given on days 7, 14, 21, and 28 post-tumor.

Figure 4. Cytotoxicity of adenovirus-delivered intrabodies. A, cells seeded in 96-well plate were infected with different recombinant adenoviruses. Three days after infection, the inhibition of cell proliferation was examined by a \(\left[{^3}H\right]\)thymidine incorporation assay. Points, mean; bars, SD. B, differences in ER morphology of HUVEC were examined by TEM. Nuclear and mitochondrial morphologies are normal in all cells. Swollen ERs (arrows) can be seen in HUVEC infected with scFv-containing adenovirus (JC7U, 1S05, and 2S03) but not in HUVEC treated with GFP-encoded adenovirus (GFP). Bar, 200 nm.
cell inoculation. The mean tumor size of all treated mice was monitored from days 6 to 45. As depicted in Fig. 5, all injected animals developed palpable tumors within 6 days. Beginning at day 18, a reduced tumor growth rate was observed in the pAd-2S03-treated group when compared with the control group of mice treated with pAd-GFP. On average, 69% reduction in tumor growth was observed by day 45 in the pAd-2S03-treated group relative to the pAd-GFP-treated group of mice. Repeated inoculation of human Tie-2-specific intrabody pAd-1S05 in mice bearing SLK tumors did not result in lower tumor sizes compared with the control group. The observed antitumor activity of pAd-JC7U intrabody could be due to specific action of the \( \alpha_v\beta_3 \) down-regulation on tumor cells. No side effects, such as loss of body weight, were observed in mice treated with any of the antibodies. Tumor weights were determined at sacrifice in all animals on day 45 post-tumor injection and confirmed the reduction in tumor growth during intrabody treatment.

The ability of the pAd-2S03 vector to modulate tumor-induced angiogenesis was also evaluated using \( \alpha_v\beta_3 \)-negative tumor cell line SW1222 (Fig. 5). For this study, nude mice were xenografted s.c. on day 0 with \( 2 \times 10^6 \) SW1222 cells into the right flank. After the establishment of tumor, the animals were peritumorally treated with the same intrabodies as above, except three injections of \( 10^8 \) plaque-forming units were given on day 7, 14, and 21 post-tumor cell inoculation. Xenografts raised from SW1222 tumor cells grew significantly faster than SLK tumors (Fig. 5). The treatment with recombinant adenovirus encoding the cross-reactive pAd-2S03 intrabody significantly inhibited tumor growth when compared with the control adenovirus pAd-GFP. In addition, no inhibition of tumor growth was observed for the pAd-1S05 intrabody targeting human Tie-2 or for \( \alpha_v\beta_3 \)-specific pAd-JC7U intrabody, confirming that the antitumor effect of the cross-reactive pAd-2S03 intrabody could be attributed to the surface depletion of Tie-2 from mouse endothelial cells infiltrating the human tumor.

In view of our findings and work showing that inhibition of Tie-2 signaling prevents tumor growth (23), it is tempting to speculate that reduced expression of Tie-2 may contribute to growth inhibition in angiogenesis-dependent tumors. These results suggest that inhibition of tumor angiogenesis by pAd-2S03 limited tumor growth.

**Determination of Tumor Microvessel Density and Tumor Cell Apoptosis.** To establish the mechanism underlying the inhibition in tumor growth, the effect of Tie-2 receptor down-regulation on tumor vascularization was evaluated by the determination of microvessel density. Cryosections were stained for murine CD31, and the number of discrete microvessels was compared in areas showing the highest vascularity (hotspots) of the tumor stroma. Strong CD31 expression was observed at the edge of the SLK tumors as well as in neovessels penetrating the substance of the tumor (Fig. 6A). A similar pattern of CD31 localization was also observed in human SW1222 tumor, in which the strongest CD31 expression was at the tumor boundary in regions of extensive tumor neovascularization (Fig. 6B). These results are consistent with capillary sprouting from surrounding mouse normal tissues, tumor vessel elongation, and/or formation of connections between tumor vessels and host vessels. CD31 expression was remarkably inhibited in both tumors when treated with 2S03 intrabody, whereas 1S05 and JC7U intrabody treatments did not affect angiogenesis in surrounding mouse normal tissues. The tumor vascularization index was measured from photomicrographs of

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**Figure 5.** pAd-2S03 inhibition of the growth of primary tumors. Cultured SLK or SW1222 cells were inoculated s.c. into nude mice on day 0. After a palpable tumor was observed (day 6), the mice were divided randomly into four groups of five mice each. Adenovirus encoding 1S05, 2S03, and JC7U scFv or control adenovirus (GFP) was administrated peritumorally according to a schedule described in MATERIALS AND METHODS. A, tumor volume from five growing tumors per group measured at 3-day intervals from 6 to 45 days postgrafting for SLK xenografts or from 6 to 24 days postgrafting for SW1222 xenografts. Points, mean; bars, SD. B, tumor weight. Columns, mean; bars, SD. *, \( P < 0.001 \), compared with GFP; **, \( P < 0.01 \), compared with GFP.
CD31-stained tumors. Quantification of microvessel density revealed a statistically significant (\( P < 0.01 \)) 8- to 8.5-fold reduction of microvessel density in pAd-2S03-treated tumors, whereas a similar reduction was not observed in either pAd-JC7U- or pAd-1S05-treated tumors (Fig. 6, top right). These results indicate that the Tie-2 pathway is essential for the vascularization and tumor growth of both SLK and SW1222 xenografts.

TUNEL staining of tumor sections from the four groups of SLK tumor-bearing animals revealed no difference in apoptosis and tumor necrosis among the four groups (Supplementary Fig. S3). Therefore, apoptosis was not a factor in tumor growth inhibition. Considering the strong link between tumor growth and tumor angiogenesis, the ability to measure any decrement in tumor vascular density strongly suggests that the primary action of the 2S03 intrabody was to inhibit tumor neovascularization. Thus, intracellular expression of 2S03 led to reduced tumor vascularization through reduced Tie-2 surface expression.

**Discussion**

Because angiogenesis is required for the growth and metastasis of solid tumors, we hypothesized that an ER-targeted intrabody with Tie-2 specificity could be beneficial to the inhibition of tumor angiogenesis and should provide a practical approach to

**Figure 6.** Effect of Tie-2 down-regulation on the vascularization of transplanted tumors. Cryostat sections of tumor grown from SLK (A) or SW1222 (B) xenografted mice were stained for the mouse endothelial marker CD31. Control (GFP, JC7U, and 1S05) treated tumors developed a substantial intratumor vasculature and an obvious peritumor-hypervascular border zone. In contrast, 2S03-treated tumors possessed a sparse vasculature with no obvious hypervascular border zone. Angiogenic index of tumor vascularization (top right) was calculated as described in MATERIALS AND METHODS and expressed as the percentage of CD31-stained area. Tumor neovascularization was significantly inhibited by 2S03 intrabody (\( P < 0.001 \) versus GFP). Mean \( \pm \) SD \( (n = 5) \). Bar, 50 \( \mu \)m for all sections.
long-term control of disease. A gene therapy strategy was designed to deliver a Tie-2-specific intrabody, pAd-2S03, and the importance of the Tie-2 receptor tyrosine kinase pathways for tumor angiogenesis in vivo was analyzed using human Kaposi’s sarcoma and colon carcinoma xenograft models. Treatment with adenovirus-delivered anti-mouse Tie-2 intrabody 2S03 resulted in a 75% reduction in Kaposi’s sarcoma tumor growth over a period of 45 days and a 63% reduction in colon carcinoma tumor growth over a period of 24 days. The inhibition of tumor growth was likely secondary to a blockade in tumor angiogenesis. This notion is supported by the decreased vascular density in the pAd-2S03-treated tumors and the ability of 2S03 antibody to recognize mouse Tie-2. These findings provide the first demonstration of the anti-Tie-2 intrabody effect on tumor angiogenesis and suggest that therapeutic approaches similarly designed to inhibit the Tie-2 pathway could be clinically useful. For example, other transcriptional or post-transcriptional approaches, such as RNA interference and zinc finger transcription factors, may be promising alternative strategies to our intrabody-based approach. It is important to note that the phenotypic outcome of receptor antagonism and down-regulation/knockout need not be the same as the integrins α5β1 and α6β1 have revealed (41, 42). Significantly, we did not find any evidence for adverse effects of Tie-2 down-regulation after peritoneal delivery of the 2S03 intrabody, suggesting that safe therapeutic regimens are possible despite the expression of Tie-2 in normal vasculature.

The molecular mechanism by which pAd-2S03 blocks tumor angiogenesis is not yet clear. However, based on several previously published results, it is likely that pAd-2S03 blocks tumor angiogenesis at a step distal to endothelial cell activation (18, 43, 44). This is supported by the results of gene-targeting experiments of Tie-2 (18, 43) that revealed indispensable but distinct functions of the receptor pathway. In Tie-2-deficient mice, endothelial cells lack the support of underlying periendothelial mesenchymal cells, which implicates a predominant role for Tie-2 in vessel maturation and maintenance. This results in collapse of the endothelial cells into the lumen of vessels that were preformed by tissue fold and vessel occlusion (45).

Disruption of angiogenesis via Tie-2 down-regulation may be globally useful in diseases that result in pathologic neovascularization. Interestingly, both tumor cell lines used in this study produce VEGF. The ability of pAd-2S03 to inhibit angiogenesis despite the presence of VEGF suggests that disrupting the angiogenic program at stages distal to endothelial activation will provide effective inhibition of neovascularization. One potential limitation of this study is that only partial tumor growth inhibition was achieved. This result indicates that blocking Tie-2 activation alone may not be sufficient to completely halt tumor angiogenesis. Because the VEGF pathway also is critical for tumor angiogenesis, future work will test the efficacy of a combined delivery of the Tie-2 intrabody and the VEGFR2 intrabody to knockout the cell surface expression of both proteins simultaneously (35).

References


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