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Paradoxical Augmentation of Tumor Angiogenesis Combined with Down-Regulation of IP-10 after Adenovirus-Mediated Transfer of Vasohibin-1 Gene in Cancer Cells

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Abstract

Vasohibin-1 (*VASH1*) is an endogenous angiogenesis inhibitor produced by the endothelium. Here we examined the efficacy of local adenovirus-mediated *VASH1* gene transfer for anti-angiogenic cancer treatment. When non-proliferative adenovirus vector encoding the human *VASH1* gene (Adh*VASH1*) was injected locally into the peritoneal cavity and HM-1 ovarian cancer cells were inoculated into the peritoneal cavity thereafter, we observed a significant inhibition of tumor angiogenesis. However, to our surprise, when HM-1 cells were infected with Adh*VASH1* (HM-1/h*VASH1*) and then inoculated in mice, we observed a paradoxical augmentation of tumor angiogenesis and tumor growth. To explore the mechanism of this augmented tumor angiogenesis, we investigated the expression of various angiogenesis regulators. The level of angiogenesis stimulators such as VEGF and FGF-2 was unchanged; however we noticed a marked down-regulation of one angiogenesis inhibitor, namely interferon-γ-inducible protein-10 (IP-10). Moreover, this down-regulation of IP-10 and augmented tumor angiogenesis were not seen when HM-1/h*VASH1* cells were inoculated into severe combined immunodeficiency mice. Our present analysis reveals that there is a mutual interaction between 2 angiogenesis inhibitors; *VASH1* and IP-10, and the immune reaction might be responsible for this interaction through hitherto unknown mechanism. It also discloses the importance of endogenous IP-10 in tumors, as down-regulation of IP-10 results in the paradoxical augmentation of tumor angiogenesis. Care must be taken when *VASH1* gene is transiently transferred to cancer cells for the anti-angiogenesis treatment.

Keywords: Angiogenesis; Endothelial cells; Cancer cells; Vasohibin-1; IP-10

Introduction

Angiogenesis, i.e., the formation of new capillaries, is a key event in various developmental or remodeling processes that take place under physiological and pathologic conditions. Physiological conditions include organ growth and development, wound healing, and reproduction; whereas pathologic conditions include tumor growth and metastasis, proliferative retinopathy, arthritis, and so forth [1]. In fact, angiogenesis is recognized as one of the principal hallmarks of cancers, and has thus become a target for cancer treatment [2].

Angiogenesis is thought to be regulated by a local balance between endogenous stimulators and inhibitors of this process [3]. A number of angiogenesis inhibitors have been identified to date. For example, thrombospondins (TSPs) are large ECM glycoproteins containing multiple domains that regulate various biological activities. Among the 5 members of the TSP family, TSP-1 and TSP-2 are the most similar in structure and having anti-angiogenic activity [4,5]. Pigmented epithelium-derived factor (PEDF), a member of the serpin superfamily, which lacks inhibitory activity against either serine or cysteine proteinases, possesses anti-angiogenic activity [6]. Growth-related oncogene-β [7], interferon-γ-inducible protein-10 (IP-10) [8] and platelet factor 4 (PF4) [9] are chemokines that exhibit anti-angiogenic activity. Chondromodulin-I is an angiogenesis inhibitor mainly expressed in cartilage [10]. Angiostatin [11], endostatin [12] and tumstatin [13] are angiogenesis-inhibiting proteolytic fragments originating from proteins having no anti-angiogenic activities themselves.

Negative-feedback regulation is one of the most important physiological mechanisms with which bodies are endowed, and it controls a wide range of phenomena. We recently isolated vasohibin-1 (*VASH1*), a protein encoded by a vascular endothelial growth factor (VEGF)-inducible gene in endothelial cells (ECs), and found it to inhibit both migration and proliferation of ECs in culture and to exhibit anti-angiogenic activity *in vivo* [14]. The expression of *VASH1* in ECs is induced not only by VEGF but also by fibroblast growth factor 2 (FGF-2), another potent angiogenesis stimulator [14,15]. Our subsequent analysis confirmed that *VASH1* is expressed in ECs of newly formed blood vessels [16]. Moreover, *VASH1 (-/-)* mice contain numerous immature microvessels in the area where angiogenesis should have terminated in the mouse model hypoxia-induced subdermal angiogenesis [16]. Thus, *VASH1* is an intrinsic angiogenesis inhibitor synthesized by the endothelium during angiogenesis. Indeed, we observed the accelerated tumor growth and metastasis combined with the augmented tumor angiogenesis in *VASH1 (-/-)* mice [17,18].

Anti-VEGF antibody and tyrosine kinase inhibitors targeting VEGF receptors are now in clinical use for anti-angiogenic cancer therapy. However, the benefit of such anti-angiogenic drugs targeting VEGF signals does not last long, as many patients encounter

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progression of their cancers [19,20]. Moreover, as VEGF is required for the maintenance of endothelium, such anti-angiogenic drugs cause adverse effects due to the damage of normal endothelium [21]. Thus, additional or alternative approaches for controlling tumor angiogenesis need to be considered. Endogenous angiogenesis inhibitors can be used for such a purpose. Especially, *VASH1* does not cause any damage of normal endothelium [22,23].

We reported that the stable transfection of *VASH1 gene* in cancer cells had no effect on their growth in culture but inhibited their growth when they are inoculate in mice via the anti-angiogenic activity [14]. Moreover, we and the other group reported that the transient transfection of this gene in the liver by the tail vein injection of adenovirus vector encoding human *VASH1 gene* (Adh*VASH1*) delivered *VASH1* protein systemically and inhibited tumor growth through anti-angiogenesis [17,24,25]. Thus, *VASH1 gene* can be used for the development of anti-angiogenic treatment. Here we examined the efficacy of local adenovirus-mediated *VASH1* gene transfer for anti-angiogenic cancer treatment.

Materials and Methods

Cell culture

Murine ovarian carcinoma cells OV2944-HM-1 (HM-1) were obtained from the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Murine melanoma cells (B16F1) were obtained from Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer at Tohoku University. HM-1 and B16F1 cells were cultured in Dulbecco's modified Eagle's Medium (Wako Pure Chemical Industries, Ltd. Osaka, Japan) supplemented with 10% fetal bovine serum (biowest, Nuaillé, France).

MTT assay

HM-1 cells infected with non-proliferative adenovirus vectors encoding the human *VASH1* or *β-galactosidase* (LacZ) gene (Adh*VASH1* or AdLacZ) were plated in 96-well plates (2000 cells/well) and incubated for 96 hours, after which cell proliferation was measured with a Cell Proliferation Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instruction. Absorbance was measured by use of an Ultrospec 2000 spectrophotometer (Amersham Pharmacia Biotech Inc. Piscataway, NJ, USA).

Tumor-bearing mouse model

All of the animal studies were examined and approved by the committee for animal study at Tohoku University in accordance with established standards of research animal handling. Female B6C3F1 mice, CB17/lcr-Prkdc^{SCID} (severe combined immunodeficiency; SCID) mice, and C57BL/6 mice were purchased from Charles River Japan. All mice were used at 6-8 weeks of age.

For evaluation of the effect of *VASH1* on the peritoneal dissemination of ovarian carcinoma, mice were given an intraperitoneal injection of AdLacZ or Adh*VASH1* (1×10^9 plaque-forming units (pfu)/body). Twenty-four hours later, 2×10^6 HM-1 cells were inoculated intraperitoneally. Alternatively, 2×10^6 HM-1 cells were infected with AdLacZ or Adh*VASH1* and then inoculated intraperitoneally. Eleven days after the inoculation, mice were sacrificed; and tumors in the peritoneal cavity were then excised.

To evaluate solid-tumor growth, 2×10^6 of HM-1 cells or B16F1 cells were subcutaneously inoculated in the left flank of B6C3F1 or C57BL/6 mice. Tumor size was measured by a micrometer caliper, and tumor volume was calculated by using the following formula:

$V=\text{long diameter}\times(\text{short diameter})^2\times0.52$.

Quantitative RT-PCR

Total RNA was extracted by use of ISOGEN (Nippongene, Tokyo, Japan) according to the manufacturer's instructions. First-strand cDNA was generated by using ReverTra Ace (Toyobo, Osaka, Japan), as specified by the manufacturer. Quantitative PCR was performed with a CFX96™ real-time PCR detection system (Bio-Rad Laboratories, Inc, California, USA.) according to the manufacturer's instructions. The primer pairs used were the following: mouse β-actin, 5'-TCGTGCGTGACATCAAAGAG-3' and 5'-TGGACAGTGAGGCCAGGATG-3'; mouse *VASH1*, 5'-AGATTCCCATACCAAGTGTG-3' and 5'-GAGCCTCTTTGGTCATTTCC-3'; human *VASH1*, 5'-AGATCCCCATACCGAGTGTG-3' and 5'-GGGCCTCTTTGGTCATTTCC-3'; *β-galactosidase*, 5'-CCTGGCGTTACCCAACTT AA-3' and 5'-GTGCATCTGCCAGTTTGAGG-3'; mouse IP-10, 5'-CCCACGTGTTGAGATCATTG-3' and 5'-CACTGGGTAAAGGGGAGTGA-3'; mouse VEGF-A, 5'-TGGCTTTACTGCTGTACC TC-3' and 5'-TGGTGAGGTTTGATCCGCAT-3'; and mouse CD3ε, 5'-GATGCGGTGGAACACTTTCT-3' and 5'-ACTGTCCTCGACTTCCGAGA-3'.

Angiogenesis antibody array

HM-1 cells infected with AdLacZ or Adh*VASH1* were inoculated subcutaneously into the left flank of B6C3F1 or SCID mice. At day 17, the tumor was excised; and then 10 mg of the tumor tissue was lysed in PBS containing 1% Triton-X100 (SIGMA ALDRICH Inc., St. Louis, MO, USA) plus protease inhibitors (Roche Applied Science, Mannheim, Germany). The samples were then applied to mouse Angiogenesis Antibody Array membranes (Panomics, Fremont, CA, USA) according to the manufacturer's instructions. The signals were visualized by use of an LAS-4000 mini device (FUJI Photo Film).

Western blot analysis

The samples were prepared by the same procedure described in Angiogenesis Antibody Array. Then the samples were separated by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories, Inc, California, USA.). Blotting was performed according to the standard procedures. The primary antibodies were Rabbit polyclonal to IP-10 (Abcam, Cambridge, MA, USA) (1:200), Monoclonal Anti-β-Actin,antibody produced in mouse (SIGMA ALDRICH Inc. St. Louis, MO,USA) (1:1000), and the secondary antibodies were Anti-Rabbit IgG-Peroxidase produced in goat (SIGMA) (1:1000), Anti-Mouse IgG-Peroxidase produced in goat (SIGMA) (1:1000). Immunoreactive protein bands were detected using ECL Western Blotting Detection Reagents (GE Healthcare) or Immobilon Western HRP Substrate (Millipore) by use of an LAS-4000 mini device (FUJI Photo Film).

Immunohistochemical analysis

Tumor tissues were embedded in O.C.T compound, frozen, sectioned at 7 μm, and fixed with methanol for 20 min at -20°C. After 3 washes in PBS for 5 min each time, the sections were blocked with 1% BSA in PBS for 1 h at room temperature, and then stained with rat anti-mouse CD31 antibody (1:500) (Research Diagnostics Inc. Flanders, NJ, USA) or goat anti-mouse CD3ε antibody (1:500) (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) overnight at 4°C in a humidified chamber. This action was followed by staining with

secondary antibodies, Alexa Fluor® 488 donkey anti-rat IgG (1:500) (Invitrogen Corp., Eugene, OR, USA), Alexa Fluor® 568 donkey anti-goat IgG (1:500) (Invitrogen Corp.) and 4,6- diamidino-2-phenylindole (DAPI) (Invitrogen Corp.) (1:500) for 1 h at room temperature. After 3 washes with PBS for 5 min each time, the sections were covered with fluorescence mounting medium (Dako, Carpinteria, CA, USA) and observed under a fluorescence microscope (BZ-9000; KEYENCE, Osaka, Japan). The CD31-positive area was quantified by using the software Dynamic cell count BZ- II analyzer (KEYENCE).

ELISA for *VASH1*

Conditioned medium for 24 hours were collected from HM-1 cells infected with AdLacZ or Adh*VASH1*. The concentration of human *VASH1* protein in the conditioned medium was determined by ELISA. ELISA for human *VASH1* protein was described previously [24].

Statistical analysis

Data are expressed as means and SD. The statistical significance of the differences was evaluated by Student *t*-test. The significance between survival curves was analyzed by Kaplan-Meier survival analysis with log-rank testing. A value of $P<0.05$ was considered as statistically significance.

Results

Local expression of hVASH1 inhibited tumor angiogenesis in the peritoneal cavity

The local gene delivery in cancer tissue may introduce gene in both interstitial cells and cancer cells. To distinguish the effect on interstitial cells from cancer cells, we injected Adh*VASH1* or AdLacZ locally into the peritoneal cavity of mice, and then HM-1 cells were injected into the peritoneal cavity 24 hours later as a model of peritoneal dissemination. Eleven days after the inoculation, we inspected the peritoneal cavity. The HM-1 cells had disseminated throughout the peritoneum, which had become thicker in the AdLacZ-injected mice (Figure 1A and B). We compared the microvessel density in the disseminated peritoneum and found, as expected, a significant decrease in microvessel density in the Adh*VASH1*-injected mice (Figure 1C and D).

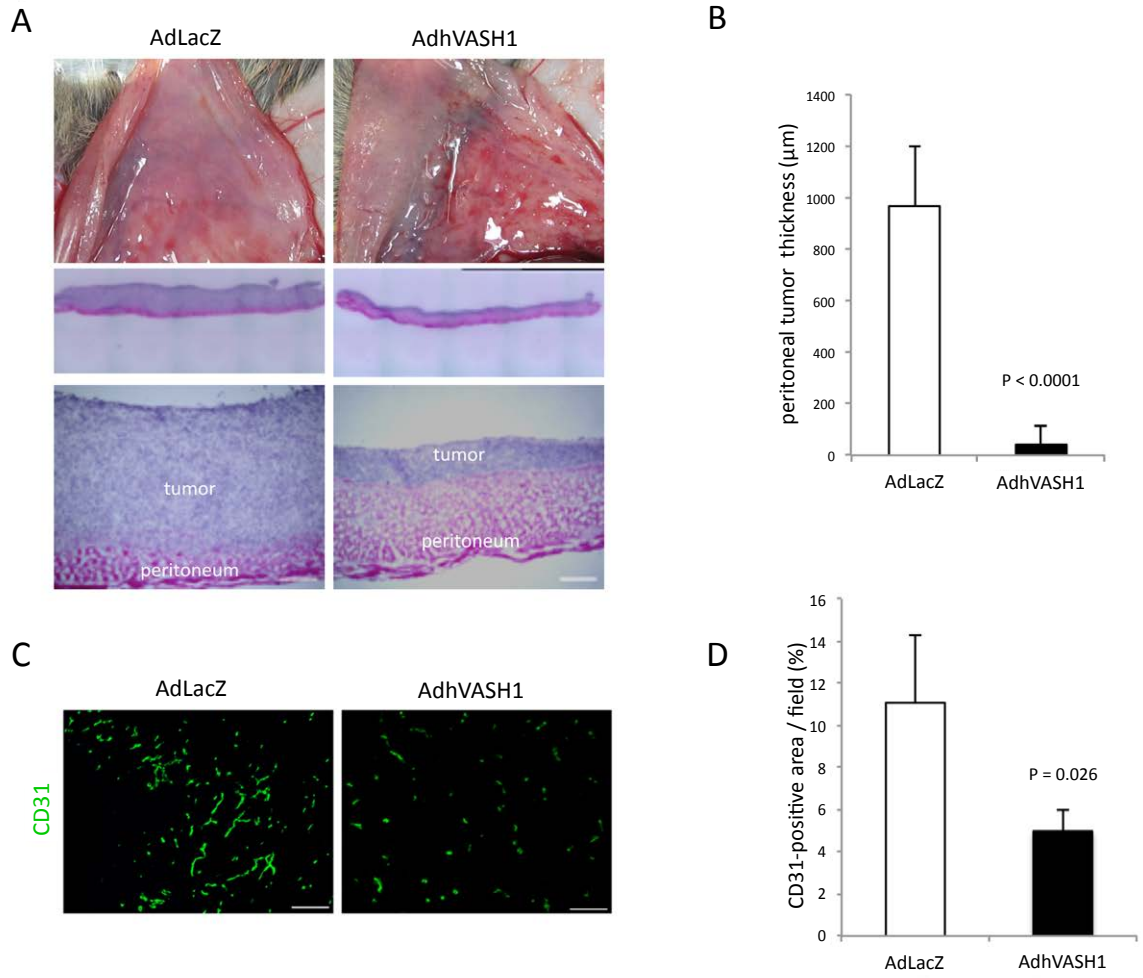

Figure 1: Local infection by Adh*VASH1* inhibited tumor angiogenesis in the peritoneum. A: Adh*VASH1* or AdLacZ ($1x10^9$ pfu) viruses were injected in the peritoneal cavity, and $2×10^6$ HM-1 cells were subsequently inoculated intraperitoneally. Eleven days after the inoculation of HM-1 cells, the peritoneum was inspected. The sagittal section of peritoneum was stained with hematoxylin and eosin. B: The thickness of peritoneal tumor was measured and compared between AdLacZ (N=6) and Adh*VASH1* (N=6) groups. Scale bar=200 μm. Means and SDs are shown. C: Microvessels of the peritoneum were immunostained with antibody against CD31. Scale bar=200 μm. D: The vascular area positive for CD31 was quantified and compared between AdLacZ (N=4) and Adh*VASH1* (N=4) groups. Means and SDs are shown.

Paradoxical augmentation of tumor angiogenesis when h*VASH1* was transiently over-expressed in cancer cells

To know the possible effect of adenovirus-mediated *VASH1* gene delivery in cancer cells, we infected HM-1 cells with Adh*VASH1* (HM-1/h*VASH1*) or AdLacZ (HM-1/LacZ). This infection did not cause any changes in proliferation rate or morphology of the cancer cells (Supplementary Figure 1A and B). After confirming the synthesis and secretion of h*VASH1* protein by the infected cancer cells (Supplementary Figure 1C), we inoculated those cells into the peritoneal cavity. Eleven days after the inoculation, we inspected the peritoneal cavity. To our surprise, we noticed that the peritoneum had become thicker in the mice inoculated with HM-1/h*VASH1* cells (Figure 2A and B). The histological analysis revealed a significant increase in the area occupied by microvessels in the peritoneum of the HM-1/h*VASH1*-injected mice (Figure 2C and D). We noticed that mice inoculated with HM-1/h*VASH1* contained more ascites and died earlier than mice inoculated with HM-1/LacZ (Figure 3A and C). Importantly, the expression of

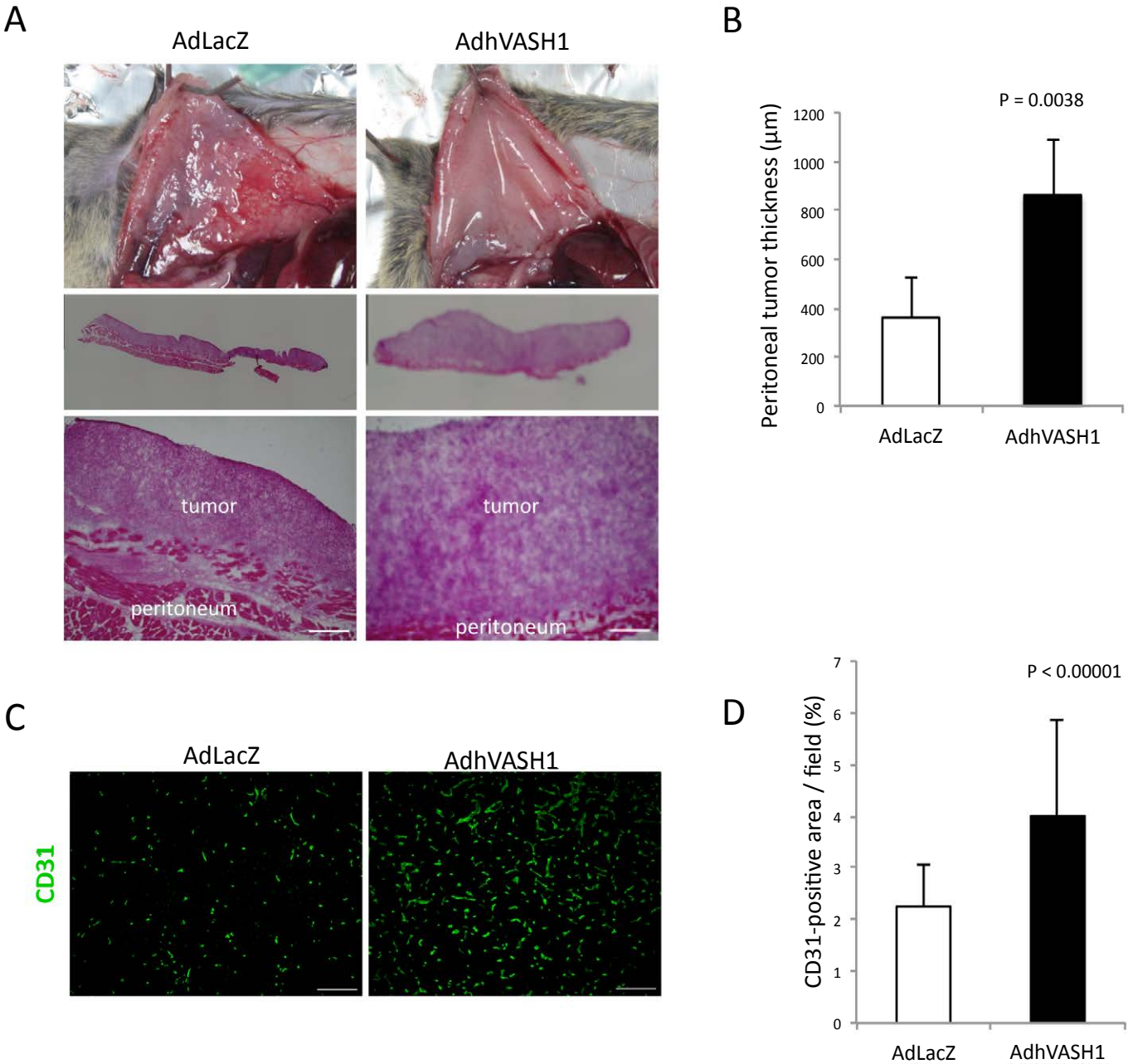

Figure 2: Infection of Adh*VASH1* in HM-1 augmented tumor angiogenesis in the peritoneum. A: HM-1/LacZ or HM-1/h*VASH1* cells were inoculated into the peritoneal cavity of mice (N=4). Eleven days after the inoculation, the peritoneum was inspected. The sagittal section of peritoneum was stained with hematoxylin and eosin. Scale bar=200 μm. B: The thickness of peritoneal tumor was measured and compared between HM-1/LacZ (N=5) and HM-1/h*VASH1* (N=5) groups. Means and SDs are shown. C: Microvessels of the peritoneum were immunostained with anti-CD31 Ab. Scale bar=200 μm. D: CD31-positive vascular area was quantified and compared between HM-1/LacZ (N=6) and HM-1/h*VASH1* (N=4) animals. Means and SDs are shown.

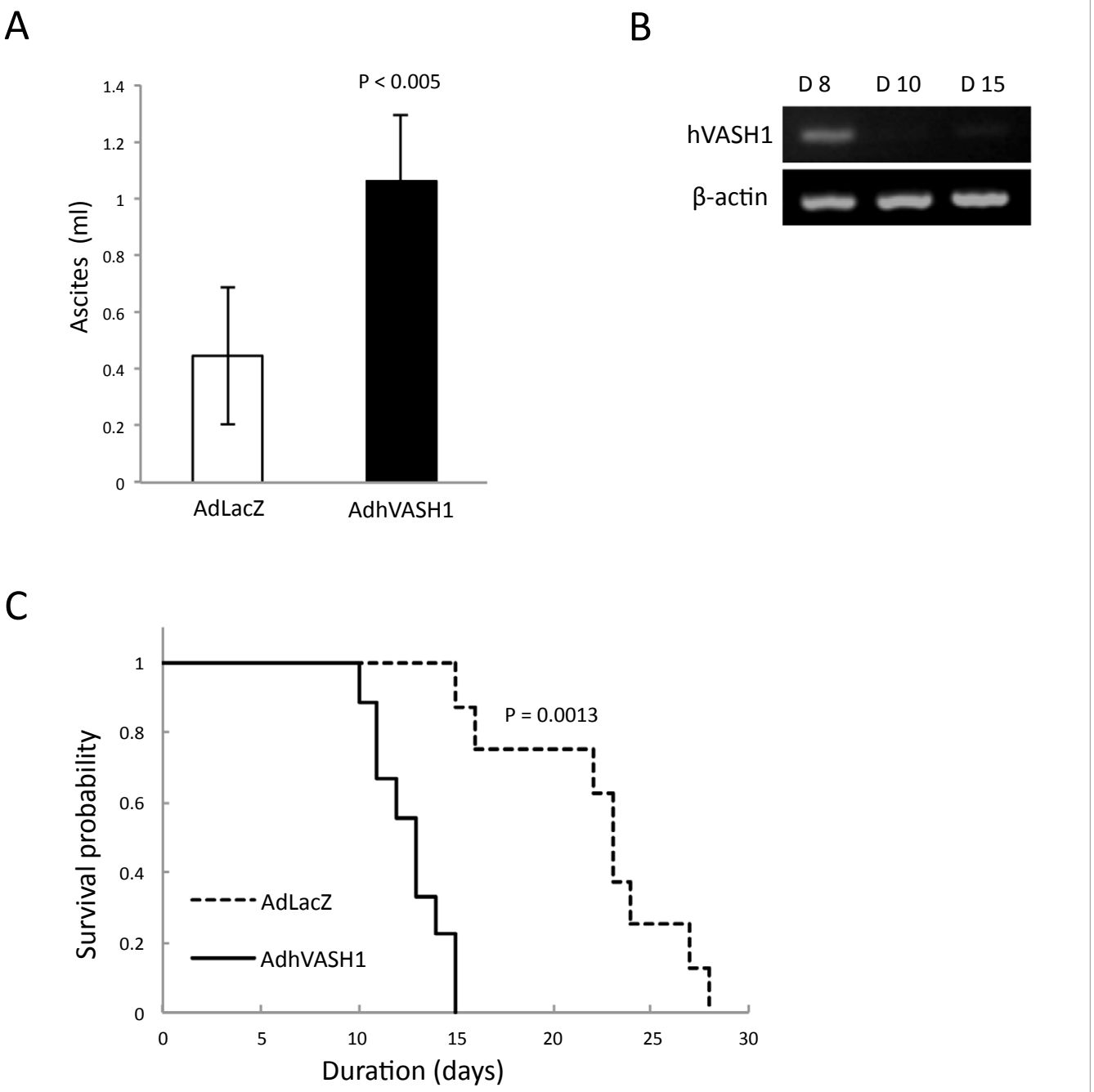

Figure 3: HM-1/h*VASH1* tumors resulted in a worse outcome when inoculated into the peritoneal cavity. A: HM-1/LacZ or HM-1/h*VASH1* cells were inoculated into the peritoneal cavity of mice (N=4). Eleven days after the inoculation, the ascites fluid was collected and its volume measured. Means and SDs are shown. B: The time course of expression of h*VASH1* in the inoculated HM-1/h*VASH1* cells was examined by RT-PCR. C: Survival probability was compared between mice with HM-1/LacZ (N=8) and mice with HM-1/h*VASH1* (N=8).

h*VASH1* in the transfected HM-1 cells was disappeared by 10 days after the inoculation (Figure 3B), and this was accompanied by the decrease of survival probability (Figure 3C).

We next inoculated adenovirus infected HM-1 cells subcutaneously and examined the tumor growth. Likewise, HM-1/h*VASH1* tumors grew bigger (Figure 4A), and the histological analysis again revealed a significant increase in the microvessel area in the HM-1/h*VASH1* tumors (Figure 4B). The expression of h*VASH1* in the transfected HM-1 cells was again disappeared by 10 days after the inoculation (data not shown).

Moreover, we substituted these cancer cells with B16F1 cells to assess the reproducibility of this paradoxical augmentation of tumor growth and tumor angiogenesis. When B16F1 cells were infected with Adh*VASH1* or AdLacZ and then inoculated subcutaneously, again we observed that the B16F1-h*VASH1* tumors grew bigger (Figure 4C), and contained more microvessels than the control (Figure 4D).

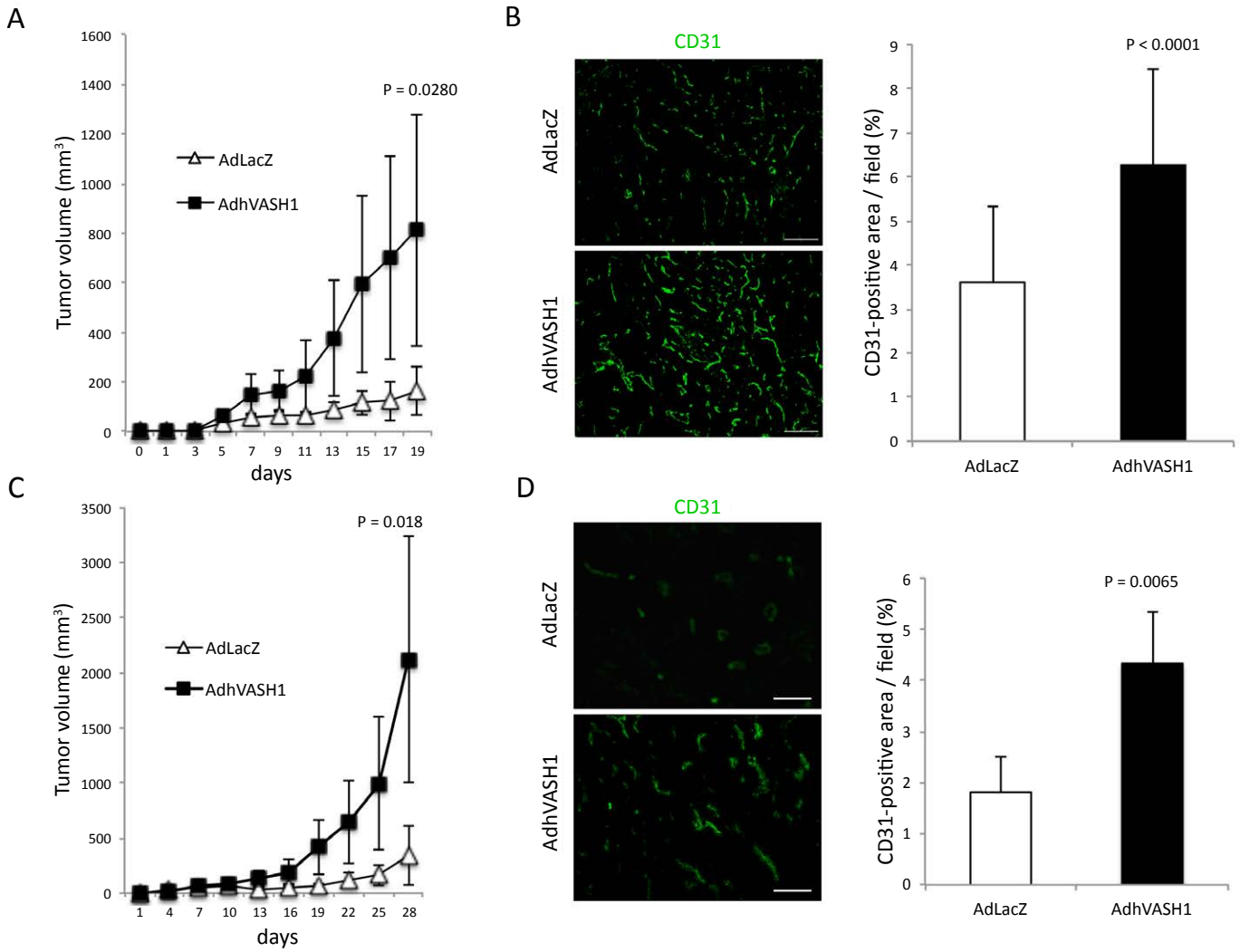

Figure 4: Enhanced tumor growth and tumor angiogenesis with subcutaneously inoculated HM-1/h*VASH1* cells or B16F1/h*VASH1* cells. A: Syngeneic mice were subcutaneously inoculated with HM-1/LacZ (N=4) or HM-1/h*VASH1* (N=4) cells, and the tumor size was measured. B: Microvessels in the subcutaneous tumor were immunostained with anti-CD31 Ab. Scale bar=200 μm. CD31-positive vascular area was quantified and compared between HM-1/LacZ (N=4) and HM-1/h*VASH1* (N=4) mice. Means and SDs are shown. C: Syngeneic mice were subcutaneously inoculated with B16F1/LacZ (N=4) or B16F1/h*VASH1* (N=5), after which the tumor size was measured. D: Microvessels in the subcutaneous tumor were immunostained with anti-CD31 Ab. Scale bar=200 μm. CD31-positive vascular area was quantified and compared between B16F1/LacZ (N=4) and B16F1/h*VASH1* (N=5) tumors. Means and SDs are shown.

Altered expression of endogenous angiogenesis inhibitors

To explore the mechanism of this paradoxical augmentation of tumor angiogenesis, we obtained subcutaneous tumor tissue and compared the expression of various endogenous angiogenesis regulators. We noticed that the transient over-expression of human *VASH1* decreased the expression of endogenous mouse *VASH1* expression. Moreover, an Angiogenesis Antibody Array revealed that one of angiogenesis inhibitors, IP-10, was absent from the HM-1/h*VASH1* tumors (Supplementary Figure 2). This aborted expression of IP-10 was confirmed by Western blotting (Figure 5A) and quantitative RT-PCR (Figure 5B). In contrast, the levels of angiogenesis stimulators including VEGF and FGF-2 were unchanged (Supplementary Figure 2). IP-10 is an anti-angiogenic molecule, but it also has an effect on T-cell trafficking. Indeed, we observed that infiltration of CD3ε-positive T-lymphocytes was significantly decreased in the HM-1/h*VASH1* tumors (Figure 5C). We assumed that this decreased infiltration of T-lymphocytes was the consequence of decreased IP-10 activity in the HM-1/h*VASH1* cells.

The decreased infiltration of T-lymphocytes might suggest that the immune system was modified in the mice bearing the HM-1/h*VASH1* tumors. To exclude the possible influence of an altered immune system, we employed SCID mice. Intriguingly, the expressions of IP-10 was identical between HM-1/LacZ and HM-1/h*VASH1* tumors in SCID mice (Figure 6A), and the paradoxical augmentation of tumor growth and tumor angiogenesis did not occur when the HM-1/h*VASH1* cells were inoculated in SCID mice (Figure 6B and C).

Discussion

Here we transiently over-expressed *VASH1* gene in cancer microenvironment by the use of a non-proliferative adenovirus vector, and examined the efficacy of local *VASH1* gene delivery on anti-angiogenic cancer treatment. The local gene delivery in cancer

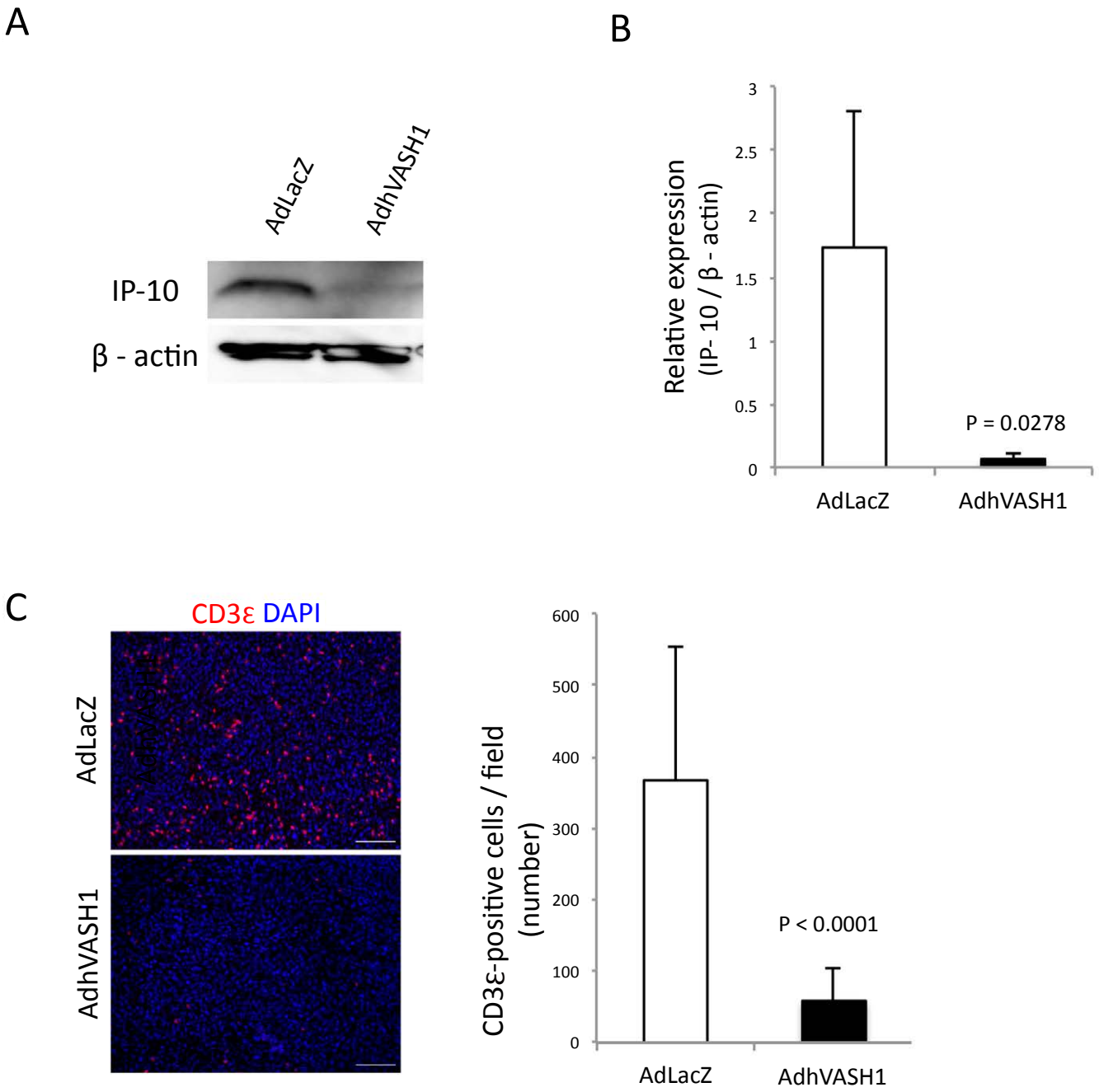

Figure 5: Down-regulation of IP-10 in the HM-1/h*VASH1* tumors. A: The protein level of IP-10 in the tumors of HM-1/LacZ (N=3) and HM-1/h*VASH1* (N=3) mice were compared by Western blot analysis. Means and SDs are shown. B: The expression of IP-10 mRNA in the tumors of HM-1/LacZ (N=3) and HM-1/h*VASH1* (N=3) mice were compared by quantitative RT-PCR. Means and SDs are shown. C: Infiltrated T-lymphocytes that had infiltrated into the tumor were immunostained with anti-CD3ε Ab. Scale bar=100 μm. DAPI (blue) was used for nuclear staining. The number of CD3ε-positive T-lymphocytes in the HM-1/LacZ (N=5) and HM-1/h*VASH1* (N=5) tumors was compared. Means and SDs are shown.

tissue may introduce gene in both interstitium and cancer cells. As expected, tumor angiogenesis and tumor growth were inhibited when the *VASH1* gene was over-expressed in local environment and then cancer cells were inoculated there. We next wanted to clariy the effect of local *VASH1* gene delivery on cancer cells. To our surprise, when *VASH1* gene was transiently over-expressed in cancer cells and then those cancer cells were inoculated in mice, we observed the paradoxical enhancement of tumor angiogenesis and tumor growth.

We previously reported that the stable transfection of *VASH1* gene in cancer cells inhibited tumor growth through anti-angiogenic activity [14]. The obvious difference in the present study from the previous one was the adenovirus-mediated transient transfection, as the expression of h*VASH1* in cancer cells disappeared by 10 days after the inoculation (Figure 3B). Angiogenesis is determined by the local balance between angiogenesis stimulators and angiogenesis inhibitors. We therefore speculated that the balance tilted to pro-angiogenesis, and found that

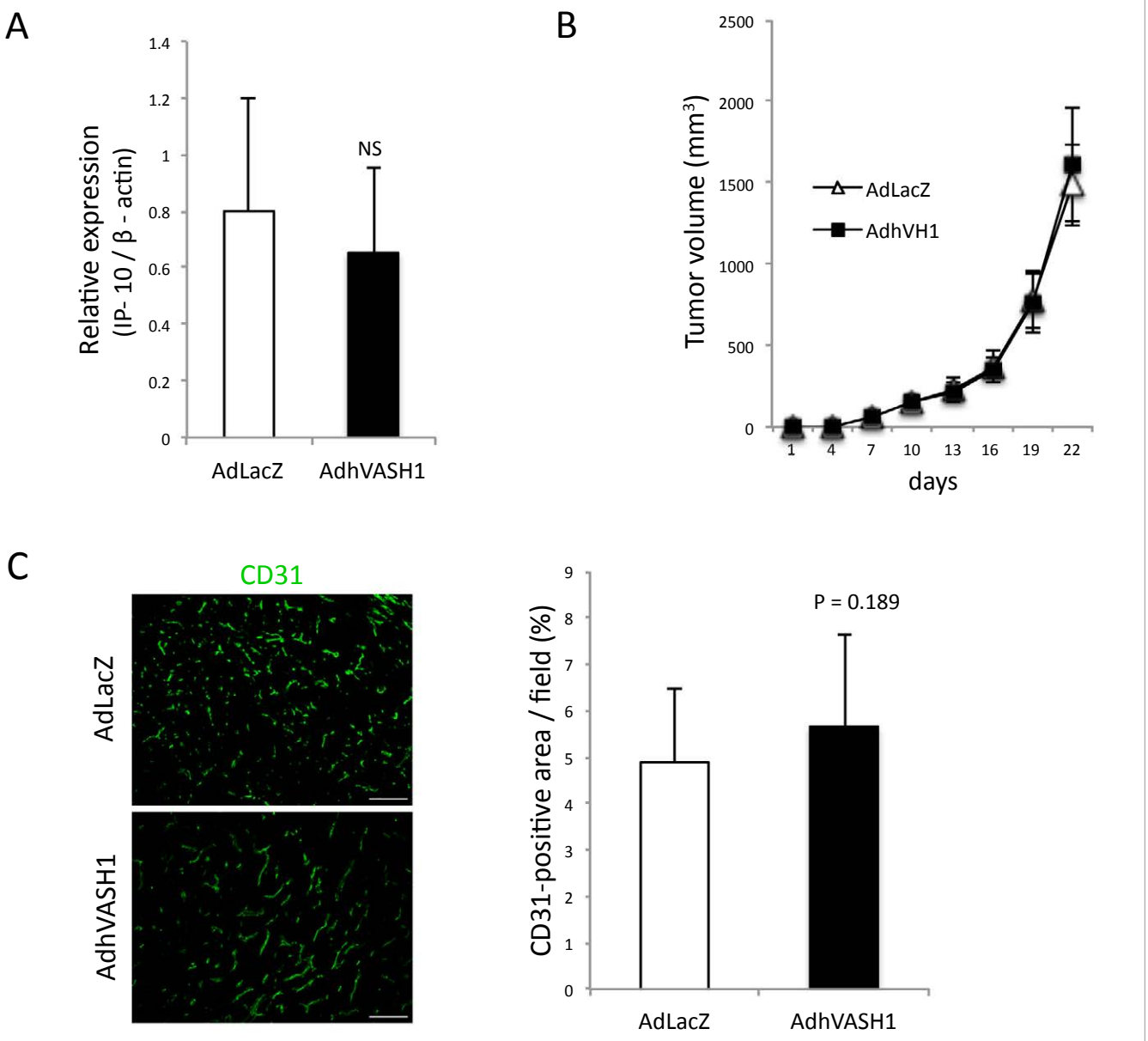

Figure 6: Tumor growth and tumor angiogenesis were unchanged when HM-1/h*VASH1* cells were subcutaneously inoculated in SCID mice. A: SCID mice were subcutaneously inoculated with HM-1/LacZ (N=5) or HM-1/h*VASH1* (N=5) cells. The expression of IP-10 in the tumors was compared by quantitative RT-PCR. Means and SDs are shown. B: SCID mice were subcutaneously inoculated with HM-1/LacZ (N=5) or HM-1/h*VASH1* (N=5) cells, after which the tumor size was measured. C: The tumor microvessel density was assessed on day 28 post inoculation by immunostaining with anti-CD31 Ab. Scale bar=200 μm. H: The CD31-positive vascular area was quantified and compared between HM-1/LacZ (N=4) and HM-1/h*VASH1* (N=4) tumors. Means and SDs are shown.

the paradoxical enhancement of tumor angiogenesis was associated not with the up-regulation of angiogenesis simulators such as VEGF and FGF-2 but with the down-regulation of one angiogenesis inhibitor, namely IP-10. IP-10, also known as CXCL10, is secreted by several cell types including monocytes, endothelial cells and fibroblasts [26]. IP-10 is one of the ligands of CXCR3, and plays roles in angiogenesis inhibition as well as in T-cell trafficking [27,28]. Hence the influence of IP-10 down-regulation was evident in both augmented tumor angiogenesis and decreased T-cell infiltration in HM-1/h*VASH1* tumors. This down-regulation of IP-10 was not evident when HM-1/h*VASH1* cells were inoculated into SCID mice, and tumor angiogenesis was not enhanced in that condition. From these results, we propose that the deregulation of IP-10 is at least one of the causes for the enhancement of tumor angiogenesis after the adenovirus-mediated transfection of *VASH1* gene into cancer cells.

We recently observed that when *VASH1* gene was transiently introduced in ECs of tumor vessels in vivo, it inhibited tumor growth and tumor angiogenesis (Horie et al. unpublished observation). Thus, the most intriguing question is the mechanism how IP-10 down-regulated when *VASH1* is transiently over-expressed in cancer cells. We observed that the down-regulation of IP-10 disappeared when the h*VASH1*-synthesizing cancer cells were inoculated into SCID mice. We therefore speculate the immune system is somehow involved the down-regulation of IP-10 when *VASH1* is transiently over-expressed

in cancer cells. However we could not disclose the precise mechanism at this moment. Further study is required to clarify this mechanism.

In terms of endogenous angiogenesis inhibitors expressed in cancer environment, many studies have focused on TSPs [29]. Nevertheless, a limited number of studies reported IP-10 to be a favorable prognostic marker in certain cancers [30,31]. Here we unexpectedly disclosed the impact of IP-10 in cancers, as down-regulation of endogenous IP-10 accelerated tumor growth and tumor angiogenesis. It would be worthy to extend the analysis of IP-10 on various cancers and see whether its expression would modulate their prognosis.

In summary, we observed paradoxical augmentation of tumor angiogenesis by the transient transfection of cancer cells with the *VASH1* gene. We speculate that the immune reaction somehow deregulates endogenous angiogenesis inhibitors after the transfection. We believe that the cell type specific gene delivery system needs to be established. At least, the *VASH1* gene should not be transiently transferred to cancer cells for the anti-angiogenesis treatment.

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