METH-1, a Human Ortholog of ADAMTS-1, and METH-2 Are Members of a New Family of Proteins with Angio-inhibitory Activity

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We have studied two related proteins that contain a repeated amino acid motif homologous to the anti-angiogenic type 1 repeats of thrombospondin-1 (TSP1). Complete sequence analysis revealed no other similarities with TSP1, but identified unique signal sequences, as well as metalloprotease and disintegrin-like domains in the NH2 termini. We named these proteins METH-1 and METH-2 due to the novel combination of metalloprotease and thrombospondin domains. Overall amino acid sequence identity between METH-1 and METH-2 is 51.7%, yet transcript distribution revealed non-overlapping patterns of expression in tissues and cultured cell lines. To characterize these proteins functionally, we isolated full-length cDNAs, produced recombinant protein, and generated antisera to the recombinant proteins. Both METH-1 and METH-2 represent single copy genes, which encode secreted and proteolytically processed proteins. METH proteins suppressed fibroblast growth factor-factor-induced vascularization in the cornea pocket assay and inhibited vascular endothelial growth factor-induced angiogenesis in the chorioallantoic membrane assay. Suppression of vessel growth in both assays was considerably greater than that mediated by either thrombospondin-1 or endostatin on a molar basis. Consistent with an endothelial specific response, METH-1 and METH-2 were shown to inhibit endothelial cell proliferation, but not fibroblast or smooth muscle growth. We propose that METH-1 and METH-2 represent a new family of proteins with metalloprotease, disintegrin, and thrombospondin domains. The distinct distribution of each gene product suggests that each has evolved distinct regulatory mechanisms that potentially allow for fine control of activity during distinct physiological and pathological states.

Thrombospondin-1 (TSP1) is a modular protein that associ-
related proteins. We therefore propose that METH-1, METH-2, pNPI, and potentially other genes belong to a unique family of proteins, which we have referred to as metallospindins.

In this study, we describe the cDNA structure of human METH-1 and METH-2, characterize their pattern of expression, and provide functional evidence that the encoded proteins represent novel inhibitors of angiogenesis. We demonstrate that the proteins are proteolytically processed and secreted from cells consistent with their proposed activity as extracellular signaling factors. Recombinant METH-1 and METH-2 inhibited angiogenesis in two independent bioassays, and both proteins exhibited higher potencies of inhibition than was mediated by either TSP1 or endostatin. In addition, both proteins block endothelial cell proliferation in a reversible fashion. The mechanism of inhibition appears to be specific, since METH-1 and METH-2 do not suppress either smooth muscle or fibroblast growth.

EXPERIMENTAL PROCEDURES

Identification and Cloning of METH-1 and METH-2—A large human cDNA data base of expressed sequence tags (ESTs) was screened for sequences homologous to the second type 1 repeat of TSP1. Several cDNA clones were found to encode proteins with TSP-like domains. Two clones from human heart (METH-1) and lung libraries (METH-2), respectively, were further sequenced and selected for functional analysis.

The amino-terminal end of METH-1 was obtained using standard cDNA library screening and 5'-rapid amplification of cDNA ends (Marathon cDNA amplification kit, CLONTECH). The amino-terminal end of METH-2 was obtained partially by 5'-rapid amplification of cDNA ends polymerase chain reaction and confirmed by isolation of a genomic fragment. For the genomic screen, BAC clones (Genome Systems, St. Louis, MO) were initially identified by polymerase chain reaction. Positive BAC clones containing 150–200 kb of sequence were subsequently subcloned into a pGEM vector (Promega, Madison, WI) as small fragments and sequenced in both directions at either the sequencing facility of Beth Israel Deaconess Medical Center or Harvard Medical School (Boston, MA), or at the Molecular Biology Core Facility of Human Genome Sciences (Rockville, MD).

Northern and Southern Blot Analysis—Total RNA was purified from cells by guanidine-isothiocyanate extraction, as described previously (17). Poly(A)+ RNA was extracted using oligo(dT) linked to magnetic beads (Roche Molecular Biochemicals) according to the manufacturer’s specifications. Some poly(A)+ RNA blots, which include the human and fetal organs were purchased from CLONTECH. TSP1, METH-1, and METH-2 probes corresponded to the entire human cDNAs. A 1.3 kb PstI fragment of the glyceraldehyde-3-phosphate dehydrogenase (GPDH) was used to normalize for loading and transfer efficiency.

For Southern blots, human genomic DNA was purchased from Promega (Madison, WI) and digested with EcoRI and EcoRI I. In Vitro Translation—In vitro translation was performed with a transcription/translation kit from Promega. pGEM vectors containing full-length METH-1 and METH-2 were initially used for transcription. The products were then introduced into a reticulocyte lysate for translation, signal cleavage, and glycosylation. Translation was performed in the presence of [35S]methionine for visualization of the protein product.

Expression Vectors, Purification of Recombinant Protein, and Generation of Antibodies—Recombinant constructs for expression of Histagged fusion proteins were generated for expression in bacteria. METH-1 nucleotides 605–1839 (from ATG) was amplified by polymerase chain reaction using primers containing BamHI and PstI sites and subcloned into the pRSET vector (Invitrogen, Carlsbad, CA). The construct was sequenced to verify frame and sequence fidelity and were then translated into BL21 (Strategene Cloning Systems, La Jolla, CA). Purification was performed by affinity chromatography on Ni-NTA columns. Recombinant protein was eluted with 500 mM imidazole in phosphate-buffered saline. Fractions containing recombinant protein were dialyzed against phenol-red free DMEM and used to generate antiserum.

Antiserum was generated by intramuscular injection of a 1:1 mixture of recombinant protein (500 μg/ml) and Freund’s adjuvant. Eight animals (five guinea pigs and three rabbits) were injected every 15 days for three cycles. After the third injection, serum was evaluated for presence of anti-METH-1 antibodies; only two of the guinea pigs showed significant titers. The antibodies recognized recombinant protein on Western blots and were able to immunoprecipitate METH-1 protein from cell extracts and recognize the protein by immunocytochemistry (data not shown). Preimmune serum was always included as control. One of the guinea pig antibodies was also able to recognize METH-2. For mammalian expression, full-length METH-1 and METH-2 cDNA were cloned into pcDNA1 expression vector (Invitrogen). Recombinant protein was obtained by transient transfection of NIH 3T3 cells using standard calcium phosphate precipitation. Upon transfection, cells were incubated for 16–8 h in serum-containing medium and then switched to serum-free medium for 36 h for accumulation of recombinant protein. As control, pcDNA3.1 vector alone was transiently transfected in parallel plates. Purification of the protein included 30% ammonium sulfate precipitation followed by dialysis in HS buffer (10 mM HEPES, 150 mM NaCl, 1 mM CaCl2, and 1 mM MgSO4). Samples were then subjected to heparin-affinity chromatography. Elution from heparin columns was achieved with HS buffer containing 550 mM NaCl. Fractions were then loaded on 5–30% sucrose gradients and spun at 100,000 rpm. Separation on sucrose gradients was assessed by Western blotting, and purity was determined by Coomassie Blue and silver nitrate staining.

Cornea Pouch Assay—Swiss Webster mice were purchased from Charles River (Boston, MA) and used between 8 and 10 weeks of age for implantation of Hydrod pellets. Cornea pouches were performed as described by Kenyon and colleagues (18). Generation of the pellets was as follows: a solution of 10 μg of recombinant basic fibrinogen growth factor (bFGF) plus 1 μg of suralactone (a gift from Dr. Joyce Bishop, Children’s Hospital, Boston) was mixed with 10 μl of Hydrod (200 mg/ml in ethanol, New Brunswick, NJ) and the recombinant protein of interest (concentrations in the figure legends) (pore size 500 μm; Tetko Inc., Briarcliff Manor, NY) and allowed to dry for 30 min. The fibers of the mesh were pulled to produce pellets of 500 μm2 that were stored at −20 °C. Uniformly sized pellets were selected under a microscope and used for the assays. Five days after pellet implantation, corneal angiogenesis was evaluated and photographed.

CAM Assay—CAM assays were performed on Leghorn chicken embryos (SPAFAS, MA) at 12–14 days of embryonic development. Matrigel (750 μg/ml), vascular endothelial growth factor (VEGF; 250 ng/mesh alone or mixed with TSP1, endostatin, or metallospindins, at concentrations specified in the figure legends, were placed onto nylon mesh (pore size 250 μm; Tetko Inc.) and incubated at 37 °C for 30 min and at 4 °C for 2 h for polymerization. Positive (Matrigel and VEGF) and negative (VEGF alone) controls were also prepared for each CAM. Polymerized meshes were placed onto the outer region of the CAM and incubated for 24 h as described (5, 6). To visualize vessels, 400 μl of 0.1% fluorescein sodium salt (100 μg/ml) in phosphate-buffered saline was injected in the chick blood stream. After 5–10 min of incubation, the chick was fixed with 3.7% formaldehyde for 5 min. The meshes were then dissected and mounted on slides. Fluorescence intensity was analyzed with a computer-assisted image program (NIH Image 1.59). TSP1 purified as described previously (5, 6). Recombinant endostatin was a kind gift from Dr. Vikas Sukhatme (Beth Israel Deaconess Medical Center, Boston) and digested with Endo and PstI.

Proliferation Assays—Human dermal endothelial cells (HDEC) were isolated as described previously (20). Cells were grown on Vitrogen*-coated Petri-dishes in EB (Clonetics, San Diego, CA) supplemented with 15% fetal calf serum, 25 μg/ml eAmp, and 1 μg/ml hydrocortisone-21-acetate and were used from passages 3 to 6. Vascular smooth muscle cells were a gift from Elaine Raines (Department of Pathology, University of Washington, Seattle, WA). Human dermal fibroblasts were isolated from neonatal foreskin by enzymatic dissociation. Both fibroblasts and smooth muscle cells were maintained in DMEM supplemented with 10% fetal calf serum. For proliferation assays, cells were made quiescent by incubation of confluent monolayers with phenol red-free EB containing 0.2% bovine serum albumin for 48 h. Quiescent HDEC were then plated on Vitrogen*-coated 24-well plates in EB supplemented with 0.2% bovine serum albumin, 0.1% fetal calf serum, and 1 ng/ml FGF-2 in the presence or absence of the recombinant protein (for dosage, see figure legends) and incubated at 37 °C for 48 h. Vascular smooth muscle and fibroblasts were incubated under the same conditions using DMEM instead of EB. A pulse of [3H]thymidine (1 μCi/ml) was added during the last 4 h prior to harvesting. Cells were washed and fixed in 10% trichloroacetic acid. Incorporation of [3H]thymidine was determined by scintillation counting, as described previously (21).

Statistical analysis was done using In-Stat (Graph Pad Software) for Macintosh. Assuming normal distributions, data were analyzed by one-way analysis of variance, followed by either t test for comparisons between groups, or Student-Newman-Keuls test for multiple comparisons between groups.
RESULTS

Identification of Novel cDNAs with TSP Anti-angiogenic Domains—In an effort to identify new inhibitors of angiogenesis, we screened expressed sequence tag sequences for novel cDNAs containing the anti-angiogenic motif of TSP1. Two previously unknown partial clones were found in human adult heart and lung libraries. Complete predicted amino acid sequences of both proteins is presented in Fig. 1.

Analysis and comparison of the deduced amino acid sequence with the GenBank, EMBL, and SwissProt data bases suggested that these genes belong to a new subfamily of metalloproteases (double underlined), the zinc-binding-site (dotted line) in the metalloprotease domain, and the putative disintegrin loops (arrows). GenBank data base accession numbers are AF060152 for METH-1 and AF060153 for METH-2.

Deduced amino acid sequence and primary structure of METH-1 and METH-2. Comparison of the amino acid sequence of METH-1 and METH-2 with that of their closest homologue, a bovine metalloprotease (pNPI). Identical amino acids are boxed. Functional domains predicted by sequence and structural homology are labeled, including the signal peptide (single line), the potential cleavage site for mammalian subtilisin (double underlined), the zinc-binding-site (dotted line) in the metalloprotease domain, and the putative disintegrin loops (arrows). GenBank data base accession numbers are AF060152 for METH-1 and AF060153 for METH-2.
proteins are secreted. There are no clear transmembrane domains as deduced from the hydrophilicity plots (data not shown). Adjacent sequences in the NH2-terminal half of each coding region show motifs consistent with a zinc metalloprotease. This region can be subdivided into a prodomain, a metalloprotease domain, and a cysteine-rich region.

The sequences RKKR in METH-1 and RTKR in METH-2 (double underlined in Fig. 1), localized at the boundary between the prodomain and the metalloprotease domain, are potential cleavage sites for mammalian convertases, such as furins (22). Proteolytic processing occurs in snake venom metalloproteases to yield soluble metalloproteases and disintegrins (23) and has also been detected in some ADAMs (13). In fact, our results would indicate that proteolytic processing occurs in both METH-1 and METH-2 (see below). Additionally, both proteins contain a conserved Zn2+--binding site (dotted upper line in Fig. 1) that is presumed to be part of the catalytic active site, based on the conservation of certain functionally important amino acids in metalloproteinases (12). These observations suggest that these proteins may be active proteases.

Following the metalloprotease domain, there is a cysteine-rich region that contains two putative disintegrin loops (marked by upper arrows in Fig. 1). Disintegrin domains are found in some members of the superfamily of metalloproteinases, which includes snake venom metalloproteinases and ADAMs (12). In snake venom metalloproteinases, the disintegrin region has been shown to inhibit binding of integrins to their ligands. Conversely, the ADAM-disintegrin-like domain, as part of membrane-anchored proteins, have been shown to promote rather than disrupt, cell-cell interactions (13).

All three metallospondin genes contain a variable number of TSP-like domains located in the COOH-half of the coding region. METH-1 contains one TSP domain, a spacer region, followed by two additional TSP domains. METH-2 presents a similar structure, but only one TSP domain after the spacer region (Fig. 2A). The alignment of the TSP-like domains of METH-1 and METH-2 with those of TSP1 and TSP2; cysteines are numbered 1–6, and tryptophans are marked by asterisks.

The overall homology ranges between 19.2% and 52% amino acid identity among all the TSP repeats. The presence of 6 cysteines (C), numbered 1–6, and 2–3 tryptophans (W), labeled by asterisks, is highly conserved.

Southern blots of human genomic DNA provided evidence that METH-1 and METH-2 are present as single copy genes. METH-1 and METH-2 probes revealed non-overlapping patterns in multiple restriction digests, suggesting that they are transcribed from different genes (Fig. 3, and data not shown).

Tissue and Cell Type Distribution of METH-1 and METH-2 mRNAs—The expression pattern of METH-1 and METH-2 was examined in human adult and fetal tissues by Northern analysis of poly(A)-enriched blots under high stringency conditions. METH-1 and METH-2 transcripts revealed single bands of 4.6 and 3.7 kb, respectively. While expression of METH-1 mRNA was seen in all tissues analyzed, abundant METH-1 mRNA
expression was observed in adrenal, heart, and placenta, followed by skeletal muscle, thyroid, and stomach (Fig. 4, A and B). Of the embryonic tissues analyzed, kidney showed the highest expression of METH-1 mRNA (Fig. 4C). Distribution of METH-2 mRNA was more restricted and less abundant than that of METH-1. The highest expression was seen in lung, both embryonic and adult (Fig. 4, B and C). Interestingly, in several tissues including brain, liver, and pancreas, METH-1 and METH-2 expression do not appear to overlap.

For purpose of comparison, we also assessed the expression levels of TSP1 transcripts in the same blots. Highest expression of TSP1 mRNA was seen in the adult placenta (Fig. 4). In contrast to METH-1 and METH-2, we observed relatively ubiquitous and higher levels of TSP1 transcript in the embryonic tissues examined.

The cell type distribution was also studied by Northern blot analysis of poly(A) RNA (Fig. 5). METH-1 mRNA was detectable in dermal fibroblasts and at low levels in vascular smooth muscle, endometrial stromal, and in some endothelial cells. Two cancer cell lines, HeLa and G631, expressed relatively high levels of METH-1 mRNA; both cell lines are of epithelial origin. METH-2 mRNA was detected only in SW480, a colon carcinoma cell line. Expression of METH-2 appeared negligible or absent from all other cell lines and primary cell cultures analyzed (Fig. 5, A and B).

Expression of Recombinant METH-1 and METH-2—Generation of recombinant protein was initially done in bacteria. A METH-1 expression vector was generated containing an amino-terminal His tag to aid on the purification. The resulting protein coded for all METH-1 translated sequence except the prodomain. Affinity chromatography on Ni\(^{2+}\) beads showed an unique band of 68 kDa (Fig. 6A). Isolation and purification was always performed under denaturing conditions, and attempts to refold the protein met with little success, probably due to a significant number or intramolecular disulfide bonds associated with the high number of cysteines. Nonetheless, the protein was used to generate antibodies. From eight animals injected, only two were able to mount an immune response and generate specific antibodies, possibly due to the high conserva-

Fig. 4. Tissue distribution of METH-1, METH-2, and TSP1 mRNA. Five micrograms of poly(A)-enriched mRNA from various human adult (A and B) and fetal (C) organs were hybridized with METH-1, METH-2, TSP1, and GPDH cDNA radiolabeled probes. The METH-1 probe identified an mRNA species of 4.6 kbp, while METH-2 mRNA corresponds to a 3.7-kb transcript. Hybridization with GPDH transcript do not reflect loading efficiency, since this transcript will vary in adult tissues according to metabolic activity of each organ.

Fig. 5. Expression of METH-1, METH-2, and TSP1 mRNA in normal (A) and transformed cell lines (B). A, cell strains were grown in culture and poly(A)\(^{+}\) RNA was isolated as described under “Experimental Procedures.” MS, myometrial smooth muscle; HDF, human dermal fibroblasts; VSM, vascular smooth muscle; HESC, human endometrial stromal cells; KER, keratinocytes; BAEC, bovine aortic endothelial cells; HUEC, human umbilical vein endothelial cells; HEEC, human endometrial endothelial cells. B, blots of specific cell lines were purchased from Clonetics. Blots were probed with full-length cDNAs for METH-1, METH-2, and TSP1. GAPDH was used to demonstrate presence of RNA in all lanes.
Molecular weights of protein standards are indicated on the right of endostatin (Fig. 8 Lane 1 of enolase, used as loading control. The *UP* arrowhead indicates a nonspecific band demonstrating loading levels in both lanes. C, Western blot of mammalian cell lysates probed with anti-METH-1 polyclonal serum. The *thin arrow* indicates the mobility for METH-1. *Arrowhead* shows mobility of enolase, used as loading control. Lane 1, human dermal fibroblasts; lane 2, human endometrial stromal cells; lane 3, vascular smooth muscle M; lane 4, human umbilical vein endothelial cells; lane 5, human endometrial endothelial cells; lane 6, keratinocytes. Note that relative levels of protein parallel transcript levels observed in Fig. 5.

**FIG. 6. Purification of recombinant fusion proteins from bacteria.** A, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of unpurified cell lysate (UP), unbound proteins/wash (W), and purified *E. coli* recombinant METH-1 protein (P) stained with Coomassie Blue. Molecular weights of protein standards are indicated on the right. The purified protein (P) was used to generate a polyclonal antibody in guinea pigs. B, Western blot of unpurified cell lysate from bacteria transfected control (C) or METH-1 (M1). The *arrow* indicates the mobility for METH-1 recombinant bacteria protein. The *arrowhead* indicates a nonspecific band demonstrating loading levels in both lanes. C, Western blot of mammalian cell lysates probed with anti-METH-1 polyclonal serum. The *thin arrow* indicates the mobility for METH-1. *Arrowhead* shows mobility of enolase, used as loading control. Lane 1, human dermal fibroblasts; lane 2, human endometrial stromal cells; lane 3, vascular smooth muscle M; lane 4, human umbilical vein endothelial cells; lane 5, human endometrial endothelial cells; lane 6, keratinocytes. Note that relative levels of protein parallel transcript levels observed in Fig. 5.

Recombinant METH-1 and METH-2 were eluted with washing buffer containing 550 mM NaCl. Fractions contained both pro-METH-1 and the processed forms. Because it was unclear whether processing was relevant for function of the proteins, we separated both forms on sucrose gradients (Fig. 7, D and E). Both full-length and processed forms were used in angiogenesis assays.

**METH-1 and METH-2 Disrupt Angiogenesis in Vivo**—In the CAM assay, the angiogenic response is determined by measuring the number of vessels that grow within a matrix polymer containing the angiogenic growth factor. To determine whether recombinant METH-1 and METH-2 inhibited growth factor-mediated neovascularization, we implanted a Matrigel polymer containing VEGF (an angiogenesis stimulator) in combination with our recombinant proteins as well as other known inhibitors of angiogenesis (Fig. 8A). A fine network of vessels was induced after 24 h in the presence of VEGF (Fig. 8, A, b and c). Incorporation of inhibitors in the polymer, however, significantly blocked neoangiogenesis. Equal molar amounts (50 nm) of endostatin (Fig. 8A, d), TSP1 (Fig. 8A, e), METH-1 (Fig. 8A, f), and METH-2 (Fig. 8A, g) were all able to interfere with VEGF-mediated angiogenesis. Quantitative analysis of five independent polymers per treatment revealed that METH-1 was the most effective inhibitor (angiogenesis suppression was 65%) followed by METH-2, TSP1, and endostatin (yeast recombinant) (Fig. 8B). Furthermore, the anti-angiogenic effect of METH-1 and METH-2 were dose-dependent with a complete inhibition of blood vessel growth at 15 μg/ml for METH-1 and 35 μg/ml for METH-2 (data not shown). CAM assays performed with the unprocessed form of METH-1 and METH-2 provided similar results (data not shown). It was unclear whether processing is not required for function or if the CAM tissue led to processing of our proteins. Thus, we incubated the intact protein parallel transcript levels observed in Fig. 5.

**FIG. 7. Purification of recombinant METH-1 and METH-2 from mammalian cells.** A, SDS-PAGE of [35S]methionine labeled in vitro translation reactions for METH-1 and METH-2. Electrophoretic mobility indicates that METH-1 is 110 kDa and METH-2 98 kDa. B and C, 293T cells were transfected with an expression vector containing the METH-1 (B) or METH-2 (C) coding sequence. Unpurified conditioned medium (UP) was stained with Coomassie Blue. Purified medium (M) and cell layer (CL) were subjected to SDS-PAGE, transferred to Nytran, and probed with anti-METH-1 and METH-2 antibodies. Arrows in both B and C indicate mobility of full-length protein, while *arrowhead* indicates mobility of the processed forms. METH-1 (D) and METH-2 (E) purified recombinant conditioned medium was subjected to a sucrose gradient at 40,000 rpm to separate the full-length (arrow) and processed forms (arrowhead) of each protein. Purity was assessed by Coomassie Blue staining.

Anti-angiogenic activities of METH-1 and METH-2 were also tested in the cornea pocket assay. In the presence of FGF-2 alone, capillaries grew from the corneal limbus into the pellet within 5 days. In contrast, addition of full-length METH-1, METH-2, or TSP1 significantly suppressed blood vessel growth (Fig. 9 and Table 1). In Table 1, a summary of the results obtained from 41 assays is presented. Intact TSP1 purified from platelets was used as an inhibitory control. All assays were performed at identical molar concentrations, as noted under "Experimental Procedures."

**Effect of METH-1 and METH-2 on Cell Proliferation**—To gain insight into the mechanism by which METH-1 and METH-2 inhibit neovascularization, we tested their effect on endothelial cell proliferation. Recombinant proteins (5 μg/ml)
were added at the time of plating. After 2 days, a 65% (METH-1), 46% (METH-2), or 34% (TSP1) inhibition in 3H incorporation was observed. No significant effect was seen when fibroblasts or smooth muscle cells were used (Fig. 10A). This result rules out the presence of any potential nonspecific inhibitor of cell growth in our recombinant protein preparations and also supports the concept of a cell-specific effect for these proteins in the inhibition of endothelial cell growth. The effect on proliferation was also seen by total cell counts after 4 days exposure to METH-1 and METH-2 (5 μg/ml) (Fig. 10B). In addition, the inhibitory effect was reversible and not toxic, since the removal of the recombinant protein and subsequent addition of growth factor alone led to the resumption of endothelial cell proliferation (Fig. 10B). Finally, suppression of proliferation mediated by METH-1 or METH-2 was dose-dependent and significant with as little as 500 ng/ml (Fig. 10C).

DISCUSSION

The present study describes two novel inhibitors of angiogenesis termed METH-1 and METH-2. The cDNAs were identified in a search for novel genes with the anti-angiogenic domain (TSP repeats/type 1 repeats) of TSP1. Recombinant METH-1 and METH-2 were shown to be effective in blocking angiogenesis in the CAM and cornea pocket assays. Furthermore, they inhibited proliferation of endothelial cells in a dose-dependent manner, while they did not suppress growth in fibroblasts or smooth muscle cells.

The consensus sequence for the TSP type 1 repeat includes 16 residues with 6 perfectly conserved cysteines. Typically consensus begins with the motif WSXWS, a sequence that binds heparin (24). The affinity of this region for heparin has been proposed to contribute to the anti-angiogenic activity of TSP1 (6, 9). Among the five members of the TSP family of proteins, only TSP1 and TSP2 inhibit angiogenesis and contain the TSP/type 1 repeats (7, 25, 26). More recently, BAI-1 (brain angiogenesis inhibitor-1), a protein isolated from a human brain library for its ability to be regulated by p53, has also been shown to contain the TSP repeats that provide anti-angiogenic potential to this molecule (27). Nevertheless, it appears that additional sequences are important, since certain proteins containing TSP repeats appear not to have clear anti-angiogenic properties. These include properdin, F-spondin, and certain members of the complement family (28–30).

The similarity between metallospindins and TSPs is restricted to the type 1 repeats. Interestingly, the proteins also have sequence and structural homology to members of the ADAM family of metalloproteases. These features led Kuno and colleagues to name the mouse sequence ADAMTS1 (14, 15). In addition to METH-1, METH-2, and pNPI, there are four additional members to this family represented as partial ESTs, making a total of seven metallospindins. At this point, it is not clear whether pNPI, or any of the new members, participates in the regulation of angiogenesis, or whether METH-1 and/or
METH-2 are effective in the cleavage of the amino-terminal pro-peptide of \(\alpha_1(II)\) procollagen, as was shown for pNPI (16). The possibility that groups of angiogenic and anti-angiogenic factors regulate vascular network formation in specific organs has been frequently discussed. We were puzzled by the expression patterns displayed by METH-1 and METH-2, which are clearly distinct and at least partially non-overlapping. TSP1 and TSP2 also share identical structure, high levels of amino acid similarity, yet their pattern of expression differs significantly (31). As previously suggested, these differences are likely based on dissimilar cis-acting elements in the TSP1 and TSP2 promoters and different regulatory mechanisms (32–34). Although we have not characterized the promoters for METH-1 and METH-2, it is likely that they will demonstrate unique regulatory features. Nevertheless, the possibility that one motif, the anti-angiogenic/TSP repeat is present in several proteins with different tissue specificity is appealing and supports the hypothesis that specific angiogenic inhibitors might regulate vascular networks in different organs and/or in specific physiological responses. Alternatively, small differences in sequence between closely related proteins could possess significance beyond functional redundancy. ADAMTS-1 was identified in a screen of genes associated with induction of cachexia.

**FIG. 9. Inhibition of FGF-2-induced angiogenesis by recombinant METH-1 and METH-2.** A Hydron pellet (P) containing 10–20 ng of FGF-2, in the presence or absence of the recombinant protein (50 nM), was implanted in a cornea micropocket. Five days after implantation, eyes were examined for vessel growth and photographed. A representative example of the corneas in the region of the pellet implant is shown. A, FGF-2 alone; B, FGF-2 + TSP1; C, FGF-2 + METH-1; D, FGF-2 + METH-2. Arrows point to blood vessels growing from the limbus of the cornea. The results of all experiments performed in the cornea are summarized in Table I.

**TABLE I**

| Recombinant protein | No. of corneas evaluated/positive response |
|--------------------|------------------------------------------|
| 1) METH-1          | 17/0                                     |
| 2) METH-2          | 17/4                                     |
| 3) Thrombospondin  | 21/6                                     |
| 4) Endostatin      | 6/2                                      |
| 5) Vehicle         | 27/27                                    |

**FIG. 10. Effect of METH-1 and METH-2 recombinant proteins on growth factor stimulated cell proliferation.** A, cells were cultured on 24-well plates in media containing FGF-2 alone (growth factor alone) or in the presence of TSP1, METH-1, or METH-2 (5 \(\mu\)g/ml). Treatment was performed for 48 h. During the last 12 h of the experiment, a pulse of 1 \(\mu\)Ci/ml \([^{3}H]\)thymidine was added to the wells. Incorporated counts were determined by liquid scintillation. Experiments were performed in quadruplicate. HDF, human dermal fibroblasts; SMC, smooth muscle cells. All counts were normalized to 100% of FGF-2 alone. S.D. bars can not be provided, since the results are given in percentage; however, the maximum variation was 17% (in METH-2 treatment). B, equal number (50,000) of endothelial cells were incubated with growth factor alone (FGF-2 and VEGF) or in the presence of METH-1 or METH-2 (5 \(\mu\)g/ml). After 4 days cells were counted and growth presented as a histogram. Alternatively, cells were incubated 2 days with METH-1 or METH-2, washed and treatment was continued with either growth factors alone (GF) or growth factors and METH-1 or METH-2 (last four bars). Bars, \(\pm\) S.D. C, dose response of METH-1 and METH-2 on HDEC proliferation. Experiments were performed as in A. Each treatment was done in triplicate. Values represent percentage of control; maximum variation was 9%. Asterisk indicates proteins that were previously heated at 100 °C for 10 min prior to addition.
and appears to be regulated by inflammatory cytokines (14). METH-2 does not appear to share these features.²

Another region of functional interest corresponds to the disintegrin loops within the cysteine-rich domain. This loop is found in members of the replosyn subfamily of metalloproteases (35, 36). The disintegrin motif consists of 13–15 amino acids, which can contain an RGD (or RG*) motif with a negatively charged residue at the X position. The RGD sequence, or equivalent, binds to integrins and serves as ligand or an antagonist of ligand binding (13). Some proteins with these domains have been shown to act as functional adhesion molecules, particularly proteins with transmembrane domains (13). METH-1 and METH-2 present two putative disintegrin loops in the cysteine-rich region. This may serve an important role in the tertiary structure of this region and its ability to interact with integrins. In addition, METH-2 contains an RGD motif located amino-terminal to the disintegrin loop. Inactivation of αβ₂ and β₁ integrins with antibodies has been shown to inhibit neovascularization both during development and in tumors (37–39). The possibility that METH-1 and METH-2 might function as disintegrins would be consistent with their anti-angiogenic properties.

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