Combinatorial anti-angiogenic gene therapy in a human malignant mesothelioma model

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Abstract. Anti-angiogenic gene therapy represents a promising strategy for cancer; however, it has rarely been tested in malignant mesothelioma, a highly aggressive tumor associated with asbestos with poor prognosis. In the present study, we investigated whether anti-angiogenic factors such as angiostatin, endostatin and the soluble form of vascular endothelial growth factor receptor 2 (sFlk1) were able to inhibit endothelial cell proliferation via lentivirus-mediated gene transfer into malignant mesothelioma cells in culture. We also assessed whether a dual-agent strategy had greater therapeutic benefit. Human malignant pleural mesothelioma MSTO-211H cells were transduced using lentiviral vectors that individually expressed angiostatin, endostatin and sFlk1 and linked to enhanced green fluorescent protein (EGFP) marker gene expression via an internal ribosome entry site. The lentivirus expressing EGFP alone was used as a control. The resultant cells designated as MSTO-A, MSTO-E, MSTO-F and MSTO-C were confirmed by western blot analysis and fluorescence microscopy to stably express the corresponding proteins. No differences were observed in the *in vitro* growth rates between any of these cells. However, co-culture of MSTO-A, MSTO-E and MSTO-F showed significant suppression of human umbilical endothelial cell growth *in vitro* compared with that of MSTO-C. Furthermore, a combination of any two among MSTO-A, MSTO-E and MSTO-F significantly enhanced efficacy. These results suggest that combinatorial anti-angiogenic gene therapy targeting different pathways of endothelial growth factor signaling has the potential for greater therapeutic efficacy than that of a single-agent regimen.

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Abbreviations: VEGF, vascular endothelial growth factor; EGFP, enhanced green fluorescent protein; HUVEC, human umbilical vein endothelial cells; sFlk1, soluble form of vascular endothelial growth factor receptor 2; LV-A, lentivirus vector expressing angiostatin; LV-E, lentivirus vector expressing endostatin; LV-F, lentivirus vector expressing sFlk1; LV-C, lentivirus vector expressing EGFP alone

Key words: anti-angiogenesis, angiostatin, endostatin, sFlk1, mesothelioma

Introduction

Malignant mesothelioma is a rare asbestos-induced aggressive cancer that arises from the mesothelial cells lining the pleural, peritoneal and pericardial cavities (1,2). Conventional therapies for this malignancy include surgical resection, chemotherapy and irradiation; however, these measures are generally non-curative (2-4). Consequently, novel therapeutic paradigms are urgently required for the effective treatment of this aggressive and currently incurable malignancy.

Recent advances in understanding the regulation of physiological and pathological angiogenesis have revealed the processes by which tumors elicit an angiogenic response and have underscored their requirement for angiogenesis to sustain growth and metastasis (5). Proteins such as angiostatin (6-8) and endostatin (8,9) or vascular endothelial growth factor (VEGF) antagonists such as truncated forms of VEGF receptors (VEGFR) (10-12) have shown antitumor effects in preclinical cancer models. Preclinical studies have shown that angiogenesis has a key role in the biology of malignant mesothelioma (13,14). Previous studies also have shown that mesothelioma cells highly express VEGF and its receptors, VEGFR-1/Flt-1 and VEGFR-2/Flk-1. VEGF stimulates mesothelioma cells growth *in vitro* in a dose-dependent manner and this growth has been shown to be inhibited by anti-VEGF antibodies (15). Mesothelioma patients have among the highest circulating VEGF levels of any solid tumor and high VEGF levels are a poor prognostic factor in this disease (16,17). Hence, anti-angiogenic therapies could be effective in the treatment of patients with malignant mesothelioma.

Bevacizumab (Avastin®), a VEGF-blocking monoclonal antibody, is widely used to treat metastatic colon and non-small cell lung cancer and ocular vascular proliferative disorders (18-20). Systemic administration of bevacizumab has been tested as a therapeutic for malignant pleural mesothelioma. Two non-randomized phase II trials of bevacizumab as maintenance strategy after six cycles of platinum-pemetrexed plus bevacizumab induction did not demonstrate an improvement in median progression-free survival compared with that of historical controls treated with pemetrexed/platinum

combinations (21,22). In a double-blind, placebo-controlled, randomized phase II trial, the addition of bevacizumab to gemcitabine-cisplatin followed by bevacizumab did not improve progression-free or overall survival rate in previously untreated patients with malignant pleural mesothelioma. These limited successes in clinical trials for mesothelioma are probably caused by the inability to achieve long-term and sustained therapeutic levels of anti-angiogenic protein at the tumor via systemic administration of bevacizumab, unlike the application of bevacizumab for the treatment of macular degeneration in which bevacizumab is injected directly into the eye (18). Therefore, persistent *in situ* production of anti-angiogenic factors via gene therapy would be an ideal strategy. We speculated that continuous production of anti-angiogenic factors at the tumors (*in situ*) by gene therapy would achieve maximum therapeutic effect of anti-angiogenic therapies.

In the present study, we investigated whether anti-angiogenic factors such as angiostatin, endostatin and the soluble form of VEGFR-2 (sFlk1) were able to inhibit endothelial cell proliferation via lentivirus-mediated gene transfer into MSTO-211H human malignant mesothelioma cells in culture. We also examined whether a dual-agent strategy could yield greater therapeutic benefit.

Materials and methods

Cell lines. Human malignant pleural mesothelioma MSTO-211H cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone, Logan, UT, USA). Normal human umbilical vein endothelial cells (HUVEC) and their specific media, EGM-2, were purchased from Lonza Japan, Inc. (Tokyo, Japan). The cells were cultured in humidified 5% CO_2 at 37°C.

Vector plasmids and virus production. Self-inactivating lentivirus vectors that individually expressed angiostatin (LV-A) and endostatin (LV-E) have been described previously as Sin-Ang (8) and Sin-End (8), respectively. The lentivirus vector, LV-F, was generated by cloning sFlk1 (provided by J. Folkman, Children's Hospital, Boston, MA, USA) into the Sin-GFP (8) vector construct. Each lentivirus vector contains an EGFP marker gene linked to the transgene expression cassette via an internal ribosome entry site. The lentivirus vector expressing EGFP alone (LV-C), which was described previously as Sin-Ang (8), was used as a control. The preparations of lentivirus vectors pseudotyped with vesicular stomatitis virus G were produced by transient cotransfection of 293T cells, as described previously (23,24). The titers of these vectors were determined by fluorescent protein expression by using a FACSCalibur flow cytometer (Becton-Dickinson Japan, Tokyo, Japan) and expressed in terms of transducing units/milliliter.

Transduction of MSTO-211H mesothelioma cells with lentivirus vectors. MSTO-211H cells were cultured in growth medium to 30-50% confluency at the time of transduction ($1x10^6$ cells/well in 6-well plates). Then, the cells were incubated with lentiviral vectors (LV-A, LV-E, LV-F or LV-C) at a multiplicity of infection of 10 for 72 h; the cells were then subcultured three times to amplify by conventional culture methods and named MSTO-A, MSTO-E, MSTO-F cells and MSTO-C, respectively.

In vitro growth curves. The MSTO-C, MSTO-A, MSTO-E and MSTO-F cells were seeded in triplicate at $5x10^3$/well in 12-well culture plates. Cells in triplicate wells were harvested daily by trypsinization and the number of viable cells was determined by the trypan blue exclusion assay (Sigma-Aldrich Japan, Tokyo, Japan).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis. To confirm the production and secretion of peptides after lentivirus infection, MSTO-C, MSTO-A, MSTO-E and MSTO-F cells were plated on two 10-cm dishes at a density of $5x10^6$/dish. The next day, the media were replaced with 10 ml of serum-free media and incubated for 48 h. The conditioned media were collected and concentrated 20-fold on a Centricon YM-10 column (Merck-Millipore Japan, Tokyo, Japan); Ten microliters of each sample was subjected to SDS-PAGE using 5-20% linear gradient gels (e-PAGEL; ATTO, Tokyo, Japan) and proteins were transferred to polyvinylidene fluoride membranes (Immobilon-P; Merck-Millipore Japan). For western blot analysis, rabbit anti-mouse angiostatin ab2904 (1:1000), rabbit anti-mouse endostatin ab58774 (1:250) or rabbit anti-human VEGF receptor 2 (Flk1) ab39638 (1:250) (Abcam, Cambridge, UK) were used as the primary antibodies and peroxidase-conjugated goat anti-rabbit antibody A6154 (1:2,000) (Sigma-Aldrich Japan) was used as the secondary antibody. Chemiluminescent detection of bound antibodies was performed by using the ECL system (ImmunoStar; Wako, Osaka, Japan).

Co-culture assay. MSTO-C, MSTO-A, MSTO-E and MSTO-F cells were harvested at near confluence using 0.05% trypsin/EDTA solution and were subsequently counted. Then, $1x10^5$ cells were seeded into Transwell chambers for 12-well culture plates with a 0.4 μm pore-size (Corning Costar, Cambridge, MA). HUVEC cells ($5x10^3$) were plated on 12-well plates with the appropriate culture medium. Following 24 h the transwell chambers were assembled on the 12-well plates for co-culture. After 70 h, the cells were collected by trypsinization and counted.

To assess the combination effect of anti-angiogenic factors, MSTO-C, MSTO-A, MSTO-E and MSTO-F cells were harvested at near confluence; the cells were subsequently counted and mixed with each other at a ratio of 1:1 to create the following cell mixtures: MSTO-C/C, C/A, C/E, C/F, A/E, A/F and E/F. These mixed cells ($1x10^5$) were seeded in Transwell chambers. HUVEC cells ($5x10^3$) were plated on 12-well plates with the appropriate culture medium. The Transwell chambers were then assembled on the 12-well plates for co-culture 24 h later. After 70 h, the cells were collected by trypsinization and counted.

Subcutaneous tumor models. MSTO-C, MSTO-A, MSTO-E and MSTO-F cells were harvested at near confluence using 0.05% trypsin/EDTA solution; the cells were subsequently counted and mixed with each other at a ratio of 1:1 to create the following cell mixtures: MSTO-C/C, C/A, C/E, A/E, A/F and E/F. One million of the mixed cells in 100 μl of Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution were

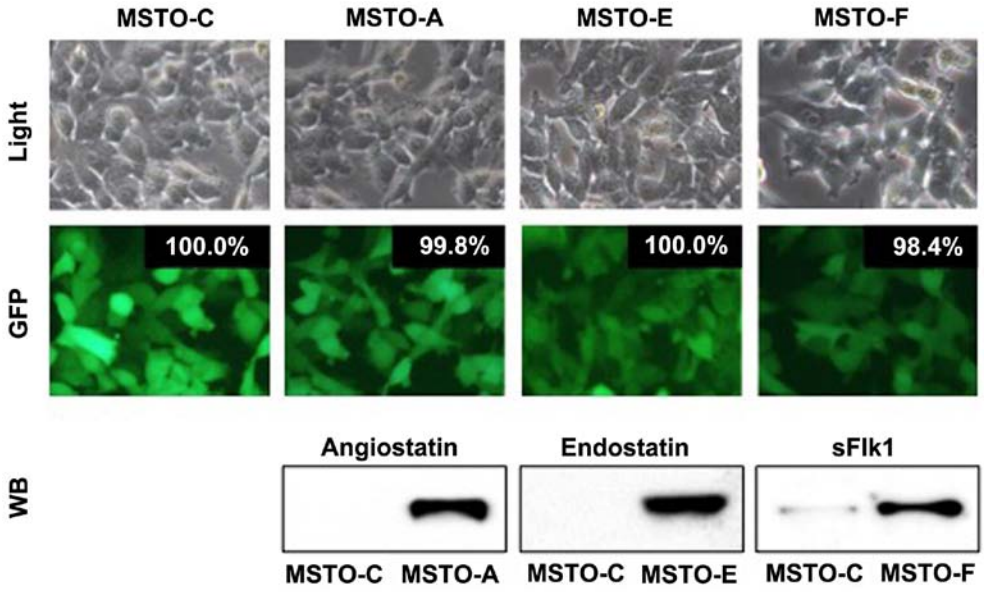

Figure 1. Transduction of human mesothelioma cells by lentivirus vectors expressing anti-angiogenic factors. Human malignant pleural mesothelioma MSTO-211H cells were transduced by using lentiviral vectors individually expressing angiostatin (LV-A), endostatin (LV-E) and soluble-Flk-1 (LV-F) and linked to EGFP marker gene expression via an internal ribosome entry site. The lentivirus expressing EGFP alone was used as a control (LV-C). The resultant cells, MSTO-A, MSTO-E, MSTO-F and MSTO-C, were analyzed for EGFP expression by fluorescence microscopy (% of EGFP-positive cells are indicated as inset numbers). The conditioned media from the lentivirus vector-transduced cells were concentrated and the corresponding anti-angiogenic proteins were analyzed by western blot analysis (WB).

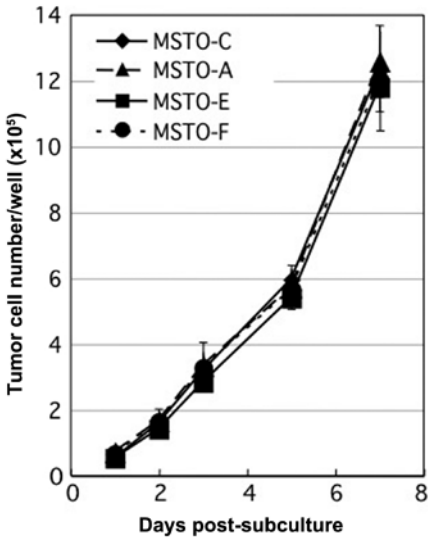

Figure 2. *In vitro* growth of MSTO-211H cells expressing anti-angiogenic factors. The growth rates at 7 days of the MSTO-A, MSTO-E, MSTO-F and MSTO-C cells were compared as determined by comparison of their proliferation in monolayer cultures. Data shown are the mean ± SD calculated from triplicates.

injected subcutaneously on the dorsal flank of 6- to 7-week-old female BALB/c-nu/nu (nude) mice (Charles River Japan, Inc., Yokohama, Japan) (n=10/group). The mice were observed closely and tumors were measured by using a caliper every week over 12 weeks. Tumor volume was calculated as: $axb^2x0.5$, where *a* and *b* were the large and small diameters, respectively.

Statistical analysis. The results were presented as the mean ± standard deviation (SD). The statistical significance of differences was calculated by using Student's t-test and a P-value of <0.05 was considered statistically significant.

Results

Transduction of human mesothelioma cells by lentivirus vectors expressing anti-angiogenic factors. Human malignant pleural mesothelioma MSTO-211H cells were transduced by using the lentiviral vectors LV-A, LV-E and LV-F and linked to EGFP marker gene expression via an internal ribosome entry site. LV-C was used as the control. As shown in Fig. 1, the resultant cells, MSTO-A, MSTO-E, MSTO-F and MSTO-C, were nearly 100% EGFP-positive even after subculturing three times, which indicated highly efficient and stable transduction of MSTO-211H cells by the lentivirus vectors. The conditioned media from the lentivirus vector-transduced cells were concentrated and the corresponding anti-angiogenic proteins were analyzed by western blot analysis. In addition, all anti-angiogenic proteins were observed to be stably expressed and secreted into the cell culture medium after transduction by their respective vectors.

In vitro biological properties of MSTO-211H cells expressing anti-angiogenic factors. We investigated whether anti-angiogenic factors have any biological properties on MSTO-211 cells. Firstly, there was no difference in cell morphology among the MSTO-A, MSTO-E, MSTO-F and MSTO-C cells (Fig. 1). Secondly, no differences were observed in the *in vitro* growth rates after 7 days among the MSTO-A, MSTO-E, MSTO-F and MSTO-C cells (P>0.05) (Fig. 2). These results showed that the expression and secretion of anti-angiogenic factors in MSTO-211H culture did not affect the morphology and growth of MSTO-211H by themselves *in vitro*.

Inhibition of HUVEC growth by co-culture with transduced MSTO-211H human mesothelioma cells expressing anti-angiogenic factors. To investigate whether anti-angiogenic factors secreted from tumor cells inhibit the growth of

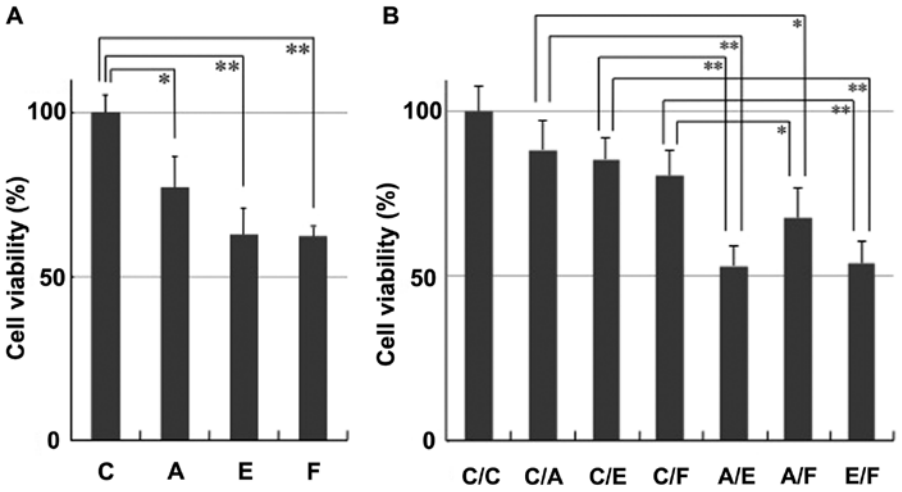

Figure 3. Inhibition of HUVEC proliferation by co-culture with transduced MSTO-211H human mesothelioma cells expressing anti-angiogenic factors. (A) Effect of a single anti-angiogenic factor on HUVEC growth. MSTO-C, MSTO-A, MSTO-E and MSTO-F cells on Transwell chambers were co-cultured with HUVECs on 12-well plates for 48 h. HUVEC growth was then evaluated by cell count. Data were normalized to the value in MSTO-C without expressing any exogenous anti-angiogenic factors and shown as the mean ± SD calculated from triplicates. *P<0.05 and **P<0.01. (B) Combination effect of anti-angiogenic factors on HUVEC growth. MSTO-C, MSTO-A, MSTO-E and MSTO-F cells were harvested and mixed with each other at a ratio of 1:1 to create the following cell mixtures: MSTO-C/C, C/A, C/E, C/F, A/E, A/F and E/F. These mixed cells on Transwell chambers were then co-cultured with HUVECs on 12-well plates for 48 h. HUVEC growth was then evaluated by cell count. Data were normalized to the value in C/C without expressing any exogenous anti-angiogenic factors and shown as the mean ± SD calculated from triplicates. *P<0.05 and **P<0.01.

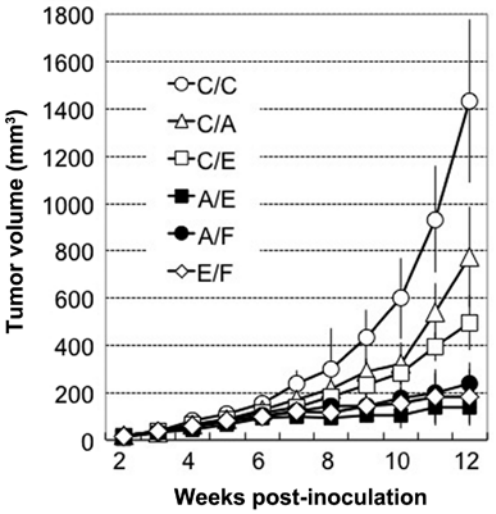

Figure 4. Combination effect of anti-angiogenic factors on subcutaneous mesothelioma tumor growth. MSTO-C, MSTO-A, MSTO-E and MSTO-F cells were mixed with each other at a ratio of 1:1 to create the following cell mixtures: MSTO-C/C, C/A, C/E, A/E, A/F and E/F. One million of the mixed cells were injected subcutaneously on the dorsal flank of nude mice (n=10/group). Tumor volumes were measured every week and data are shown are the mean ± SD.

adjacent endothelial cells, HUVEC cells were co-cultured with MSTO-C, MSTO-A, MSTO-E and MSTO-F cells in a dual-chamber assay (Fig. 3A). Co-culture of HUVEC cells with either MSTO-A (77.3±11.6%, P=0.0191), MSTO-E (62.7±8.3%, P=0.0009) or MSTO-F (62.2±1.5%, P=0.0002) cells resulted in significant inhibition of HUVEC cell proliferation compared with that of MSTO-C cells expressing no exogenous anti-angiogenic factors (100.0±5.2%). These data demonstrate the ability of anti-angiogenic lentivirus vectors to mediate anti-proliferative effects on HUVEC cells indirectly via paracrine secretion from transduced cells.

For assessing the combination effect of anti-angiogenic factors secreted from tumor cells, HUVEC cells were co-cultured with transduced cells mixed with MSTO-C, MSTO-A, MSTO-E and MSTO-F cells at a ratio of 1:1 in a dual-chamber assay (Fig. 3B). Inhibition effects on HUVEC cells in co-culture with any of the cells expressing single anti-angiogenic factors were MSTO-C/A (88.5±8.8%, P=0.1404), MSTO-C/E (85.6±6.3%, P=0.0557) or MSTO-C/F (80.6±7.7%, P=0.0266), and that in co-culture with cells expressing no exogenous anti-angiogenic factors was MSTO-C/C (100.0±7.6%). These inhibition effects on HUVEC cells decreased (MSTO-C/A, C/E and C/F) as compared with those in the previous experiment (MSTO-A, E and F) (Fig. 3A), probably because of a decrease in the percentages of MSTO-A, MSTO-E and MSTO-F cells by mixing with MSTO-C. Notably, any combination of anti-angiogenic factors resulted in enhanced inhibition (A/E, A/F and E/F; Fig. 3B), which suggested that the combinatorial anti-angiogenic therapy has the potential for greater therapeutic efficacy than that of a single-agent regimen.

In vivo combination effect of anti-angiogenic factors on subcutaneous mesothelioma tumor growth. To confirm the therapeutic advantage of combinatorial anti-angiogenic therapy, we examined *in vivo* antitumor efficacy in a subcutaneous MSTO xenograft model in athymic nude mice, which received subcutaneous injection of the following cell mixtures: MSTO-C/C, C/A, C/E, A/E, A/F and E/F. Tumors injected with the cells expressing single anti-angiogenic factors (C/A, C/E) resulted in a significant (46.1-65.4%) reduction in tumor volume compared with that in the C/C group (P<0.05) (Fig. 4). This reduction was unexpectedly high with respect to the *in vitro* co-culture data (Fig. 3B), which suggested that these anti-angiogenic factors possess an indirect effect *in vivo*. Furthermore, the combination of any two among MSTO-A, MSTO-E and MSTO-F significantly enhanced the antitumor

efficacy compared with that of the single anti-angiogenic factor group (MSTO-C/A and C/F), which indicated the feasibility of the combinatorial anti-angiogenic therapy *in vivo*.

Discussion

Tumors elicit an angiogenic response and have been shown to require angiogenesis to sustain growth and metastasis (5). For example, VEGF is produced by a variety of tumors, including malignant mesothelioma and stimulate neovascularization of tumors (4,25). Endothelial cells engaged in angiogenesis express VEGFR-1 and VEGFR-2; however, they produce only low levels of endogenous VEGF for an autocrine/paracrine signal required for endothelial cell survival and vascular homeostasis mainly through intracellular VEGFR-2 (26,27). In addition, VEGFR-2 appears to play a more important role in initiating signal transduction pathways within endothelial cells because of its greater kinase activity and thus, in activating multiple signaling networks that lead to increased proliferation, sprouting, migration and tube formation of endothelial cells (28). Similar to the case described above, anti-angiogenic factors may function as an autocrine or paracrine growth factor for endothelial cells or several types of tumor cells, including mesothelioma. Therefore, continuous *in situ* production of anti-angiogenic factors at the tumors by gene therapy would achieve maximum therapeutic effect of anti-angiogenic therapies. As expected, in the present study, even a single factor significantly suppressed vascular endothelial growth. However, the anti-angiogenic factors, angiostatin, endostatin and sFlk1, did not affect the morphology and growth of any MSTO-211H cells producing anti-angiogenic factors (Fig. 1 and 2). This observation is partially consistent with a previous observation that MSTO-211H expressed VEGFR-2 at the mRNA level but did not produce detectable VEGFR-2 at the protein level (29). Therefore, other growth factor receptors and their signaling pathways may be much more important for the proliferation of this cell line.

In addition, we also showed that a dual-agent strategy may yield greater therapeutic benefit. The anti-angiogenic activities of angiostatin and endostatin interfere with the $\alpha_V\beta_3$ integrin-mediated signaling in endothelial cells (6-9), although sFlk1 uses a different pathway through VEGF-signaling blockage (10-12). Because the combination of angiostatin and endostatin that have the same mechanism of anti-angiogenic action could yield greater therapeutic benefit than a single factor, the integrin pathway may not be saturated even by continuous *in situ* production of a single factor. Our data suggest that *in situ* continuous production of combined anti-angiogenic factors would be absolutely required at levels that cannot be achieved by oral or intermittent systemic administration. However, anti-angiogenic gene therapy targeting the different pathways of endothelial growth factor signaling should be better for long-term success by presumably reducing the risk of resistance.

Anti-angiogenic therapy cannot eradicate tumors by itself and therefore, is a kind of second-line maintenance therapy. Therefore, long-term and combination treatment regimens will be essential in a clinical setting. In the present study, we used lentivirus-based vectors that allowed permanent integration and hence, the potential for stable expression of the delivered transgene. Alternatively, retroviral replicating vectors (RRVs) would be feasible in combination with RRV-mediated prodrug activator gene therapy against malignant mesothelioma because they can keep producing anti-angiogenic factors while a tumor exists (30,31). As a combination, oncolytic viruses such as adenovirus and measles that can selectively kill tumor cells would be feasible because they can keep producing anti-angiogenic factors while viruses exist in the mesothelioma tumors (32-34).

In conclusion, continuous *in situ* production of anti-angiogenic factors at tumors by combinatorial anti-angiogenic gene therapy targeting the same and/or different pathways of endothelial growth factor signaling has the potential for greater therapeutic efficacy than that of a single-agent regimen.

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