Melanoma Differentiation-associated Gene 7/Interleukin (IL)-24 Is a Novel Ligand That Regulates Angiogenesis via the IL-22 Receptor

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ABSTRACT

The melanoma differentiation-associated gene 7 (mda-7), also called interleukin (IL)-24, suppresses the growth of some cancers in vitro and in vivo as a result of the ectopic expression of its protein. However, the function of the secreted form of the protein in cancer has not been previously studied. The purpose of this study was to determine the antiangiogenic function of a secreted form of the MDA-7/IL-24 protein (sMDA-7/IL-24). In vitro, sMDA-7/IL-24 inhibited both endothelial cell differentiation and migration of endothelial cells induced by vascular endothelial growth factor and basic fibroblast growth factor. The sMDA-7/IL-24-mediated inhibitory effect was 10–50 times more potent than endostatin, IFN-γ, and IFN-inducible protein 10 in vitro. Furthermore, the inhibitory effect was not mediated by IFN or IFN-inducible protein 10. IL-22 receptor mediated the antiangiogenic activity of sMDA-7/IL-24. Administration of a blocking antibody to IL-22 receptor in conjunction with sMDA-7/IL-24 led to abrogation of inhibition of endothelial differentiation. sMDA-7/IL-24 inhibited vascular endothelial growth factor-induced angiogenesis as evidenced by reduced vascularization and hemoglobin content in vivo Matrigel plug assays. In vivo, the growth of human lung tumor cells was significantly inhibited, and vascularization was reduced when the cells were mixed with 293 cells stably expressing sMDA-7/IL-24. Systemic administration of sMDA-7/IL-24 inhibited lung tumor growth in a mouse xenograft model. Associated with tumor growth inhibition was decreased tumor microvessel density and hemoglobin content, indicating the presence of antiangiogenic activity. These data demonstrate that sMDA-7/IL-24 is a novel and potent antiangiogenic effector and support the development of MDA-7/IL-24-based therapeutics.

INTRODUCTION

Angiogenesis, a complex process that involves the formation of new blood vessels, is critical for the growth of solid tumors (1). Recent studies support the concept that metastasis from solid tumors is facilitated by angiogenesis of the primary tumor (2, 3). Extensive data demonstrate that solid tumors express genes coding for angiogenic mediators such as VEGF, bFGF, platelet-derived growth factor, and IL-8 (3) in the local tumor milieu, resulting in the production of new blood vessels (4, 5). Therefore, inhibition of tumor angiogenesis should prove to be an effective means of inhibiting cancer growth and spread. A number of angiogenesis inhibitors have been identified and have been shown to inhibit angiogenesis in preclinical studies, but very few have had therapeutic effects in clinical trials (6). Thus, there is need to identify new agents with potent antiangiogenic activity in patients.

One candidate is the mda-7, which was identified by a subtraction hybridization approach from the human HO-1 melanoma cell line (7). The mda-7 gene belongs to the IL-10 family of cytokines and has recently been classified as IL-24 (8, 9). Introduction of the mda-7/IL-24 gene into a wide variety of cancer cells suppressed growth in vitro and in vivo, with minimal toxicity to normal cells (10–15). This antitumor activity has been attributed to the overexpression of MDA-7/IL-24 protein. We and others (13, 15–17) have recently demonstrated secretion of the glycosylated form of MDA-7/IL-24 in vitro. Although the secretion of MDA-7/IL-24 and the binding of this secreted protein (sMDA-7/IL-24) to two distinct heterodimeric receptors has been reported (16, 17), the functional significance of sMDA-7/IL-24 in cancer has not been evaluated. Caudell et al. (18) recently demonstrated in human PBMCs that sMDA-7/IL-24 functions as a pro-Th1 cytokine and induces production of IFN-γ, IL-6, and tumor necrosis factor α. The antitumor and cytokine activity demonstrated by MDA-7/IL-24 is similar to the activity observed with other Th1-type cytokines such as IL-12 and IFN-γ (19–21). In a more recent study (22), we demonstrated that ectopic expression of MDA-7/IL-24 inhibited endothelial cell differentiation in vitro and inhibited human lung tumor growth in a mouse xenograft model that was associated with reduced tumor vascularization. On the basis of these observations, we speculated that MDA-7/IL-24 possesses antiangiogenic activity in addition to its antitumor activity. However, it was not clear whether the intracellular MDA-7/IL-24 or the extracellular sMDA-7/IL-24 protein exerted the antiangiogenic activity.

In this study, we tested the antiangiogenic activity of sMDA-7/IL-24 in vitro and in vivo. We found it had potent activity and suggest a possible mediator. The data from this study strongly suggest that sMDA-7/IL-24 can be an effective treatment for cancer.

MATERIALS AND METHODS

Cell Culture. The human non-small cell lung cancer cell line A549 (adenocarcinoma) and human embryonic kidney cells (293) obtained from the American Type Culture Collection (Manassas, VA) were grown in Ham’s F-12 medium (A549 cells) or DMEM (293 cells) supplemented with 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY). The HUVEC and HMVEC were purchased from Clonetics (Walkersville, MD) and were grown in endothelial cell basal medium with 5% fetal bovine serum and additional reagents supplied as a bulk kit by the manufacturer. Endothelial cells were used at passage 3–9.

Production and Purification of Secreted MDA-7/IL-24 Protein. MDA-7/IL-24 protein was produced by transfecting 293 cells with a eukaryotic

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expression vector carrying the full-length mda-7 cDNA. After transfection was completed, cells were selected in hygromycin (0.4 μg/ml) for 14 days. The stable cell line (293-mda-7) was tested for production of sMDA-7/IL-24 protein by Western blot analysis and by ELISA. An aliquot of 10⁶ cells (293-mda-7), as determined by ELISA, produced ~30–50 ng/ml sMDA-7/IL-24 in 24 h. To purify the sMDA-7/IL-24 protein on a large scale, 293-mda-7 cells were grown to 90% confluence in 150-mm tissue culture plates. The tissue culture supernatant was collected and pooled for protein purification by affinity chromatography, as described previously (18). The size and purity of the sMDA-7/IL-24 protein were determined by silver stain gel and by Western blot analyses.

Cell Proliferation Assay. To test the effect of sMDA-7/IL-24 protein on cell proliferation, endothelial cells (HUVECs and HMVECs) were serum-starved overnight. The next day, cells were seeded in 2-well chamber slides (1 × 10⁵/well). The cells were allowed to adhere and spread for 4–6 h, and then we added fresh medium containing 1 ng/ml bFGF as a proangiogenic stimulator and variable concentrations of sMDA-7/IL-24 (1, 5, 10, and 50 ng/ml). Cells treated with PBS served as negative control, whereas cells treated with angiostatin served as positive control. Cells were then harvested 3 days after treatment and cell proliferation determined by trypsin blue exclusion assay as described previously (12). The effect of sMDA-7/IL-24 on lung tumor cell (H1299 and A549) proliferation was also evaluated. The experimental conditions were the same as described above for endothelial cells except that tumor cells were not stimulated with bFGF. Tumor cells treated with Ad-mdr7 (3000 vp/cell) served as positive control.

Endothelial Cell Differentiation Assay. Endothelial cell differentiation (tube formation) assays were done using the in vitro angiogenesis assay kit (Chemicon, Temecula, CA). Briefly, HUVECs and HMVECs were grown to 80% confluence, collected, resuspended in growth medium, and plated at a concentration of 2 × 10⁴ cells/well in a 96-well plate coated with Matrigel (Chemicon). Cells were treated with sMDA-7/IL-24 protein (1, 5, 10, and 50 ng/ml) or a preparation immunodepleted of sMDA/IL-24 protein for 24 h at 37°C. Cells treated with PBS served as negative controls in these experiments. The ability of sMDA-7/IL-24 to inhibit tube formation was determined 24 h after treatment and quantitated by counting the number of tubes under bright-field microscopy.

For experiments involving comparative studies, cells were treated with PBS or with equimolar concentrations of sMDA-7/IL-24 (5, 10, and 300 ng/ml), recombinant human endostatin (5.2, 10.4, and 315 ng/ml; Calbiochem, La Jolla, CA), recombinant IFN-γ (4.5, 9, and 268 ng/ml; R&D Systems, Minneapolis, MN), or recombinant IP-10 (2.4, 4.5, and 134 ng/ml; R&D Systems) and analyzed for tube formation assay as described above. All samples were tested in duplicate. Experiments were repeated at least five to six times.

For receptor blocking studies, HUVECs grown in 6-well plates were pre-treated with a 1:5 ratio of sMDA-7/IL-24 blocking antibody (1 and 5 ng/ml). After overnight incubation, cells were harvested, washed, and plated in Matrigel-coated 96-well plates. Fresh IL-22R1 blocking antibody and sMDA-7/IL-24 were added to the wells in a 1:1 ratio (1 ng/ml IL-22R1 antibody:1 ng/ml sMDA-7/IL-24) or 1:5 ratio (1 ng/ml IL-22R1 antibody:5 ng/ml sMDA-7/IL-24) and incubated at 37°C. After overnight incubation, the plates were examined for tube formation. All other experimental procedures were the same as described above.

For experiments involving endostatin or IP-10, we used higher concentrations of these proteins (endostatin, 315 ng/ml; IP-10, 134 ng/ml) that demonstrated inhibitory activity in tube formation assay. The relative amount of IL-22R1 used for experiments involving endostatin was 315 ng/ml (1:1 ratio) and 134 ng/ml for experiments involving IP-10 (1:1 ratio). All other experimental procedures were the same as described above. For blocking studies using anti-IFN-γ, anti-IFN-γ-neutralizing antibodies (R&D Systems), experiments were conducted as described above for receptor studies except that HUVECs were treated with the appropriate neutralizing antibody (1 and 5 μg/ml) before treatment with sMDA-7/IL-24 (300 ng/ml).

Endothelial Cell Migration Assay. Cell migration assays were performed using HUVECs. Cells were starved overnight in basal medium containing 0.5% fetal bovine serum, collected, resuspended in the same medium, and seeded at a concentration of 10⁶ cells/well on the upper surface of a 24-well transwell insert with a pore size of 8 μm (Millipore, Cambridge, MA). The insert was placed in a 6-well plate that contained medium plus PBS, medium plus VEGF (100 ng/ml), or VEGF plus sMDA-7/IL-24 (5, 10, or 50 ng/ml). The plates containing the transwell insert were incubated at 37°C overnight to allow migration. The next day, the wells were disassembled, membranes were fixed in crystal violet, and the number of cells that had migrated to the lower wells was counted under high-power magnification (×40).

Determination of IFN-γ and IP-10 Production. Recent studies have demonstrated that treatment of PBMCs with sMDA-7/IL-24 results in secretion of IFN-γ (18). Furthermore, IFN-γ is a potent inducer of IP-10 (23). Both IFN-γ and IP-10 have been reported to possess antiangiogenic activity (24, 25). We therefore tested whether the antiangiogenic activity of sMDA-7/IL-24 was mediated by IFN-γ or IP-10. HUVECs were seeded in 6-well plates (1 × 10⁵/well) and treated with sMDA-7/IL-24 (10 ng/ml). Cell culture supernatant was collected at 6, 24, and 48 h after treatment, centrifuged at 1200 rpm, and analyzed for IP-10 and IP-10 protein production using commercially available ELISA kits. Assays were performed as recommended by the manufacturer (R&D Systems). Cells treated with recombinant IFN-γ (4.5 ng/ml) served as positive control for the IP-10 assay, whereas cells treated with Ad-mdr7 (3000 vp/cell) served as positive control for the IFN-γ assay. Cells treated with PBS served as negative controls in these experiments. Samples were analyzed in triplicate, and the data represented as the average value for each concentration of sMDA-7/IL-24 tested.

Western Blot Analysis. Recent studies have demonstrated activation of STAT-3 expression in HACAT cells as a measure of sMDA-7/IL-24 binding to its receptors (16, 17). Therefore, we determined the activation of STAT-3 expression in endothelial cells after treatment with sMDA7/IL-24. HUVECs were seeded in 6-well plates (5 × 10⁵ cells/well) and treated with sMDA-7/IL-24 (10 ng/ml). Untreated cells served as negative control. Cells were harvested at 4 and 24 h after treatment and analyzed for STAT-3 expression by Western blot analysis as described previously (13, 26). pSTAT-3 protein was detected using rabbit antihuman pSTAT-3 antibody (1:1000; Cell Signaling Technology, Beverly, MA) and horseradish peroxidase-labeled secondary antibody (Amersham Biosciences, Piscataway, NJ). Finally, the proteins were visualized on enhanced chemiluminescence film (Hyperfilm; Amersham Biosciences) by application of Amersham’s enhanced chemiluminescence Western blotting detection system. STAT-3 protein expression level was quantitated after normalization with total STAT-3 protein expression using Image Quant software ( Molecular Dynamics; Amersham Pharmacia Biotechnology, Piscataway, NJ).

Immunofluorescence Assay. Activation of STAT-3 was also determined by immunofluorescence assay. HUVECs seeded in 2-well chamber slides (1 × 10⁶ cells/well) were treated with PBS (control) or with sMDA-7/IL-24 (10 ng/ml) for 4 h, washed in PBS, fixed in cold acetic acid, and stained for pSTAT-3 using rabbit antihuman pSTAT-3 antibody (1:1000; Cell Signaling Technology) and rhodamine-labeled antirabbit secondary antibody (1:5000; Molecular Probes, Eugene, OR). Slides were mounted using antifade mounting reagent (Vector Laboratories, Burlingame, CA). Pictures were taken through a fluorescence microscope.

In Vivo Assessment of Antiangiogenic Activity Using the Matrigel Plug Assay. To determine the antiangiogenic activity of sMDA-7/IL-24, an in vivo angiogenesis assay was performed as described previously (25). Briefly, sMDA-7/IL-24 (12.5 ng) and bFGF (60 ng) was mixed with 500 μl of Matrigel (Becton Dickinson, Bedford, MA) on ice and injected s.c. into athymic nude mice. Animals receiving Matrigel containing only bFGF (60 ng) served as positive controls and animals receiving Matrigel containing no growth factor served as negative controls. Each group comprised of 5 animals, and the experiments were performed twice. Animals were sacrificed 10 days after injection. The Matrigel plugs were recovered, photographed, and analyzed for hemoglobin content as described previously (27).

Effects on Xenograft Tumors in Nude Mice. Parental 293 cells and 293-mdar7 cells were first tested for their ability to form tumors. Aliquots of cells (10⁵) were injected subcutaneously in right flank of athymic BALB/c female nude mice and the implantation site monitored for 1 month. No tumors formed at this cell concentration, so subsequent experiments were performed using this cell number. For in vivo mixing experiments, human lung tumor cells (A549) grown to 90% confluence were trypanized, washed, and resuspended in sterile PBS at a concentration of 5 × 10⁶/ml. The tumor-cell suspension was mixed with an equal number (5 × 10⁶/ml) of parental 293 cells or with 293-mdar7 cells, gently vortexed, and injected s.c. in nude mice (10⁵ cells/animal) as described above. Each group comprised 8 animals, and the experiments were done twice. Tumor growth was monitored and measured as described previously (22). At the end of the experiment, animals were eutha-
ized by CO₂ inhalation, and tumors were harvested for histopathological analysis, Western blot analysis, and CD31 and TUNEL staining.

To evaluate the systemic effect of sMDA-7/IL-24 on tumor growth, s.c. tumors were established by injecting A549 tumor cells (5 × 10⁶ cells) into the lower right flank of nude mice. When the tumors were 50–60 mm³ in size, the animals were assigned to one of two groups of 10 mice each. One group of animals received injections of Matrigel containing parental 293 cells (1 × 10⁶), and the other group received injections of Matrigel containing 293-mda-7 cells (1 × 10⁶). The Matrigel containing the cells was injected s.c. into the upper right flank of the tumor-bearing mice. The effect of sMDA-7/IL-24 on tumor growth was monitored as described above. At the end of the experiment, animals were euthanized, and tumors were harvested for additional analyses as described above. All of the animal experiments described were performed at least twice, and the differences in the tumor growth were tested for statistical significance.

Immunohistochemical Analysis. Tumor tissues were stained for CD31 and TUNEL as described previously (22). Tissue sections stained without primary antibody or stained with an isotypic antibody served as negative controls. Staining of tissue sections was analyzed and quantitated, and the results were interpreted in a blind fashion.

Statistical Analysis. Student’s t test was used to calculate the statistical significance of the experimental results. P < 0.05 was considered to be statistically significant.

RESULTS

sMDA-7/IL-24 Inhibits Endothelial Cell Differentiation but not Cell Proliferation. In preliminary studies, we tested the inhibitory effect of sMDA-7/IL-24 on endothelial cell (HUVECs and HMVECs) and lung tumor cell (H1299 and A549) proliferation. Treatment of cells with various concentrations (1, 5, 10, and 50 ng/ml) of sMDA-7/IL-24 resulted in no significant antiproliferative activity compared with PBS-treated control cells (Fig. 1, A and B). Antiproliferative activity against tumor cells was not observed even at higher concentrations (>50 ng; data not shown). However, treatment of HUVECs and HMVECs with the above mentioned concentrations of sMDA-7/IL-24 significantly inhibited (P = 0.001) the formation of capillary tube-like structures by both types of endothelial cells (Fig. 1, C and D). The inhibitory effect was observed at all concentrations and was dose dependent, with a virtually complete abrogation of tube formation occurring at concentrations above 10 ng/ml (Fig. 1D).

To rule out the unlikely possibility that the inhibition of endothelial cell tube formation by sMDA-7/IL-24 protein was attributable to unrelated proteins in the preparation, depletion experiments were performed. Immunodepletion of sMDA-7/IL-24 protein from the test preparation before its addition to HUVEC resulted in complete restoration of endothelial cell tube formation (Fig. 1, C and D). These data show that the observed inhibitory activity in the endothelial cell assays was attributable to sMDA-7/IL-24 and suggest that sMDA-7/IL-24 possesses potent antiangiogenic activity.

sMDA-7/IL-24 Is more Potent than Endostatin in Inhibiting Endothelial Cell Differentiation. We next compared the inhibitory activity demonstrated by sMDA-7/IL-24 with endostatin in tube formation assays. HUVECs were treated with equimolar concentrations of sMDA-7/IL-24 or endostatin. sMDA-7/IL-24 but not endostatin significantly (P = 0.001) inhibited tube formation at low concentrations compared with control cells (Fig. 2). However, endostatin significantly inhibited tube formation (40–50% over control; P = 0.001) compared with control cells at high concentrations (315 ng/ml), demonstrating that the endostatin protein used was functional (Fig. 2). These results indicate that sMDA-7/IL-24 is a much more potent antiangiogenic agent than endostatin.

sMDA-7/IL-24 Inhibits Endothelial Cell Migration. To determine whether sMDA-7/IL-24 inhibited endothelial cell migration, we examined the effect of VEGF on cell migration. sMDA-7/IL-24 inhibited endothelial cell migration significantly (P = 0.001) in response to VEGF (Fig. 3). No inhibitory effect on control cell migration was observed that did not contain sMDA-7/IL-24. Inhibition occurred in a dose-dependent manner, with complete inhibition occurring at 50 ng/ml (Fig. 3). sMDA-7/IL-24 demonstrated a similar inhibitory activity when bFGF was used as an inducer (data not shown).
Inhibition of Endothelial Cell Differentiation by sMDA-7/IL-24 Is not Mediated by IFN-γ or IP-10. Production of IFN-γ by human PBMCs upon treatment with sMDA-7/IL-24 has recently been reported (18). On the basis of this study, we tested whether inhibition of tube formation by sMDA-7/IL-24 was mediated via IFN-γ, or IP-10 production. Tissue culture supernatants from PBS-treated and sMDA-7/IL-24-treated HUVECs were collected at various times and analyzed for IFN-γ and IP-10 by ELISA. sMDA-7/IL-24-induced secretion of IFN-γ (<30 pg/ml) and IP-10 (<32 pg/ml) in a 48-h period compared with control cells (Fig. 4, A and B). To further test whether the low amounts of IFN-γ or IP-10 induced by sMDA-7/IL-24 were responsible for the observed inhibitory effects on HUVEC tube formation, comparative studies were performed. A direct comparison of the inhibitory activity of sMDA-7/IL-24 with IFN-γ or IP-10 at equimolar concentrations showed that higher concentration of IFN-γ (268 ng/ml) or IP-10 (134 ng/ml) was required compared with sMDA-7/IL-24 (10 ng/ml) to significantly inhibit HUVEC tube formation (P = 0.01; Fig. 4C). Additionally, the inhibitory activity of sMDA-7/IL-24 on HUVEC tube formation was not lost in the presence of anti-IP-10 or anti-IFN-γ-neutralizing antibodies (P = 0.001; Fig. 4D). These results indicate that sMDA-7/IL-24 is more potent than IFN-γ and IP-10 in vitro and that sMDA-7/IL-24 mediated inhibitory activity on HUVEC tube formation is not because of IFN-γ or IP-10 induction.

sMDA-7/IL-24 Activates STAT-3 Expression. Recent studies have demonstrated the activation of STAT-3 in HACAT cells and in PBMCs upon receptor engagement by sMDA-7/IL-24 (16, 17). On the basis of these studies, we hypothesized that activity of sMDA-7/IL-24 on endothelial cells was receptor mediated and upon receptor binding would activate STAT-3. Western blot analysis and immunofluorescence assay (Fig. 5, A and B) showed that the addition of sMDA-7/IL-24 to HUVECs increased the expression levels of the pSTAT-3 protein in as little as 4 h (Fig. 5A), and it persisted even at 24 h after treatment. The increase in pSTAT-3 expression was two to three times higher in PBS treated control cells. There was also increased nuclear localization of pSTAT-3 protein in HUVECs after treatment with sMDA-7/IL-24 (Fig. 5B). In contrast, no changes in STAT-3 expression were observed in untreated control cells. Furthermore, STAT-3 activation was inhibited in the presence of anti-MDA-7 antibody, indicating receptor-mediated activation (data not shown).

sMDA-7/IL-24 Mediates Its Inhibitory Activity via Its Receptor. Two related receptors for sMDA-7/IL-24 have recently been identified (16, 17). sMDA-7/IL-24 can bind to either of the two receptor complexes, IL-20R1/IL-20R2 (IL-20 receptor) and IL-22R1 and IL-20R2 (IL-22 receptor). On the basis of these studies, we next determined whether the sMDA-7/IL-24-mediated inhibitory effects on endothelial cells were receptor mediated. We evaluated endothelial differentiation using a blocking antibody against IL-22R1 in the presence or absence of sMDA-7/IL-24 (Fig. 6). sMDA-7/IL-24 (5 ng/ml) alone completely inhibited tube formation in HUVECs, whereas no inhibition was observed in untreated control cells (Fig. 6A). However, pretreatment of HUVECs with IL-22R1 blocking antibody significantly (P = 0.001) abrogated the inhibitory effects of sMDA-7/IL-24 on tube formation and in a dose-dependent manner (Fig. 6A). The addition of 1 ng/ml blocking antibody to HUVECs (1:5 ratio) only partially restored tube formation (<60%), whereas the addition of 5 ng/ml of the blocking antibody (1:1 ratio) completely restored it (>90%). Blocking antibody alone did not significantly affect the ability of HUVECs to form tubes. Furthermore, pSTAT-3 protein expression increased significantly after sMDA-7/IL-24 protein was added to HUVECs, whereas sMDA-7/IL-24-mediated pSTAT-3 expression did not increase in the presence of IL-22R1 antibody (data not shown). These results indicate that sMDA-7/IL-24 mediated its inhibitory effect on endothelial cell tube formation occurs via IL-22R1.

To test the specificity of this inhibition, we treated HUVECs with high concentrations of IP-10 or endostatin in the presence of IL-22R1 antibody. Treatment with IP-10 or endostatin significantly inhibited HUVEC tube formation even in the presence of IL-22R1 antibody (P = 0.001; Fig. 6B) compared with PBS-treated control cells. These results demonstrate that IL-22R1 antibody specifically inhibited sMDA-7/IL-24-mediated activity but not that of endostatin, IFN-γ, or IP-10.

sMDA-7/IL-24 Inhibits Angiogenesis in the Matrigel Plug Model. sMDA-7/IL-24 encapsulated into Matrigel containing bFGF was implanted s.c. into nude mice. Matrigel containing bFGF alone and Matrigel containing PBS served as positive and negative controls, respectively. bFGF-induced angiogenesis was significantly inhibited in the presence of sMDA-7/IL-24 (P = 0.0001; Fig. 7) when compared with Matrigel that contained only bFGF and Matrigel that contained PBS.

sMDA-7/IL-24 Inhibits s.c. Xenograft Tumor Growth in Vivo. Human lung tumor (A549) cells were mixed (1:1 ratio) with either parental 293 cells (control animals) or 293 cells producing sMDA-7/IL-24 protein (293-mda-7) and injected s.c. into the lower right flanks of mice. Tumor growth was significantly less in the animals that...
received a mixture of A549 and 293-mda-7 cells (Fig. 8, A and C) than in the animals that received a mixture of A549 and parental 293 cells (P = 0.001). Injection of 293 or 293-mda-7 cells alone did not form tumors in nude mice (data not shown). The animals were euthanized on day 22 after implantation, and the tumors were harvested and additionally evaluated. Western blot analysis demonstrated that MDA-7/IL-24 protein was expressed in tumors that contained 293-mda-7 cells (Fig. 8B, Lanes 3 and 4). However, the level of MDA-7/IL-24 protein expression was observed to vary between samples. This variation can be explained because of differences in the tumor size that may reflect a difference in the ratio of total amount of MDA-7 protein to the amount of total cellular protein present for each tumor. No MDA-7/IL-24 protein expression was detected in the control tumors that contained parental 293 cells (Fig. 8B). Histopathological examination of the tumor tissues did not reveal any significant differences in the tumor cell proliferative index or tumor cell infiltration between the control and experimental animals (Fig. 8D). However, tumors that contained 293-mda-7 cells demonstrated less vascularization by CD31 staining than did control tumors that contained parental 293 cells (Fig. 8E). TUNEL staining of tumor tissues from experimental animals demonstrated endothelial cells and tumor cells undergoing apoptotic cell death (Fig. 8F). In contrast, no TUNEL-positive staining was observed in control tumor tissues. Additionally, hemoglobin level was significantly (P = 0.02) lower in tumors that contained 293-mda-7 cells than in the tumors that contained parental 293 cells (Fig. 8G). Reduction in CD31 staining and decreased hemoglobin levels indicated sMDA-7/IL-24 inhibited angiogenesis.

**sMDA-7/IL-24 Systemically Inhibits s.c. Xenograft Tumor Growth in Vivo.** We next evaluated whether sMDA-7/IL-24 produced by 293-mda-7 cells can systemically inhibit tumor growth. Mice were inoculated s.c. with A549 tumor cells in the lower right flank. When the tumors reached 50–100 mm³, 293 cells producing sMDA-7/IL-24 protein (293-mda-7 cells) or parental 293 cells (control) were encapsulated in Matrigel and implanted s.c. in the upper right flank. Tumor measurement was initiated after implantation of 293 cells. The growth of A549 lung tumor xenografts was significantly (P < 0.001) less in the mice treated with 293-mda-7 cells than in the control group (Fig. 9A). Compared with tumor growth in the control mice, the growth of the tumors in mice implanted with the encapsulated 293-mda-7 cells was suppressed by 40–50%. To confirm that the inhibitory effect was because of sMDA-7/IL-24, serum samples from animals were tested for MDA-7 protein by Western blot analysis and ELISA. Intense banding of sMDA-7/IL-24 at the expected Mr 40,000 size was observed in the serum of animals implanted with 293-mda-7 cells by Western blot analysis (Fig. 9B, Lanes 1, 3, 5, 7, and 9).

**sMDA-7/IL-24 inhibitory activity is not because of IFN-γ and IP-10 production by HUVECs.** HUVECs seeded in 6-well plates were treated with sMDA-7/IL-24 (10 ng/ml). Cell culture supernatant was collected at the indicated time points and analyzed for IFN-γ and IP-10 (B) by ELISA. Supernatant from HUVECs treated with IFN-γ or Ad-mda7 served as positive controls for IP-10 and IFN-γ ELISA, respectively. Supernatant from PBS-treated cells served as negative control. All treatments were assayed in quadruplicate. C. HUVECs seeded in Matrigel-coated 96-well plates were treated with equimolar concentrations of sMDA-7/IL-24, IFN-γ, or IP-10 and analyzed for tube formation. The inhibitory activity was determined by counting the number of tubes. sMDA-7/IL-24 significantly (P = 0.01) inhibited tube formation at low concentrations compared with IFN-γ or IP-10. Inhibitory activity for IFN-γ or IP-10 was observed only at high concentrations. D. HUVECs pretreated with anti-IP-10 or anti-IFN-γ-neutralizing antibody was seeded in Matrigel-coated 96-well plates and treated with sMDA-7/IL-24 and analyzed for tube formation. sMDA-7/IL-24 significantly (P = 0.001) inhibited tube formation.
Fig. 5. sMDA-7/IL-24 activates STAT-3 protein expression in HUVECs. HUVECs treated with sMDA-7/IL-24 were analyzed for activation of pSTAT-3 protein expression by Western blot analysis and immunofluorescence assay. A, sMDA-7/IL-24-activated pSTAT-3 expression as early as 4 h after treatment compared with control cells. The level of pSTAT-3 activation was two to three times increased over control cells as determined by semiquantitative analysis of the gels. B, immunofluorescence analysis demonstrated nuclear localization of pSTAT-3 in HUVECs treated with sMDA-7/IL-24 compared with control cells.

Fig. 6. sMDA-7/IL-24 inhibits endothelial cell differentiation via the IL-22R1. HUVECs were either untreated or treated with two different concentrations of IL-22R1 blocking antibody for 24 h before seeding in Matrigel-coated 96-well plates that contained PBS or the indicated concentrations of sMDA-7/IL-24, endostatin, or IP-10. The next day, the cells were examined for tube formation under bright-field microscopy and quantitated. A, tube formation was inhibited in sMDA-7/IL-24-treated HUVECs but not in PBS-treated control cells. However, in the presence of IL-22R1 blocking antibody, the inhibitory effect of sMDA-7/IL-24 on HUVEC tube formation was abrogated in a dose-dependent manner. B, endostatin or IP-10 inhibited tube formation of HUVECs pretreated with IL-22R1 antibody. Bars, SE.

Fig. 7. Inhibition of angiogenesis by Matrigel-encapsulated sMDA-7/IL-24 in vivo. sMDA-7/IL-24 (12.5 ng) encapsulated in Matrigel containing 60 ng of bFGF was implanted s.c. into athymic nude mice. Matrigel that contained bFGF only served as positive control, and Matrigel that contained PBS served as negative control. After 10 days, Matrigel was harvested and analyzed for hemoglobin levels as described in “Materials and Methods.” A significant (P = 0.0001) reduction in hemoglobin level was observed in Matrigel containing sMDA-7/IL-24 compared with controls.

DISCUSSION

In this study, we provide evidence for the first time that sMDA-7/IL-24 has potent antiangiogenic activity in vitro and in vivo. In vitro, sMDA-7/IL-24 demonstrated no inhibitory effect on endothelial and tumor cell proliferation. Evaluation of sMDA-7/IL-24 activity against VEGF-stimulated endothelial cell proliferation also demonstrated no significant antiproliferative activity (data not shown). Similarly, sMDA-7/IL-24 demonstrated no antiproliferative activity against tumor cells at concentrations higher (>50 ng) than that used in this study (data not shown). However, sMDA-7/IL-24 inhibited endothelial cell differentiation and cell migration in a dose-dependent manner, with complete inhibition occurring at concentrations >10 ng/ml. The inhibitory effects of sMDA-7/IL-24 were similar to those observed with other antiangiogenic agents (28, 29). On the basis of these studies, we compared the inhibitory effect of sMDA-7/IL-24 to that of endostatin, IFN-γ, and IP-10. The antiangiogenic activity of endostatin, IFN-γ, and IP-10 has been demonstrated previously (24, 25, 30). At equimolar concentrations, the inhibitory effect of sMDA-7/IL-24 on endothelial cell differentiation was 10–50 times more potent than endostatin, IFN-γ, and IP-10 (Figs. 2 and 4). However, endostatin, IFN-γ, or IP-10 demonstrated inhibitory activity at high concentrations in this study. The requirement for higher concentrations of these proteins to inhibit HUVEC tube formation is not surprising and is in agreement with previous reports (25, 31). Similarly, sMDA-7/IL-24 treatment was 20–30 times more effective in inhibiting HUVEC tube formation than sMDA-7/IL-24 (Fig. 6)
formation when compared with treatment with recombinant human IL-10 (unpublished data). The antiangiogenic activity of IL-10 has been demonstrated previously (32). These results demonstrate that sMDA-7/IL-24 has a potent antiangiogenic activity in vitro.

Pretreatment of HUVECs with sMDA-7/IL-24 for 24 h before plating on Matrigel significantly increased the inhibitory effects over that seen when sMDA-7/IL-24 treatment was done at the time of plating the cells on Matrigel (unpublished data). Additionally, sMDA-7/IL-24 inhibited tube formation only on newly forming tubes and not on established tubes (unpublished data). Thus, sMDA-7/IL-24 could block the initiation of differentiation but could not reverse the phenotype of differentiated endothelial cells. These results are similar to the effects mediated by IFN-γ on endothelial cells (33). Induction of IFN-γ/H9253 by sMDA-7/IL-24 in PBMCs has previously been demonstrated (18). Thus, it is possible that in endothelial cells, sMDA-7/IL-24 induces IFN-γ/H9253, which in turn activates its downstream targets, namely IFN-γ-inducible Mr 10,000 protein and monokine induced by IFN-γ, which are known inhibitors of angiogenesis (24, 34). How-
ever, in this study, treatment of HUVECs with sMDA-7/IL-24 did not result in significant levels of IFN-γ or IP-10 production. To exclude the possibility that the sMDA-7/IL-24-mediated inhibitory effect was attributable to IFN-γ or IP-10 produced by HUVECs, in vitro experiments were performed using neutralizing antibodies against these proteins. Loss of sMDA-7/IL-24-mediated inhibitory activity on HUVEC tube formation was not observed even in the presence of neutralizing antibody. Furthermore, as described above, IFN-γ or IP-10 demonstrated significant inhibitory activity in tube formation assay only at high concentrations but not at low concentrations when compared with sMDA-7/IL-24. These results indicate that sMDA-7/IL-24-mediated antiangiogenic activity is not attributable to IP-10 or IFN-γ and occurs via a novel mechanism. Although, we have provided evidence demonstrating IP-10 and IFN-γ does not play a role in sMDA-7/IL-24-mediated antiangiogenic activity in vitro, a note of caution is that in vivo these proteins may be induced by unknown factors produced by the tumor microenvironment as a consequence of MDA-7/IL-24 treatment and may participate in the sMDA-7/IL-24-mediated antiangiogenic activity. We are currently investigating the underlying mechanism of the antiangiogenic activity of sMDA-7/IL-24 in vitro and in vivo.

We next examined whether sMDA-7/IL-24-mediated antiangiogenic activity was receptor mediated in a fashion similar to IFN-γ and thrombospondin. Receptor-mediated antiangiogenic activity has previously been reported for IFN-γ and thrombospondin (35, 36). More recently, receptors for endostatin have been reported (37). In this study, utilization of the receptors for sMDA-7/IL-24 was demonstrated by activation of STAT-3 expression. However, activation of STAT-3 was inhibited in the presence of a blocking antibody to IL-22R1. This inhibition correlated with restoration of tube formation by HUVECs. Furthermore, the IL-22R1 blocking antibody inhibited the antiangiogenic activity of sMDA-7/IL-24 but not that of endostatin or IP-10, demonstrating its specificity. These results strongly suggest that sMDA-7/IL-24-mediated antiangiogenic activity occurs specifically via the IL-22R1. Although we have demonstrated that sMDA-7/IL-24 mediates its effects via the IL-22R1, the role of other receptors (IL-20R) in sMDA-7/IL-24-mediated antiangiogenic activity was not investigated in this study. Additionally, the downstream targets of STAT-3 in sMDA7/IL-24-treated endothelial cells have not been examined. One potential downstream target is IFN-γ (38). However, a role for IFN-γ in mediating the sMDA-7/IL-24 antiangiogenic activity is ruled out as very low levels of IFN-γ were produced by HUVECs upon sMDA-7/IL-24 treatment. Thus, additional STAT-3 targets may exist, a question that warrants additional investigation.

The antiangiogenic activity of sMDA-7/IL-24 was next investigated in vivo. sMDA-7/IL-24 systemically inhibited xenograft tumor growth that was associated with decreased microvessel density. These results demonstrate the antiangiogenic activity of sMDA-7/IL-24 in vivo and correlated with our in vitro studies. However, the mechanism by which sMDA-7/IL-24 inhibited tumor growth in vivo has not been studied. It is possible that sMDA-7/IL-24 may inhibit tumor growth in vivo in two ways. First, sMDA-7/IL-24 may directly inhibit endothelial cell differentiation in the tumor as observed in our in vitro experiments in this study. Second, sMDA-7/IL-24, as with many other antiangiogenic agents, may indirectly exert its antiangiogenic activity by inhibiting the expression of growth factors (IL-8, bFGF, and VEGF) produced by the tumor cells (39–41). Thus, inhibition of these growth factors will result in the failure to support tumor growth and neovascularization. In fact, intratumoral treatment of a s.c. human lung xenograft with an adenoviral vector expressing MDA-7/IL-24 (Ad-mda-7/IL-24) resulted in significant reduction in the expression of bFGF, VEGF, and IL-8 as revealed by immunohistochemical and reverse transcription-PCR analyses (unpublished data). On the basis of these observations, we speculate that sMDA-7/IL-24 in addition to its direct antiangiogenic activity on the tumor endothelial cells may also exert its activity indirectly on the tumor cells by inhibiting the expression of the growth factors produced by the tumor cells. It is therefore interesting to examine the effect of sMDA-7/IL-24 on the regulation of various growth factors produced by the tumor cells in vitro and in vivo that promote angiogenesis. We are currently investigating these possibilities in our laboratory.

Although we have provided evidence that sMDA-7/IL-24 is an antiangiogenic agent in which its effects are receptor mediated, there is little evidence to explain the selective activity for endothelial cells as opposed to tumor cells. It is possible that the difference in activity results from differences in receptor expression in the different types of cells. Alternatively, the downstream signaling mechanism may differ between endothelial and tumor cells. We are currently examining these possibilities in our laboratory.

The source and exact role of MDA-7/IL-24 in normal tissues is not known. However, it is known that MDA-7/IL-24 is expressed in melanocytes and that its expression is lost during tumor progression (7, 14, 42). On the basis of this observation, one may speculate that MDA-7/IL-24 produced by normal tissues inhibits tumor growth and metastasis by affecting the tumor vasculature and by suppressing the signals initiated by the tumor cells. Thus, the ability to inhibit angiogenesis and tumor growth may be one of several functions associated with the sMDA-7/IL-24 protein. Other known functions for sMDA-7/IL-24 include activation of Th1 cytokines (18). Given that sMDA-7/IL-24 functions as a Th1 cytokine, we speculate that sMDA-7/IL-24 may play a role in regulation of the acquired or innate immune system. However, additional studies are required to investigate this possibility.

The data presented here demonstrate that sMDA-7/IL-24 is a potent antiangiogenic agent. Thus, delivery of the mda-7 gene into tumors using gene delivery vectors will have two advantages: first, it will produce direct tumoricidal effects mediated by ectopic expression of MDA-7/IL-24. Second, it will produce sMDA-7/IL-24-mediated antiangiogenic activity as demonstrated in this study. Thus, MDA-7/IL-24 may be an effective treatment for cancers. To this end, a Phase I gene therapy trial evaluating the safety and activity of an adenoviral vector carrying the mda-7 gene (Ad-mda7; INGN-241) in patients with solid tumors has been initiated (43).

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Melanoma Differentiation-associated Gene 7/Interleukin (IL)-24 Is a Novel Ligand That Regulates Angiogenesis via the IL-22 Receptor

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