Proteomic Screening of Anaerobically Regulated Promoters from Salmonella and Its Antitumor Applications*§

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Solid tumors often contain hypoxic and necrotic areas that can be targeted by attenuated Salmonella typhimurium VNP20009 (VNP). We sought to develop a hypoxia-inducible promoter system based on the tumor-specific delivered strain VNP to confine expression of therapeutic gene specifically or selectively within the tumor microenvironment. A hypoxia-inducible promoter - adhE promoter was screened from the hypoxia-regulated endogenous proteins of Salmonella through two-dimensional gel electrophoresis and matrix-assisted laser desorption ionization-time-of-flight/time-of-flight MS-based proteomics approaches. The efficiency and specificity of the selected adhE promoter were validated first in both bacteria and animal tumor models. The adhE promoter could specifically drive GFP gene expression under hypoxia, but not under normoxia. Furthermore, luciferase reporter expression controlled by the system was also confined to the tumors. Finally, we investigated the anticancer efficacy of VNP delivering human endostatin controlled by our adhE promoter system in both murine melanoma and Lewis lung carcinoma models. Our results demonstrated that by the dual effects of tumoricidal and anti-angiogenic activities, the recombinant Salmonella strain could generate enhanced antitumor effects compared with those of unarmed VNP treatment or untreated control. The recombinant VNP could retard tumor growth significantly and extend survival of tumor-bearing mice by inducing more apoptosis and more severe necrosis as well as inhibiting blood vessel density within tumors. Therefore, VNP carrying the endostatin gene under our tumor-targeted expression system holds promise for the treatment of solid tumors. Molecular & Cellular Proteomics 10: 10.1074/mcp.M111.009399, 1–11, 2011.

Tumor targeting VNP20009 (VNP)¹ was a genetically modified strain of Salmonella typhimurium (S. typhimurium) YST2 with partial deletion in the msbB and purF genes for reducing toxicity (1–4). VNP has a propensity to multiply preferentially in solid tumors and consequently retards tumor growth and prolongs survival of tumor-bearing mice (1, 3). The mechanisms behind this phenomenon remain unclear (1, 3, 5). Histological studies show that Salmonella preferentially replicates within and adjacent to necrotic areas within tumors, and the distribution pattern of Salmonella within a tumor may reflect oncolysis and tumor necrosis formation (3, 6–8). However, bacterial growth alone in tumors is not sufficient to overcome recalcitrant malignant tumors. To enhance tumor suppression efficacy of the bacteria, investigators have explored an alternative strategy of delivering specific therapeutic genes or proteins to tumors but not other tissues by exploiting Salmonella. However, they found that, following the administration of attenuated Salmonella to tumor-bearing animals, the bacteria colonized not only tumor sites but also normal tissues (1). Because the recombinant Salmonella strains are mostly engineered to carry a strong constitutive promoter to drive the therapeutic gene of interest, this biodistribution pattern is likely to cause undesired side effects and compromise the specificity of the Salmonella-mediated gene transfer system (9, 10). High levels of expression of heterologous proteins of therapeutic interest from the strong constitutive promoter, on the other hand, may lead to accumulation of toxic levels of this agent in bacterial cells, which should result in dramatic plasmid loss during infection (11). One way to address these issues and to obtain limited gene expression when using attenuated Salmonella is to exploit the unique hypoxic tumor microenvironment.

Targeting angiogenesis to kill tumor cells is one of the promising therapeutic approaches for cancer. Endostatin, a

¹ The abbreviations used are: VNP, VNP20009; 2-DE, two-dimensional electrophoresis; adhE, alcohol dehydrogenase E; CAM, Chick chorioallantoic membrane; H&E, hematoxylin and eosin; TUNEL, terminal deoxynucleotidyl transferase-mediated d-UTP nick and labeling; VEGF, vascular endothelial growth factor; SpA, staphylococcal protein.
Application of A Hypoxia Specific Salmonella Promoter in Tumor Therapy

C-terminal fragment of collagen XVIII, exhibits potent antiangiogenic activity and has been shown to inhibit tumor growth without leading to tumor resistance in preclinical studies (12, 13). However, in phase I clinical studies, recombinant endostatin only induced minor antitumor effect in cancer patients (14, 15). In view of the complex biologic heterogeneity of tumors, a combination of anti-angiogenic therapy with other modalities would be expected to enhance therapeutic efficacy. More recently, our group has concentrated on such a therapeutic approach by combining VNP with human endostatin. This combination therapy significantly enhanced therapeutic efficacy in murine the melanoma model (16). These results prompted us to examine the feasibility of using VNP-producing human endostatin as a potent anticancer agent in murine tumor models.

Proteomics is a promising approach in the identification of proteins that may be used as new targets for therapeutic intervention. Two-dimensional gel electrophoresis (2-DE) is a powerful proteomic technique to differentially display protein expression and post-translational modifications. 2-DE can separate thousands of proteins based on their differences in charge and size. In the present work, we sought to profile protein expression in Salmonella under anaerobic and aerobic conditions by two-dimensional gels. Among several proteins that were found to be up-regulated under anaerobic growth, alcohol dehydrogenase (AdhE), a protein of the oxidoreductase family, was up-regulated by more than a hundred-fold. We therefore chose AdhE for further validation and functional analysis.

We first developed this hypoxia-inducible, Salmonella-endogenous promoter, adhE promoter, and defined its tumor-selective delivery and expression of target gene. We then sought to investigate the anticancer efficacy of VNP-expressing human endostatin controlled by the hypoxia-inducible promoter on tumor-bearing animals. We found that by the dual effects of tumoricidal and anti-angiogenic activities, the bacteria armed with endostatin expression in tumors could produce potent anticancer activity, suggesting that the system could be of potential clinical interest for targeted tumor therapy.

EXPERIMENTAL PROCEDURES

Expression Vectors—Enzymes for vector construction were purchased have been sent to Joan Chessboard and Jeanne Gladfelter from Takara (Kusatsu, Japan). Primers used in this study are listed in supplemental Table 1. Oligonucleotide primers AP-1/AP-2 were used to amplify the region upstream of the adhE (described below) gene of VNP. The promoterless GFP gene was amplified from pEgFP (Clontech, Mountain View, CA) with specific primers GFP1 and GFP2 and then cloned downstream of the adhE promoter region, generating the adhE-GFP fusion gene. To construct suicide vector pDMSB-GFP, mutant msbB fragment amplified from VNP chromosomal DNA was cloned into suicide plasmid pDS132 (kindly provided by Dr. Schneider) (17) and subsequently interrupted by the adhE-GFP fusion gene. The coding sequence of the Flu gene from plasmid pGL3-basic (Promega, Madison, WI) and SpA secretion signals from plasmid pEZZ18 (Clontech, Walkersville, MD) were amplified using two separated PCR reactions. These two products were pooled and fused by overlapping PCR method. Then, the overlapping fragment was inserted into the low-copy plasmid pBR322 (Takara) to generate pLuc (promoterless) or sequentially linked downstream of the adhE promoter region by second-round overlapping PCR. The adhE-luciferase fusion was finally cloned into pBR322, yielding plasmid padhEluc. Similarly, human endostatin amplified from pET23a-rhEndostatin was fused to the gene fragment encoding Spa secretion signal and placed it under the control of the adhE promoter regulator cassette by means of two rounds of overlapping PCR. The final DNA fragment was further inserted into pBR322 generating vector padhEEndostatin.

Bacteria, Transformation and Colony Formation Assays—Lipid A-modified (msbB`) auxotrophic (pur`) S. typhimurium strain VNP20009 was obtained from American Type Culture Collection (ATCC, Rockville, MD) and grown at 37 °C to the mid-logarithmic phase in LB broth. The bacteria (2.0 × 10⁹ cfu) were harvested at 4 °C and dissolved in 40 μl 10% glycerol and then mixed with 1 μl appropriate vectors (0.5 μg/μl) for electroporation using a Gene Pulser apparatus (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions and subsequently cultured on LB plates with appropriate antibiotics at 37 °C. For colony formation assays, the number of colony forming units (CFUs) was determined as previously described (18). For induction of the expression of genes of interest, anaerobic LB medium was prepared by boiling to remove dissolved oxygen and residual oxygen was further driven out by nitrogen for 15 min in boiling water. Then, 100 μl 0.05% sodium sulfide and 0.05% cysteine were added to maintain the reducing environment in anaerobic jars (Oxid, London, England). VNP20009 carrying appropriate expression vectors was inoculated and grown to the mid-logarithmic phase.

Two-dimensional Gel Electrophoresis and Matrix-assisted Laser Desorption Ionization-time-of-flight (MALDI-TOF) MS—Ten milliliters VNP cultures were grown under aerobic and anaerobic conditions, respectively, to 10⁹ cells/ml and harvested by centrifugation at 4 °C. The bacterial pellets were rinsed three times with ice-cold phosphate buffered saline (PBS) and pelleted at 2000 × g for 5 min. Then, the pellets were lysed in a cell lysis solution containing 7 M urea, 2 M thiourea, 40 mM dithiotreitol, and 2% IPG buffer (pH 3–10 L, GE Healthcare) at a volume ratio of 1:10 frozen at liquid nitrogen and thawed for three times. The lysates were sonicated in short bursts to disrupt nucleic acids, and then were clarified by centrifugation at 40,000 × g at 4 °C for 60 min. The protein concentration in the supernatant was determined by Bradford method. All procedures were performed on ice. The supernatant containing 120 μg protein was first isoelectric focused on a gel strip with 3–10 linear pH gradient, and then resolved on an SDS poly acryl amide gel as previously reported (19). The separated proteins were visualized by silver dianne-staining as described before (20). After destaining with double distilled water, gels were scanned at 300 dpi resolution, and the images were processed using the Adobe Photoshop software (Adobe Systems) and analyzed using the Image Master Platinum™ software (GE Healthcare) according to the manufacturer’s procedures. Over a twofold change in protein concentration was considered to be significant (p < 0.01 by Mann-Whitney test). The differentially expressed proteins were cut and digested essentially as described by Yang et al. (21).

MALDI samples were prepared according to a thin layer method described before (21, 22). Mass spectra were recorded on an Ultraflex II MALDI-TOF-TOF mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) under the control of FlexControl™ 3.0 software (Bruker Daltonics GmbH). MALDI-TOF spectra were recorded in the positive ion reflector mode in a mass range from 700–4000 Da, and the ion acceleration voltage was 25 kV. Acquired mass spectra were processed using the software FlexAnalysis TM 3.0 (Bruker Daltonics, ETX, Bruker Daltonics, Germany). The MALDI samples were prepared by mixing the purified protein samples at appropriate concentrations with 2-μl matrix (cysteine, 1 mg/ml) in the MALDI sample matrix. MALDI samples were prepared from the bacterial lysate by mixing the bacterial lysate with an equal volume of 3-μl MALDI sample matrix.

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Bremen, Germany): Peak detection algorithm: SNAP (Sort, Neaten, Assign, and Place); signal-to-noise (S/N) threshold: 3; Quality Factor Threshold: 50. The tryptic auto-digestion ion picks (trypsin, [108–115], MH+ 842.509, trypsin, [58–77], MH+ 2211.104) were used as internal standards. Matrix and/or auto-proteolytic tryptic fragments or known contaminant ions (keratins) were excluded. The resulting peptide mass lists were used to search NCBI nr 20101105 (101942 sequences) with Mascot (v2.3.02) in automated mode. Using the following criteria for search parameters: significant protein MOWSE score at p < 0.05, minimum mass accuracy 120 ppm, trypsin as enzyme, 1 missed cleavage site allowed, alkylation of cysteine by carbamidomethylation as fixed modification and oxidation of methionine as variable modification. In addition, the Mascot Score and expectation of the first nonhomologous protein to the highest ranked hit were checked.

**Chromosomal Expression of Green Fluorescent Protein (GFP)—** For construction of single copy GFP gene fusion, the adhE-GFP fusion gene was integrated into the chromosome of VNP by using a suicide plasmid system as previously described (17). Briefly, the suicide vector pDSMSB-GFP carrying the adhE-GFP fusion was introduced into VNP by electroporation (25 μF, 400 Ω and 1.8 kV). Resolution of the pDS132 sequence and the origin copy of the msbB deletion present in VNP resulted in a clone that contained the adhE-GFP fusion inserted into the chromosome at the msbB deletion locus. The integrity of the adhE-GFP fusion in the engineered strain was confirmed by PCR and the resulting strain was designated as mutVNP-GFP.

To estimate the expression levels of GFP, mutVNP-GFP was grown to the mid-logarithmic growth (OD600≈0.4) phase under aerobic or anaerobic condition as described above. One-milliliter aliquots of bacterial culture were harvested by centrifugation, and bacterial pellets were washed with PBS and air dried for 20 min, which were then examined under a fluorescence microscope. Furthermore, the remaining bacterial suspension was lysed by sonication and filtered through a 0.22 μm-pore-size filter to remove residual intact bacteria. The absorbance spectrum at 485 nm was used to calculate the amount of GFP as previously described (11).

Luciferase Assays—Tumors, spleens, and livers from mice bearing tumors were homogenized in cold PBS at a ratio of 1:1 (weight:volume). The tissue homogenates were then lysed in 50 mM Tris-HCl (pH 8.3), 4 mM dithiothreitol, 20% (v/v) glycerol, 2% (v/v) Triton X-100, and 2 mg/ml lysozyme for 10 min at 25 °C. Fluor activity was determined using the Luciferase Assay System (Promega) according to the manufacturer’s instructions. Emission was measured in a Lumat LB9507 luminometer (Berthold Detection Systems, Oak Ridge) and calculated using the formula: tumor volume = length × width² × 0.52 (25). Tumor doubling time was the time for a tumor to double in volume, and tumor growth delay was the difference between the time required for tumors in treated mice and control mice to reach at least 1000 mm³ in volume as described before (26).

H&E, Terminal Deoxynucleotidyl Transferase Mediated d-UTP Nick and Labeling (TUNEL) and Immunohistochemical Assay—Tissue sections (5 μm in thickness) were prepared according to standard protocols for hematoxylin/eosin (H&E) staining. Apoptotic cells in tumor sections (three sections per mouse, totaling three mice) were stained according to standard immunohistochemistry protocols. The number of microvessels in the field with the highest vessel density (“hot spots”) was determined according to the method as previously described (27–29). Three fields with the highest vessel density per section were counted. Microvessel density was determined by averaging the number of microvessel in counted fields.

**Statistical Analysis**—Data were expressed as mean ± S.D. and analyzed using the SPSS software. Paired Student’s t test was performed to assess statistical significance. Differences between experimental groups were considered significant if the p value was less than 0.05.
The alcohol dehydrogenase (adhE) gene is specifically induced in Salmonella under anaerobiosis. A, Bacterial lysate (120 μg protein) was first isoelectrically focused on a gel strip with 3–10 linear pH gradient, and then separated on an SDS-polyacrylamide gel. In the sections of the representative gels, three protein spots (as shown by the red arrows) with differences in protein expression patterns beyond twofold under anaerobic or aerobic conditions were determined by mass spectrometry. Zoomed sections of the anaerobically inducible protein, AdhE, as indicated by the red frame, and B, The regulated elements in the adhE promoter region.

RESULTS

The Alcohol Dehydrogenase (adhE) Gene Is Specifically Induced in Salmonella Anaerobically—Tumor-specific promoter is critical and urgently needed for tumor-targeted therapy. Considering the unique hypoxic microenvironment in tumor, we sought to screen protein candidates that were specifically expressed under hypoxia by the high throughput proteomic approach. Proteomic profiling of Salmonella under anaerobic growth and aerobic culture was performed in parallel by two-dimensional analyses. The locations of informative spots on two-dimensional images of silver-stained two-dimensional gels are shown in Fig. 1A. Ten proteins with at least a twofold expression difference on two-dimensional gels consistently in at least three paired samples were selected for MS analysis (Table I). As shown in Fig. 1 and Table I, among the ten identified proteins, alcohol dehydrogenase (adhE), exhibited a 120.3-fold increase in expression under anaerobic growth compared with growth under aerobic condition. The tandem MS (MS/MS) data supporting this assertion appeared to be reliable (MASCOT score, 308; sequence coverage, 53%). In view of the fact that adhE is an important factor specifically expressed in Salmonella under anaerobic growth, we subsequently focused on this target gene in designing hypoxia-specifically induced promoter for tumor-targeted therapy.

The adhE Promoter Functions Effectively and Efficiently in S. typhimurium—Based on the published sequence of the Salmonella adhE gene, we screened for the effective adhE promoter region (about 1200 bp in length) (data not shown) in VNP20009 and determined its in vitro activities. To allow accurate measurement of its activities in vitro, we first integrated a single-copy of the fused adhE-GFP gene into the bacterial chromosome via homologous recombination (Fig. 2A) and then monitored GFP expression under inducing conditions. We found that Salmonella only growing under anaerobic condition exhibited intense fluorescence (Fig. 2B), demonstrating that the adhE promoter possessed excellent anaerobic specificity by presenting its activity under hypoxia, but not under normoxia. Our unpublished work has found that inability of Salmonella to secrete expressed proteins leads to low or even no therapeutic efficacy. In this study, we added the Spa secretion signal peptide sequence in front of endostatin (Fig. 2C). Western blotting assays showed that endostatin was effectively secreted by our recombinant strain VNPpadhLEndostatin as demonstrated by the presence of a 20 kDa protein band in both the extracellular and intracellular fractions of recombinant Salmonella growing under anaerobiosis, which however, was not observed under aerobic condition (Fig. 2D).

The adhE Promoter Effectively Drives Target Gene Expression in the Tumor where S. typhimurium Strain VNP20009 Preferentially Accumulate—We injected VNP carrying the plasmids padhELuc or pLuc intraperitoneally into mice bearing B16F10 melanoma xenograft. Examination of colony formation in the tumor, spleen, and liver of mice 3 days after inoculation revealed that the bacteria carrying padhELuc or pLuc were enriched over a 1000-fold in the tumors (Fig. 3A), suggesting a preferential localization of the bacteria in the tumor. Furthermore, virtually no detectable luciferase activity was seen in the liver and spleen whereas intense luciferase activities were observed in the tumor at day 3 and 6 post inoculation (Fig. 3B). We further examined the growth of the bacteria carrying padhELuc at day 5 and 15 post inoculation and found that the bacteria carrying padhELuc grew well in the inoculated mice and similar numbers of colony forming units (CFUs) were found of the bacteria carrying padhELuc and bacteria carrying no plasmid, (Fig. 3C), indicating that the presence of padhELuc in the bacteria did not compromise the growth of the bacteria and the plasmids were stably maintained in the bacteria over 15 days. Fluorescence microscopy further revealed that mutVNP-GFP preferentially accumulated in the hypoxic, necrotic area of the tumor (Fig. 3D). These findings together suggested that S. typhimurium strain VNP preferentially accumulated in the tumor tissue, particularly in the hypoxic necrotic areas of the tumor, allowing localized expression of GFP by the adhE promoter.
Promoter AdhE-mediated Tumor-targeted Therapy with Endostatin Significantly Suppresses Melanoma or Lung Cancer Xenograft Growth in Mice—Given that S. typhimurium strain VNP2009 preferentially accumulated in tumor tissues, allowing selective expression of genes of interest in the region driven by the adhE promoter, we evaluated the effect of VNPpadhEEndostatin on tumor growth in the mouse xenograft model. We inoculated mice bearing Lewis lung cancer xenograft with VNPpadhEEndostatin or VNP. Mice inoculated with VNPpadhEEndostatin or VNP showed markedly reduced tumor growth over time compared with controls \( (p < 0.01 \text{ versus control at day 6 to 12}) \) (Fig. 4). Furthermore, tumor doubling time was significantly prolonged from 3.46 days (CI, 3.23–2.68 days) in control mice or 4.44 days (CI, 4.27–4.61 days) in mice receiving VNP to 5.21 days (CI, 4.99–5.42 days) in mice receiving VNPpadhEEndostatin \( (p < 0.001) \) (Fig. 4 and Table II). In addition, tumor growth delay was also markedly increased from 8.45 days (CI, 8.27–8.64 days) in control mice or 11.43 days (CI, 11.22–11.64 days) in mice receiving VNP to 14.54 days (CI, 14.22–14.86 days) in mice receiving VNPpadhEEndostatin \( (p < 0.001) \) (Fig. 4 and Table II). In mice bearing melanoma xenograft, VNPpadhEEndostatin significantly suppressed the growth of melanoma mice with a markedly reduced tumor volume than that in mice receiving VNP or control mice (Fig. 4). Furthermore, tumor doubling time was significantly prolonged in mice receiving VNPpadhEEndostatin (6.96 days; CI, 6.86–7.07 days) compared with that in mice receiving VNP (5.86 days; CI, 5.70–5.98 days) or control mice (2.96 days; CI, 2.85–3.08 days) \( (p < 0.001) \) (Fig. 4D and Table II). In addition, tumor growth delay was significantly increased in mice receiving VNPpadhEEndostatin (17.07 days; CI, 16.97–17.17 days) compared with that in mice receiving VNP (12.90 days; CI, 12.67–13.05 days) or control mice (7.56 days; CI, 7.48–7.64 days) \( (p < 0.001) \) (Fig. 4D and Table II).

Promoter adhE Mediated Tumor-targeted Therapy with Endostatin Markedly Extends the Survival of Mice Bearing Lung Cancer or Melanoma Xenograft—We further analyzed the effect of Salmonella-mediated targeted therapy with the
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**DISCUSSION**

Hypoxia is a natural and typical characteristic of many solid tumors, and appears to be strongly associated with malignant progression or resistance to radiotherapy and most chemotherapeutics. Hence, approaches that can specifically target hypoxic areas of solid tumors are of considerable interest. We have previously shown that in vitro and in vivo expression of the hypoxia-specific promoter adhE in Salmonella typhimurium can be induced in hypoxic environments, thus yielding a potential tool for targeting hypoxic areas of solid tumors.

Some studies have shown that the tumor hypoxic area is a natural and typical characteristic of many solid tumors and displays specific features. These features include preferential accumulation of drugs, increased retention of nanoparticles, and a unique cellular environment that can affect the efficacy of tumor therapy. Therefore, it is important to develop targeted therapy strategies that can specifically target hypoxic areas of solid tumors.

It is well known that hypoxia plays a critical role in tumor progression and resistance to therapy. Hypoxic areas of solid tumors can exhibit unique cellular properties, such as increased retention of nanoparticles and preferential accumulation of drugs. This suggests that targeting hypoxic areas of solid tumors may be an effective strategy for improving tumor therapy.

**REFERENCES**

1. Meier survival analysis showed that inoculation with VNPpadhEEndostatin significantly improved survival (p < 0.01) and the 30-daysurvival rate was 86% versus 71% for VNPpadhEEndostatin and VNP, respectively (Fig. 5A and Table II).

2. In addition, the median survival for mice bearing melanoma receiving VNPpEndostatin was 28.5 days (CI, 16.18–32.82 d), which was significantly longer than of control mice (11.5 days; CI, 9.42–13.08 days) (p < 0.01) or mice receiving VNP (19.5 days; CI, 13.60–26.65 days). Moreover, the 30-day survival rate was 25% versus 0% for VNPpEndostatin and VNP, respectively (Fig. 5B and Table II).

3. VNPpadhEEndostatin Inhibits Neoangiogenesis Both In Vitro and In Vivo—To determine whether endostatin produced by VNPpadhEEndostatin inhibited angiogenesis in vivo, we performed CAM assays at the 8th day of chick embryo development. As shown in Fig. 6A, an avascular zone around the filter disk was observed with VNPpadhEEndostatin treatment in a dose-dependent manner compared with controls or VNP alone, indicating that indeed VNPpadhEEndostatin was capable of inhibiting in vivo angiogenesis. We were also interested in whether VNPpadhEEndostatin inhibited tumor angiogenesis. We examined the expression of VEGF by ELISA. We found that VNPpadhEEndostatin markedly inhibited the expression of VEGF compared with VNP treated alone (p < 0.05) (Fig. 6B). Immunohistochemical studies further revealed that VNPpadhEEndostatin markedly reduced the expression of CD31, a marker of neoangiogenesis, compared with controls (Figs. 6C and 6D). Our findings indicated that VNPpadhEEndostatin likely suppressed tumor growth and improved survival of melanoma-bearing mice by inhibiting neoangiogenesis.

4. VNPpadhEEndostatin Induced Necrosis of Melanoma, Promoted VNP Tumor Colonization and Melanoma Cells Apoptosis—We further examined whether VNPpadhEEndostatin therapy was associated with enhanced necrosis or apoptosis of tumor cells. Our light microscopy revealed that melanoma tissues in mice receiving VNPpadhEEndostatin displayed more severe necrosis than control or VNP treatment. VNP alone or untreated controls displayed tissue necrosis interspersed with viable tumor cells, whereas VNP padhEEndostatin induced large areas of continuous necrosis within tumors (Figs. 7A and 7B). TUNEL analysis of the B16F10 tumors was performed to assess whether the antitumor effect by VNPpadhEEndostatin was attributed to the induced tumor cell death via apoptosis in vivo. The results showed an increase in the amount of cells undergoing apoptosis within the tumors after treatment with VNPpadhEEndostatin, as compared with those of other groups (Figs. 7A and 7B). These findings suggest that VNPpadhEEndostatin treatment inhibited B16F10 tumors through induction of programmed cell death in vivo. We further analyzed tumor colonization by VNPpadhEEndostatin. We found that the hypoxic and necrotic environment attracted more Salmonella intratumoral proliferation or colonization (Figs. 7C).

5. **FIG. 3.** Preferential accumulation of S. typhimurium strain VNP20009 in B16F10 tumors allowed targeted expression of genes in the tumor. A. Colonization of host tissue by VNP bearing plasmids with inducible luciferase. Tissue homogenates were plated at consecutive time points during the course of induction. (Mean ± S.D., n = 3, 1p < 0.001 tumor versus liver or spleen). B. Luciferase reporter of the adhE promoter in tumor bearing C57BL/6 mice. Schematic diagram of luciferase reporter vectors in the studies is shown and promoter in vivo activity was determined from tissue homogenates. For each time point three animals were used and experiments were performed three times with similar results. (Mean ± S.D., n = 3, 1p < 0.001). C. Stability of padhEEndostatin in tumors. The number of total VNP was quantified on LB agar (open symbols, Amp−) and the number of VNPpadhEEndostatin isolated on LB agar containing antibiotic (closed symbols, Amp+). Each point represents one single mouse. D. Fluorescent microscopy demonstrated preferential accumulation of mutVNP-GFP in the necrotic and hypoxic area in B16F10 tumor and the adhE promoter could be induced in this area. V: vital tumor cells; N: necrotic tumor area. Each experiment was performed in triplicate.

endostatin gene on the survival of mice bearing the lung cancer or melanoma xenograft. The median survival for mice bearing human lung cancer xenograft that received VNPpadhEEndostatin was 49.0 days (CI, 33.46–52.54 days), which was significantly longer than that of control mice (18.0 days; CI, 13.59–26.41 days) (p < 0.001) or mice receiving VNP (25.92 days; CI, 25.92–42.08 days). Kaplan-Meier survival analysis showed that inoculation with VNPpadhEEndostatin significantly improved survival (p < 0.001) and the 30-daysurvival rate was 86% versus 71% for VNPpadhEEndostatin and VNP, respectively (Fig. 5A and Table II).
therapeutic drugs (30). The unique microenvironment in solid tumor could be turned to advantage, and provides an opportunity for alternative therapeutic approaches. Hypoxia and its accompanying necrosis can be served as a niche for some species of obligate as well as facultative anaerobic bacteria, which are capable of selective tumor colonization and exerting tumoricidal effect. Meanwhile, hypoxia may provide a short cut to target gene expression selectively to solid tumors. One promising way to achieve this is to introduce or explore a hypoxia-inducible promoter. Therefore, it is possible to take advantage of tumor hypoxia coupled with tumor-targeting bacteria to obtain tumor-selective delivery of therapeutic protein. In this study, we used 2-DE coupled mass spectrometry to identify endogenous hypoxia-inducible proteins in *Salmonella*, and found that the adhE was anaerobically expressed in tumor-targeted *Salmonella* but not under aerobic growth conditions. The promoter sequence of the adhE gene contains a Fur binding box and a NarL binding site, which, consistent with previous reports (31, 32), contribute to the anaerobia-induced property of *Salmonella*. We fo-

![Fig. 4. Antitumor effects of VNPpadhEEndostatin on mice bearing Lewis lung carcinomas (A, B) and melanomas (C, D). Tumor volumes and animal survivals among different groups were compared in mice bearing (A) Lewis lung carcinomas (n = 7) and (C) B16F10 melanomas (n = 8) (Mean ± S.D., *p < 0.05). Tumor doubling time and growth delay in mice bearing (B) Lewis lung carcinomas (n = 7) and (D) B16F10 melanomas (n = 8) were determined by growth curve of each group (Mean ± S.D., †p < 0.001, VNPpadhEEndostatin versus VNP).](image)

### TABLE II

Regression analysis for treatment effects on tumor growth and Kaplan-Meier analysis for treatment effects on survival. Data are listed as mean with their 95% confidence intervals in parentheses.

| Treatment          | n  | Growth curve* \(v(d)\) | \(r\)  | Tumor doubling time(d)* | Tumor growth delay(d)* | Median survival | 30-days survival |
|--------------------|----|------------------------|-------|-------------------------|------------------------|-----------------|-----------------|
| LLC                |    | \(\ln(v) = -0.2009d+1.69\) | 0.9509 | 3.46 (3.23–3.68)         | 8.45 (8.27–8.64)       | 18.0 (13.59–26.41) | 0.00            |
| VNP                |    | \(\ln(v) = 0.1583d-1.81\) | 0.9733 | 4.44 (4.27–4.61)         | 11.43 (11.22–11.64)    | 35.0 (25.92–42.08)** | 0.71            |
| VNPpadhEEnd        |    | \(\ln(v) = -0.1341d+1.95\) | 0.9652 | 5.21 (4.99–5.42)         | 14.54 (14.22–14.86)    | 49.0 (33.46–52.54)** | 0.86            |
| B16F10             |    | \(\ln(v) = -0.2342d+1.77\) | 0.9814 | 2.96 (2.85–3.08)         | 7.56 (7.48–7.64)       | 11.5 (9.42–13.08)  | 0.00            |
| Control            |    | \(\ln(v) = -0.1186d+1.53\) | 0.9586 | 5.84 (5.70–5.98)         | 12.90 (12.67–13.05)    | 19.5 (13.60–26.65)** | 0.00            |
| VNP                |    | \(\ln(v) = -0.0999d+1.70\) | 0.9833 | 6.96 (6.86–7.07)         | 17.07 (16.97–17.17)†   | 28.5 (18.18–32.82)** | 0.25            |
| VNPpadhEEnd        |    | \(\ln(v) = -0.0896d+1.70\) | 0.9833 | 6.96 (6.86–7.07)         | 17.07 (16.97–17.17)†   | 28.5 (18.18–32.82)** | 0.25            |

* Regression growth curves summarize volume \(V, \text{cm}^3\) dependence on time \(d, \text{days}\) from initial treatment, with correlation coefficients indicated by \(r\).

** Tumor doubling time was derived from exponential growth curves.

† Growth delay was determined by assessing the time interval to 1000 mm\(^3\), (mean ± S.D., †p < 0.0001, VNPpadhEEndostatin vs. VNP, **p < 0.01). Median survival time was analyzed by software MedCalc and is listed with their 95% confidence intervals in parentheses. Significance is indicated as **p < 0.01 (in Lewis lung carcinoma (LLC) treatment: VNP vs. control, in B16F10 treatment: VNP vs. control, VNPpadhEEndostatin vs. VNP), †p < 0.0001 (in LLC treatment: VNPpadhEEndostatin vs. VNP).
focused on the promoter region for vector construction. Reporter protein downstream of the adhE promoter, as a single-copy integrant into the VNP chromosome or constructed into relatively low-copy plasmid pBR322, could be anaerobically induced for expression in vitro and in vivo, respectively. These implied that the prokaryotic gene-delivery promoter from attenuated Salmonella could be genetically engineered to spatially control gene expression in the tumor. This opens a great number of possibilities for improvement of tumor-specific delivery of therapeutic molecules by Salmonella.

More recently, our group has defined enhanced antitumor effects mediated by the combination of VNP with recombinant human endostatin in B16F10 murine melanoma model (16). To extend the earlier study, we investigated in this work the efficacy of a combination bacteriolytic therapeutic approach in which VNP was capable of preferentially accumulating within tumors and selectively delivering the endostatin protein to the tumor site. The therapeutic promise of this approach was based on the hypoxia-induced promoter system as described previously. We assumed that this therapeutic strategy would target two important tumor components—the hypoxic tumor tissue and the life line of a tumor—thus achieving a durable control of established tumors. That is, bacteria can destroy a solid tumor from its center, which is less accessible to other therapeutic agents, and angiogenic inhibitor derived from bacteria within the hypoxic region can attack the tumor.

FIG. 5. Salmonella-mediated targeted therapy with padhEEndostatin markedly extended the survival of mice bearing Lewis lung carcinomas (LLC (A)) or melanoma (B). A, VNPpadhEEndostatin (VNPpadhEEnd) treatment significantly prolonged survival compared with untreated controls. Data are expressed as mean ± S.D., n = 7, \( p < 0.001 \) VNPpadhEEnd versus Control by Kaplan-Meier analysis. B, VNPpadhEEndostatin prolonged the survival of mice bearing B16F10 melanoma compared with untreated control. Data are expressed as mean ± S.D., n = 8, \( **p < 0.01 \) VNPpadhEEnd versus Control.

FIG. 6. VNPpadhEEndostatin inhibited neoangiogenesis in vitro and in vivo. A, CAM assay. Variable amount of bacteria was applied to the CAMs of 8-day-old chick embryos. After 48 h, the avascular zone (~1 cm in diameter) of each CAM was observed. The representative appearance of chick CAMs implanted with a coverslip containing VNPpadhEEndostatin (200 \( \mu \)g/embryo or 100 \( \mu \)g/embryo), VNP with empty vector (200 \( \mu \)g/embryo) or PBS are shown. The marked square indicates the location of the implanted filter disk. An avascular zone around the filter disk was observed only with VNPpadhEEndostatin treatment. B, VEGF expression following VNPpadhEEndostatin therapy. Tumors were dissected 12 days post-treatment. Tumor weights were measured and VEGF levels in tumor extracts were determined by ELISA. (Mean ± S.D., n = 3, \( *p < 0.05 \), \( **p < 0.01 \)). C, Tumor microvessel in section with CD31 staining is indicated with red arrows (×400). D, Quota of CD31 staining in tumor sections and microvessel density assay were performed as described in Material and Methods. (Mean ± S.D., n = 3, \( *p < 0.05 \), \( **p < 0.01 \)).
periphery by shutting down its blood supply, leaving the tumor minimal chances to survive. Our results demonstrated, on two murine tumor models, that the combination bacteriolytic therapy could induce enhanced therapeutic efficacies as compared with those from bacteria treatment alone or untreated control by retarding tumor growth and extending the life span of the tumor-bearing mice.

As has been tested by several laboratories, Salmonella alone could induce modest antitumor effects in animal tumor models although the mechanisms responsible for this are not yet fully understood (1, 3, 5). When VNP was armed with the construct encoding human endostatin, endostatin and bacteria may cooperate to inhibit tumor angiogenesis. It has been widely recognized that angiogenesis is a critical process required by solid tumors to support their growth. As an endogenous angiogenesis inhibitor, endostatin seems to show an antiproliferative effect on endothelial cells by several means, including its binding to α5β1 integrins, attenuation of VEGF receptor signaling and inhibition of cyclin D1 (33). Meanwhile, VNP may also affect angiogenesis. It has been defined that lipopolysaccharide is able to induce apoptosis in endothelial cells (34). More recently, we have established that VNP directly induces apoptosis in endothelial cells in a dose- and time-dependent manner in vitro (data not shown). Thus, to

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**Fig. 7.** VNPpadhEEEndostatin induced necrosis of melanoma and promoted VNP tumor colonization and melanoma cell apoptosis. 

**A,** Determination of tumor necrosis and apoptosis after treatment of VNPpadhEEEndostatin. Tumor necrosis areas are shown by H&E staining and observed under light microscope (×100). The viable tumor cells are indicated with blue arrow. TUNEL assay was used to detect apoptotic cells (original magnification ×200). Positive cells for TUNEL staining are indicated by green arrow. **B,** Quota of tumor necrosis and apoptosis. Tumor necrosis was determined by software Image J (NIH, USA). Four sections/mouse and three mice were prepared (Mean ± S.D., †p < 0.001). The ratio of apoptotic cells to total cells. TUNEL-positive cells were counted from three fields of the highest density of positive-stained cells in each section to determine the percentage of apoptotic cells (mean ± S.D., n = 4, *p < 0.05). **C,** Tumor bacterial colonization on day 12 post-treatment. Mean ± S.D., n = 4, †p < 0.05 VNPpadhEEEndostatin versus VNPpBR treatment.
some extent, this strain may have served as a vasculotoxic agent within the tumors. These existing data suggest that the decrease in VEGF expression level and vessel density in tumors after treatment with VNP could partly result from the influence of bacteria on tumor angiogenesis. As for the combination therapy, we found in the current study that VNPpadhE(Endostatin) treatment resulted in significantly profound inhibition of tumor angiogenesis and VEGF production within tumor compared with either treatment alone. This result is consistent with our previous study in which VNP was combined with recombinant human endostatin in the murine melanoma model (16). It may represent a synergic antitumor mechanism mediated by the accumulation of VNP and the local production of endostatin at the tumor site.

We noted that the tumor-targeted gene therapy of several solid tumors with S. cholerasuis delivering the endostatin gene had been previously described (35). However, there is a great difference between the published report and our study described here. In their study, S. cholerasuis was used to transfer a eukaryotic expression vector encoding murine endostatin into mammalian cells and gene transfer persisted for about 10 days in vivo, whereas VNP used in this work has been proved not suitable for gene transfer (data not shown) but could persist in the tumor tissues for more than 2 months (data not shown) and produce therapeutic polypeptide in situ. The key difference lies in the specificity of the promoter. In the previous report, although it displayed anti-angiogenic efficacy in animal models, we are puzzled as to the following: How did the eukaryotic $\beta$-actin promoter work in bacteria in vivo? Was it expressed in other tissues? Furthermore, as it did not contain secretory signal peptides, how did it exert its anti-angiogenic function within the bacteria or even mammalian host cells? All the above questions drove us to design a Salmo-nella-delivered, tumor-microenvironment selective, expression system with a clearer mechanism, a detectable and secretory expression, and an increased tumor-specificity. Despite important differences between the previous report and our study, both of them verified the feasibility of the combination therapy of tumor with Salmonella and endostatin. We also test the efficiency of this gene delivery system on other anticancer genes such as TRAIL, and similar results have been also obtained (data not shown).

In summary, by comparative proteomics, we successfully screened a hypoxia-inducible promoter, the adhE promoter, from Salmonella. Furthermore, we provided a proof-of-principle that an endogenous inducible bacterial promoter systems can be employed to target the hypoxic region of solid tumors and exert enhanced anticancer effects when driving the expression of human endostatin in animal tumor models. Because hypoxia has been shown to be present in most solid human tumors and to be a primary cause of treatment failure for conventional therapeutic agents, we believe that this approach would act in a complementary way to current radiotherapy and chemotherapy treatments, which preferentially kill well oxygenated cells. Therefore, the application of this hypoxia-driven targeting approach may be applicable to an extensive patient population.

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