**Control of angiogenesis by VEGF and endostatin-encapsulated protein
microcrystals and inhibition of tumor angiogenesis**

**Goichi Matsumoto^a, Rie Hirohata^b, Kousuke Hayashi^b, Yoko Sugimoto^b, Eiji Kotani^b,
Junji Shimabukuro^b, Tomoko Hirano^c, Yumiko Nakajima^d, Shin Kawamata^e,
Hajime Mori^{b,f,*}**

^aDivision of Oral Surgery, Yokohama Clinical Education Center of Kanagawa Dental University,
3-31-6 Tsuruya-cho, Kanagawa-ku, Yokohama 221-0835 Japan

^bDepartment of Applied Biology, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto
606-8585, Japan

^cVenture Laboratory, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585,
Japan

^dFunctional Genomics Group, COMB, Tropical Biosphere Research Center, University of the
Ryukyus, Okinawa, Japan

^eBasic Research Group for Regenerative Medicine, Foundation for Biomedical Research and
Innovation, TRI308, 1-5-4 Minatojima-Minamimachi, Chuo-ku, Kobe 650-0043, Japan

^fInsect Biomedical Research Center, Kyoto Institute of Technology. Matsugasaki, Sakyo-ku, Kyoto
606-8585, Japan

Key Words: VEGF, endostatin, polyhedra, angiogenesis, antiangiogenesis

***Corresponding author:** Insect Biomedical Research Center, Kyoto Institute of Technology.
Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan. Tel./fax: +81-75-724-7776.

E-mail address: hmori@kit.ac.jp

Running title: Control of angiogenesis by VEGF and endostatin microcrystals

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Abstract

Encapsulation of cytokines within protein microcrystals (polyhedra) is a promising approach for the stabilization and delivery of therapeutic proteins. Here, we investigate the influence of vascular endothelial growth factor (VEGF) microcrystals and endostatin microcrystals on angiogenesis. VEGF was successfully encapsulated into microcrystals derived from insect cypovirus with overexpression of protein disulfide bond isomerase. VEGF microcrystals were observed to increase the phosphorylation of p42/p44 MAP kinase and to stimulate the proliferation, migration, and network and tube formation of human umbilical vein endothelial cells (HUVECs). Endostatin was also successfully encapsulated into microcrystals. Endostatin microcrystals showed antiangiogenesis activities and inhibited the migration, and network and tube formation of HUVECs. Local administration of endostatin microcrystals in mice inhibited both angiogenesis and tumor growth with clear significant differences between treatment and control groups. Endostatin microcrystals only affected angiogenesis, but had no significant effect on lymphangiogenesis compared to controls. Local therapy using endostatin microcrystals offers a potential approach to achieve sustained therapeutic release of antiangiogenic molecules for cancer treatment.

1. Introduction

Cypovirus (CPV) infections result in the production of significant amounts of protein microcrystals (occlusion bodies) termed polyhedra. Virus particles become occluded within the polyhedra hence polyhedra are often referred to as occlusion bodies [1, 2]. Polyhedra are the main vectors by which virus particles transfer from insect to insect and are the main agents of survival of the virus from one insect generation to the next. Polyhedra stabilize virions thereby allowing them to remain viable for long periods in the environment [3, 4]. We have successfully developed a system to immobilize foreign proteins into insect virus occlusion bodies [5]. We found that the virion outer capsid protein, VP3, is implicated in the occlusion of viral particles into *Bombyx mori* CPV (BmCPV) polyhedra, and identified that the N-terminal VP3 domain (VP3 immobilization signal) was responsible for association and incorporation of foreign proteins into BmCPV polyhedra [5, 6]. The atomic structure of BmCPV polyhedra revealed that the polyhedrin H1 helix at the N-terminus of the molecule is a useful tag for incorporating foreign proteins into polyhedra such as the VP3 immobilization signal [4, 7]. In our new baculovirus expression vector system, the immobilization signal-containing foreign proteins expressed under the control of baculovirus polyhedrin promoter are occluded into BmCPV polyhedra consisting of crystallized polyhedrin expressed by another recombinant baculovirus containing BmCPV polyhedrin gene.

We have previously reported the encapsulation of fibroblast growth factor-2 (FGF-2) and FGF-7, epidermal growth factor (EGF), leukemia inhibitory factor, and secreted frizzled-related protein 4 into polyhedra [7-10]. These cytokines have biological activities as monomers. The vascular endothelial growth factor (VEGF) family plays an integral role in angiogenesis, lymphangiogenesis, and vasculogenesis [11, 12]. The VEGF family currently comprises seven members (VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and placental growth factor). All members have a common VEGF homology domain. The core region is composed of a cystine knot motif, with eight invariant cysteine residues involved in inter- and intramolecular disulfide bonds at one end of a conserved central four-stranded β -sheet within each monomer, which

dimerize in an antiparallel, side-by-side orientation [11-14]. VEGF-A is a 34- to 42-kDa, dimeric, disulfide-bound glycoprotein. VEGF-A exists in at least seven homodimeric isoforms where each subunit consists of 121, 145, 148, 165, 183, 189, or 206 amino acids [11, 12]. Before the encapsulation of VEGF-A into polyhedra, overexpression of protein disulfide bond isomerase (PDI) is required to obtain biologically active VEGF-encapsulated polyhedra.

Endostatin, a 20-kDa C-terminal fragment digested from collagen XVIII, inhibits the proliferation and migration of endothelial cells, and induces apoptosis, leading to repression of growth of a wide variety of tumors with neovascularization [15, 16]. The apoptotic effect of endostatin is associated with decreased levels of anti-apoptotic molecules [17]. Recently, Zhuo *et al.* reported that endostatin directly inhibits lymphatic endothelial cells (LEC) and lymphangiogenic vessels via the cell surface receptor nucleolin on LEC which inhibits lymphatic metastasis [18].

In this study VEGF and endostatin were encapsulated into polyhedra and the effect of both VEGF and endostatin polyhedra on angiogenesis in cell culture and *in vivo* was investigated.

2. Materials and methods

2.1 Medium

Growth medium: EGM-2 (LONZA) containing 2% fetal bovine serum (FBS), EGF, FGF-2, VEGF, R³-IGF-1, hydrocortisone, ascorbic acid, heparin, GA-100 (gentamicin, amphotericin-B). Assay medium: EGM-2 containing 0.2% FBS, hydrocortisone, ascorbic acid, heparin, GA-1000. Starvation medium: α MEM (WAKO).

2.2 Construction of VEGF and endostatin transfer vector

Recombinant VEGF-A baculovirus transfer vectors were generated using GATEWAY[®] cloning technology (Invitrogen). The VEGF-A gene (189 aa) fused to DNA encoding either VP3 immobilization signal at N-terminal or C-terminal was amplified by polymerase chain reaction (PCR) using primer sets containing *attB1* and *attB2* sequences below:

the forward primer is

5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCACCCACGACAGAAGGAGAGC-3'

and reverse primers are

5'-GGGGACCACTTTGTACAAGAAAGCTGGGTATCACCGCCTTGGCTTGTACATCTGCA-3'

for N-terminal VP3 or

5'-GGGGACCACTTTGTACAAGAAAGCTGGGTACCGCCTTGGCTTGTACATCTGCA-3' for

C-terminal VP3. The resulting *attB*-flanked PCR products were cloned into a donor vector (pDONR221) to construct entry vectors by BP reactions of GATEWAY® system. The open reading frame cloned between the *attL1* and *attL2* sites in the entry vectors was transferred to destination vectors (pDEST-N-VP3 or pDEST-C-VP3) via LR Clonase™ reactions resulting in production of the transfer vectors encoding VEGF-A fused with VP3 tag (pVP3/VEGF and pVEGF/VP3).

DNA fragment encoding C-terminal region (183 aa) of collagen XVIII was fused with H1 or VP3 immobilization signal and also amplified by PCR using primer sets containing *attB1* and *attB2* sequences below:

the forward primer is

5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCACAGCCACCGCGACTTCCAGC -3'

and reverse primers are

5'-GGGGACCACTTTGTACAAGAAAGCTGGGTACTACTTGGAGGCAGTCATGAAGCTGTT-3'

for H1 signal at N-terminal or

5'-GGGGACCACTTTGTACAAGAAAGCTGGGTACTTGGAGGCAGTCATGAAGCTGTT-3' for

VP3 signal at C-terminal. The resulting *attB*-flanked PCR products were also cloned into pDONR221 to construct entry vectors. The open reading frame cloned between the *attL1* and *attL2* sites in the entry vectors was transferred to destination vectors (pDEST-N-H1 or pDEST-C-VP3) resulting in production of the transfer vectors encoding endostatin fused with H1 or VP3 tags (pH1/Endo and pEndo/VP3).

2.3 Production of VEGF and endostatin polyhedra

The transfer vectors were co-transfected into *Spodoptera frugiperda* IPLB-Sf21-AE (Sf21) cells with linearized Baculogold Baculovirus DNA (BD Pharmingen). After incubation for 5 days at 27 °C, recombinant baculoviruses expressing VEGF-A and endostatin were harvested and stored at 4 °C. To incorporate VEGF-A into polyhedra (VEGF polyhedra), immobilization of VEGF-A using VP3 tag was accomplished by dual infection of the recombinant baculoviruses that express the VEGF-A and a recombinant baculovirus AcCP-H expressing BmCPV polyhedrin or AcCP-H/PDI expressing BmCPV polyhedrin and PDI, in which BmCPV polyhedrin and PDI were expressed under the control of the baculovirus polyhedrin promoter and p10 promoter, respectively. Endostatin polyhedra were also made by dual infections with recombinant baculoviruses expressing endostatin with H1 tag (AcH1/Endo) or VP3 tag (AcEndo/VP3) and AcCP-H, resulting in H1/Endo polyhedra and Endo/VP3 polyhedra. To generate empty polyhedra, Sf cells were inoculated with recombinant baculovirus AcCP-H or AcCP-H29/PDI. The infected cells were cultured for 10 days at 27 °C and then the cells were harvested in a conical tube by centrifugation. The cell pellet was resuspended in phosphate-buffered saline (PBS; pH 7.2) and treated with an ultrasonic homogenizer at 6% power for 30 sec. The cell homogenate was centrifuged at 1500 x g at 4 °C and the supernatant was removed. These treatments were repeated and the purification was complete. The polyhedron suspension was adjusted to the same density (5×10^4 numbers / μ l) and stored at 4 °C in distilled water containing 100 units/ml penicillin and 100 μ g/ml streptomycin.

2.4 Phosphorylation of p44/p42 mitogen-activated protein kinase (MAP kinase)

Human umbilical vein endothelial cells (HUVECs) were plated into a 6-well plate at a density of 1×10^5 cells per well and cultured to confluence in the growth medium. Cells were washed with PBS and then incubated with the starvation medium for 6 hours to synchronize cell cycles. The cultures were then treated with rhVEGF and empty or VEGF polyhedra. HUVECs were washed with PBS

twice and dissolved in 100 μ l of 1 x SDS sample buffer containing 50 mM Tris-HCl (pH 6.8), 2% SDS, 50 mM dithiothreitol, 0.1% bromophenol blue, and 10% glycerol. Cell lysates were harvested, sonicated, and cleaned by centrifugation. Samples were electrophoresed on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). After preincubation with blocking buffer containing 5% nonfat milk, 0.1% Tween 20, 25mM Tris-HCl (pH 7.6), 150 mM NaCl, the membranes were incubated with either p44/42 MAP Kinase Antibody or Phospho-p44/42 MAP kinase (Thr202/Tyr204) Antibody followed by horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (New England Biolabs). Peroxidase activity was detected and visualized by ECL Western Blotting Detection Reagents (GE Healthcare).

2.5 Proliferation assay of HUVECs

The proliferation of HUVECs was measured by Cell Counting Kit-8 using WST-8 (DOJINDO). Cells were seeded into 96-well plates at a density of 3×10^3 cells/well. After incubation in the assay medium containing rhVEGF and empty or VEGF polyhedra for 3 days at 37 °C under 5% CO₂, Cell Counting Kit-8 solution was added to the cultures and incubated for 4 hours at 37 °C under 5% CO₂, according to the manufacturer's instructions. Absorbance at 450 nm was determined with a microplate reader Model 680 (Bio-Rad).

2.6 *In vitro* scratch assay

We used the *in vitro* scratch assay to assess the activity of VEGF polyhedra and endostatin polyhedra on migration of HUVECs. HUVECs were seeded in 24-well plates (8×10^3 /well) with the growth medium and cultured to confluence in the growth medium. The cells were scraped away vertically 24 hours later by pipette tip. Each well was washed twice with PBS to remove debris, and then further incubated for 18 hours in serum-free EGM-2 medium with rhVEGF or polyhedra (empty, VEGF, endostatin). Endostatin polyhedra were assayed in the presence of 10 ng/ml rhVEGF. The distances between the 2 edges of the scratch were photographed on each

well using an inverted microscope.

2.7 Network formation of HUVECs

Subconfluent culture of HUVECs were washed with PBS and suspended in the starvation medium to synchronize cell cycles. After rhVEGF or polyhedra (empty, VEGF, endostatin) were mixed with ECMatrix (CHEMICON) solution, 50 μ l of the aliquot were added to each well of the 96-well plate and the plate was incubated for 1 hour at 37 °C to allow the matrix solution to solidify. To assay endostatin polyhedra, ECMatrix solution containing rhVEGF (10 ng/ml) was used. HUVECs (1×10^4 /well) were seeded onto presolidified ECMatrix. After incubation for 18 hours at 37°C under 5% CO₂, 50 μ l of Calcein AM (BD Biosciences) solution was added to each well and plates were incubated at 37°C under 5% CO₂ for 30 minutes. Total tube length of each well was measured using an image analyzer (ESPEC TECHNO CORP).

2.8 Tube formation of HUVECs

Normal human dermal fibroblast cells (2×10^4 /well) were seeded onto a collagen-coated 24-well plate and incubated with 500 μ L of Medium 106 supplemented with Low Serum Growth Supplement containing 2% FBS, EGF, FGF-2, hydrocortisone, heparin (Life Technologies) for 24 hours at 37°C under 5% CO₂. The medium was replaced with the starvation medium and then rhVEGF or polyhedra (empty, VEGF, endostatin) were added to each well followed by incubation for 1 hour at 37°C under 5% CO₂. Endostatin polyhedra were assayed in the presence of 10 ng/ml rhVEGF. HUVECs (2×10^4 /well) were seeded into each well and incubated with the assay medium for 11 days at 37°C under 5% CO₂. The assay medium with or without rhVEGF (10 ng/ml) was used for wells of rhVEGF or polyhedra, respectively, at each 2 days medium change. Cells were washed with PBS and fixed with cold 70% ethanol. After 30 min, ethanol was discarded and cells were incubated with 1% BSA. Fixed cells were reacted with mouse anti-human CD31 antibody and then goat anti-mouse IgG AP conjugate antibody. Tube formation of HUVEC was

detected by addition of substrate BCIP/NBT.

2.9 Tumor cell line and cell cultures

The SCC-VII tumors were cutaneous mouse squamous cell carcinomas that spontaneously arose in the C3H/He mouse strain [19]. SCC-VII cells were inoculated subcutaneously into C3H/He mice. We cultured SCC-VII cells in RPMI 1640 medium supplemented with 10% heat-inactivated FCS at 37°C in an incubator with 5% CO₂.

2.10 Tumor model and treatment

Our animal studies conformed to the Kanagawa Dental University guidelines for research animal care and were approved by ethical committees of Kanagawa Dental University. Subcutaneous injection of 5×10^5 SCC-VII cells in 100 μ l of PBS into the backs of C3H/He mice (Japan SLC, Shizuoka, Japan) aged 6 weeks generated back tumors. Tumor volume was estimated using the formula: tumor volume (mm³) = length (mm) x cross-section (mm)² x 1/2. Two weeks later, when the tumors had grown to 100-200 mm³, we randomly divided the mice into 4 treatment groups: 5×10^6 H1/Endo polyhedra with 100 μ l PBS; 5×10^6 Endo/VP3 polyhedra with 100 μ l PBS; 5×10^6 empty polyhedra with 100 μ l PBS; and no treatment as a control. On the 2nd and 4th week after the inoculation of tumor cells, we administered each polyhedron by intratumoral injection. The control group was without treatment. After SCC-VII tumor-cell inoculation, tumor volume was measured at 6 weeks. Each group contained 4 mice.

2.11 Immunohistochemical analysis

Implanted-tumor specimens from mice were embedded in OCT compound, quickly frozen in dry ice, and stored at -80 °C for immunohistochemical staining. To analyze tumor angiogenesis and lymphangiogenesis, we performed double immunofluorescence staining of excised tumors for the vascular marker CD31 and for the lymphatic-specific marker LYVE-1. Frozen sections of 6 μ m

thickness were stained using a rat anti-mouse CD31 mAb (clone MEC13.3, Pharmingen, CA) for 60 min at RT and then rinsed 3 times. Sections were then stained with anti-rat antibody conjugated with Alexa Fluor 488 (Invitrogen, CA) for 60 min at RT. After staining with CD31, the sections were rinsed 3 times in PBS and incubated a second time with anti-LYVE-1 polyclonal Ab (Abcam, Cambridge, UK) for 60 min at RT and then rinsed 3 times. Sections were then stained with anti-rabbit antibody conjugated with Alexa Fluor 546 (Invitrogen, CA) for 60 min at RT. Cell nuclei were counterstained with DAPI (InnoGenex, CA). Slides were then mounted with a fluorescent mounting medium (DAKO, CA). Images were obtained on a Zeiss microscope and analyzed using AxioImager software (Carl Zeiss, NY). Microvessel density and lymphatic microvessel density in the tumors were determined after immunostaining with antibodies against CD31- and LYVE-1- positive vessels, which were counted microscopically under x100 magnification from 3 areas of the highest vascular density per section.

3. Results

3.1 Proliferation, migration, network and tube formation of HUVECs by VEGF polyhedra

VEGF is secreted as a cysteine-linked dimer and has two binding sites at opposite poles through which it binds VEGF receptors (VEGFRs). VEGFRs are tyrosine kinases found on the surface of endothelial and other cells. VEGF-A tagged with VP3 at N-terminus or C-terminus was incorporated into polyhedra with or without the expression of PDI and biological activities of these polyhedra were studied. To examine activation of the downstream signaling pathway by polyhedra encapsulating VEGF-A, phosphorylation of p44/p42 MAP kinase was analyzed in HUVECs. Phosphorylated forms of p44 and p42 MAP kinase were detected in HUVECs incubated with rhVEGF as well as VEGF/VP3+PDI polyhedra, in which VEGF-A fused with VP3 at C-terminus and co-expressed with PDI was encapsulated (Fig. 1A). In contrast, phosphorylation was not or was poorly detected in the cells incubated with empty polyhedra, nor was detection observed for

1 other polyhedra in which VEGF-A was encapsulated by VP3 at N-terminus or without PDI
2 expression. The VEGFRs require dimerization to be active. Signaling is initiated when VEGF
3 binds first to one of the receptors, resulting in translocation of the second receptor for dimerization.
4
5 As it was considered that firstly dimerization of VEGF-A was caused by PDI and then VEGF-A
6 molecules were incorporated into polyhedra, we used these polyhedra as VEGF polyhedra for
7 assay of proliferation, migration, and network and tube formation of HUVECs.
8
9

10 We have previously reported similar results regarding encapsulation of bone
11 morphogenetic protein 2 (BMP-2) into polyhedra [20]. Native BMP-2 protein consists of two
12 polypeptide chains linked by an S–S bond between Cys78 of each chain. Co-expression of PDI
13 was needed to obtain biologically active BMP-2 polyhedra during encapsulation into polyhedra.
14 BMP-2 expressed in *Escherichia coli* is accumulated in inclusion bodies and the first step of
15 preparation is isolation of inclusion bodies from *E. coli* cells. Secondly, denaturation of inclusion
16 bodies and purification are necessary. Finally, subsequent refolding of BMP-2 is required to attain
17 the biologically active homodimeric form [21]. VEGF is usually expressed in *E. coli* cells and
18 purified by similar steps [22, 23]. Our data show that the isolation and denaturation of inclusion
19 bodies and subsequent refolding into the biologically active homodimeric form are unnecessary
20 when cytokines for encapsulated into polyhedra.
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40

41 Proliferation of HUVECs in the presence of VEGF polyhedra showed a dose-response
42 curve (Fig. 1B). VEGF activity from 8.75×10^4 numbers of VEGF polyhedra was estimated to be
43 equivalent to the activity of 1 ng rhVEGF. Endothelial cell migration is a necessary step of
44 angiogenesis. The effect of VEGF polyhedra on HUVECs migration was determined using the
45 scratch assay. As shown in Figure 2, cell migration was stimulated by VEGF polyhedra. The
46 formation of three-dimensional capillary-like structures by HUVECs is well-established in *in vitro*
47 angiogenesis assays. HUVECs were seeded on Matrigel substrate and dispersed evenly
48 throughout the culture. Thereafter, cells spontaneously aligned to form a network of capillary-like
49 structures in the presence of rhVEGF or VEGF polyhedra (Fig. 3A, B). The formation of tube-like
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

structures is an essential step in angiogenesis. Therefore, HUVEC angiogenesis was also observed using the tube formation assay. As shown in Figure 3C, capillary tube structures were formed by rhVEGF or VEGF polyhedra after HUVECs were placed in the wells, indicating that VEGF polyhedra can stimulate angiogenesis.

3.2 Antiangiogenic activities of endostatin polyhedra

We studied the antiangiogenic activities of endostatin polyhedra. Endostatin fused with H1 or VP3 tags was incorporated into polyhedra (H1/Endo polyhedra and Endo/VP3 polyhedra, respectively). The antimigratory effect of both endostatin polyhedra was observed by the inhibition of migration of HUVECs (Fig. 2). Addition of endostatin polyhedra to the culture media significantly inhibited the network and tube formation of HUVECs (Fig. 3), indicating that endostatin polyhedra had potent anti-endothelial cell activity.

3.3 Inhibition of tumor growth by endostatin polyhedra injections

Approximately 2 weeks later, when the tumors had grown to 100-200 mm³, the mice were randomly assigned to groups and therapy was initiated. H1/Endo polyhedra, Endo/VP3 polyhedra and empty polyhedra were administrated at 2 and 4 weeks after tumor-cell inoculation. At 6 weeks post-inoculation, the tumor volumes of endostatin polyhedra groups (H1/Endo polyhedra and Endo/VP3 polyhedra) were lower than those of control or empty polyhedra groups (Fig.4). We concluded that local therapy with endostatin encapsulated into polyhedra as a carrier efficiently inhibited tumor growth *in vivo*.

To identify the mechanism of tumor growth inhibition, we examined angiogenesis of tumor tissues by immunohistochemical staining. Angiogenesis within the tumor tissues was determined by directly counting the number of CD31⁺ cells on microvessels. The most highly vascularized areas of each tumor were identified and CD31⁺ vessels were counted in at least five fields (magnification: x100). Angiogenesis was inhibited in the treatment with H1/Endo polyhedra or

1 Endo/VP3 polyhedra, and there was a significant difference compared to the number of vessels
2 counted for the control and empty polyhedra groups. There was a pronounced inhibition of
3 angiogenesis in tumors treated with the H1/Endo polyhedra or Endo/VP3 polyhedra (Fig. 5B).
4
5 However for lymphatic vessel assay, no statistically significant differences in lymphatic vessel
6 densities were observed in all experimental groups compared to controls (Fig. 5C). To conclude,
7
8 endostatin polyhedra were effective in the blood vessel assay but were not effective in the
9
10 lymphatic vessel assay. Endostatin polyhedra therefore may have anti-angiogenic effect but likely
11
12 do not have anti-lymphangiogenic effect.
13
14
15
16
17
18
19
20
21

22 **4. Discussion**

23
24 Cytokines are not diffusible and bind to the cell surface or surrounding extracellular matrix (ECM)
25
26 [24, 25]. FGF-2 binds to heparan sulfate in ECM and ECM-bound FGF-2 is released by exposure
27
28 to heparin or heparan sulfate [26]. FGF-2 released from ECM is biologically active and stimulates
29
30 cell proliferation and DNA synthesis in vascular endothelial cells and 3T3 fibroblasts. VEGF and
31
32 endostatin have heparin-binding domains which are critical in angiogenesis and antiangiogenesis,
33
34 respectively [27-29]. Release of VEGF and endostatin from ECM and their activation is triggered
35
36 by the binding of heparin. Such a signaling cue originating from the extracellular
37
38 microenvironment is also essential for cellular processes including proliferation, differentiation,
39
40 migration and apoptosis. For *in vivo* study of interactions between cytokines and target cells or
41
42 tissues, reservoirs or slow-release agents are necessary for sustained release of active cytokines.
43
44 If the cytokines are unstable molecules, such a reservoir might be more important for *in vivo* study.
45
46
47
48
49

50
51 Dr. Folkman observed that endostatin has potent antiangiogenesis activity and
52
53 endostatin was effective in inhibiting tumor growth in several preclinical studies, however the
54
55 response in clinical trials was less impressive. It was difficult for other laboratories to reproduce
56
57 these experiments. There are some practical problems in expression, refolding, bioavailability,
58
59 short half-life, purification and stock of endostatin due to its instability [30]. There are various
60
61
62
63
64
65

1 methods by which to transfect endostatin cDNA into cells. One way is to use a viral vector to
2 incorporate the gene or gene fragment into the cells [31, 32]. While this method allows effective
3 and reliable supply of endostatin in the cell, there is an unavoidable risk that the virus may cause
4 harm to the host.
5
6
7
8
9

10 To mitigate these problems, we have genetically modified endostatin to improve its
11 biological activity. The modified cypovirus polyhedra can function as nano- or micro-containers
12 stabilizing incorporated proteins in a functional form [2]. One application is as a mimic of ECM
13 whereby cytokines including growth factors can be expressed in the polyhedra to mimic their
14 natural structural scaffolds such as collagen, fibronectin and heparan sulfate. In the ECM,
15 enhanced stability and activity of the embedded cytokines results in cell proliferation and
16 differentiation regulated in time and space [24, 25]. Such perspectives can drive active research
17 on the applications of polyhedra in stem cell research, tissue engineering and regenerative
18 medicine. Another application is a novel tool for “proof of concept”. Unstable cytokine molecules
19 are rapidly degraded and the biological activities are lost. However, purification of cytokines
20 becomes unnecessary when encapsulating within polyhedra. We would like to propose the use of
21 polyhedra for elucidation or identification of unknown functions of cytokines.
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37

38 Tumor growth and metastatic dissemination depend on the formation of new tumor
39 microvessels. The process of tumor angiogenesis is a balance between proangiogenic and
40 antiangiogenic factors. The use of antiangiogenic agents to restore this balance represents a
41 useful approach in cancer treatment. Inhibition of tumor angiogenesis by antiangiogenic agents
42 for anticancer therapy is widely accepted.
43
44
45
46
47
48
49

50 Generally, the lymphatic system plays important roles in maintaining fluid balance in
51 inflammation and cancer [33, 34]. In cancer, lymphangiogenesis, the sprouting of new lymphatic
52 vessels from the preexisting lymphatic system, may provide a way to facilitate the dissemination
53 of tumor cells to sentinel lymph nodes and other distant organs [34, 35]. Recently, several
54 researchers reported that endostatin inhibits lymph node metastasis via down-regulation of
55
56
57
58
59
60
61
62
63
64
65

1 VEGF-C, either by tumor cells or by mast cells in the tumor micro-environment [36, 37].
2
3 Interestingly, endostatin directly acts on lymphangiogenic endothelial cells via cell surface
4
5 receptor nucleolin on LECs and inhibits tumor lymphangiogenesis [18]. Here, we did not detect
6
7 the effects of endostatin on tumor lymphangiogenesis and lymph node metastasis. These
8
9 discrepancies may be due to the use of different tumor models. SCC-VII tumors are cutaneous
10
11 mouse squamous cell carcinomas that spontaneously arise in the C3H/He mouse strain and do
12
13 not metastasize [38].
14
15

16
17 Antiangiogenic cancer therapy with antiangiogenic agents requires the peptide to be
18
19 administered regularly or daily over a prolonged period, implying that large quantities of the
20
21 therapeutic agent are needed. These functional antiangiogenic proteins are expensive to produce,
22
23 and frequently there are technical problems related to physical properties and purity. Moreover,
24
25 some therapeutic agents that could otherwise provide effective antiangiogenic cancer therapy
26
27 have biological half-lives too short to sustain tumor regression. All of the above factors are
28
29 obstacles for effective therapies using exogenously injected antiangiogenic agents to inhibit tumor
30
31 angiogenesis. Polyhedral microcrystals represent an attractive and promising alternative method
32
33 to deliver antiangiogenic agents. We reported that polyhedron microcrystals can remain in the
34
35 connective tissue over five weeks after implantation [20]. Consequently, therapy using polyhedral
36
37 microcrystals has the potential to produce the antiangiogenic agent in high concentrations in a
38
39 tumor area over a sustained period, thereby avoiding the problems encountered with long-term
40
41 administration of recombinant proteins.
42
43
44
45
46
47
48
49

50 **5. Conclusion**

51
52 VEGF fused with VP3 tag at the C-terminus was co-expressed with PDI and encapsulated into
53
54 insect virus-derived protein microcrystals, polyhedra. The polyhedra stimulated phosphorylation of
55
56 P44/P42 MAP kinase of HUVECs. VEGF polyhedral stimulated angiogenesis as revealed by
57
58 assays of proliferation, migration, and network and tube formation of HUVECs. Endostatin fused
59
60
61
62
63
64
65

1 with H1 tag at N-terminus or VP3 at C-terminus was encapsulated into polyhedra. Endostatin
2
3 polyhedral showed potent anti-endothelial activity as they inhibited the migration and network and
4
5 tube formation of HUVECs. Our data suggest that endostatin polyhedra may have promise for the
6
7 treatment of experimental squamous cell carcinoma by inhibiting tumors angiogenesis. Further
8
9 studies are needed to establish optimal protocols including dosage and scheduling for
10
11 antiangiogenic therapy using endostatin polyhedra.
12
13
14
15
16

17 **Acknowledgments**

18
19 This work was supported in part by A-STEP exploratory research AS232Z00847F from JST,
20
21 Grants-in-Aid for Scientific Research (A) 22241052 and (C) 90237131 from JSPS, and Regional
22
23 Consortium Research Development Work and Regional Innovation Creation R&D Programs of
24
25 Kansai Bureau of Economy, Trade and Industry of Japan.
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

References

- [1] Belloncik S, Mori H. Cypovirus. In: Miller LK, Ball LA, editors. The insect viruses. New York: Plenum Press; 1998. p. 337-69.
- [2] Mori H, Metcalf, P. Cypoviruses. In: Asgari S, Johnson, K. N., editor. Insect Virology: Caister Academic Press; 2010. p. 307-24.
- [3] Rohrmann GF. Polyhedrin structure. J Gen Virol. 1986;67 (Pt 8):1499-513.
- [4] Coulibaly F, Chiu E, Ikeda K, Gutmann S, Haebel PW, Schulze-Briese C, et al. The molecular organization of cypovirus polyhedra. Nature. 2007;446(7131):97-101.
- [5] Ikeda K, Nagaoka S, Winkler S, Kotani K, Yagi H, Nakanishi K, et al. Molecular characterization of Bombyx mori cytoplasmic polyhedrosis virus genome segment 4. J Virol. 2001;75(2):988-95.
- [6] Ikeda K, Nakazawa H, Shimo-Oka A, Ishio K, Miyata S, Hosokawa Y, et al. Immobilization of diverse foreign proteins in viral polyhedra and potential application for protein microarrays. Proteomics. 2006;6(1):54-66.
- [7] Ijiri H, Coulibaly F, Nishimura G, Nakai D, Chiu E, Takenaka C, et al. Structure-based targeting of bioactive proteins into cypovirus polyhedra and application to immobilized cytokines for mammalian cell culture. Biomaterials. 2009;30(26):4297-308.
- [8] Mori H, Shukunami C, Furuyama A, Notsu H, Nishizaki Y, Hiraki Y. Immobilization of bioactive fibroblast growth factor-2 into cubic proteinous microcrystals (Bombyx mori cypovirus polyhedra) that are insoluble in a physiological cellular environment. J Biol Chem. 2007;282(23):17289-96.
- [9] Nishishita N, Ijiri H, Takenaka C, Kobayashi K, Goto K, Kotani E, et al. The use of leukemia inhibitory factor immobilized on virus-derived polyhedra to support the proliferation of mouse embryonic and induced pluripotent stem cells. Biomaterials.

2011;32(14):3555-63.

- [10] Matsushima K, Suyama T, Takenaka C, Nishishita N, Ikeda K, Ikada Y, et al. Secreted frizzled related protein 4 reduces fibrosis scar size and ameliorates cardiac function after ischemic injury. *Tissue Eng Part A*. 2010;16(11):3329-41.
- [11] Hoeben A, Landuyt B, Highley MS, Wildiers H, Van Oosterom AT, De Bruijn EA. Vascular endothelial growth factor and angiogenesis. *Pharmacol Rev*. 2004;56(4):549-80.
- [12] Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev*. 2004;25(4):581-611.
- [13] Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J*. 1999;13(1):9-22.
- [14] Ortega N, Hutchings H, Plouet J. Signal relays in the VEGF system. *Front Biosci*. 1999;4:D141-52.
- [15] O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, et al. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell*. 1997;88(2):277-85.
- [16] Folkman J. Antiangiogenesis in cancer therapy--endostatin and its mechanisms of action. *Exp Cell Res*. 2006;312(5):594-607.
- [17] Dixelius J, Larsson H, Sasaki T, Holmqvist K, Lu L, Engstrom A, et al. Endostatin-induced tyrosine kinase signaling through the Shb adaptor protein regulates endothelial cell apoptosis. *Blood*. 2000;95(11):3403-11.
- [18] Zhuo W, Luo C, Wang X, Song X, Fu Y, Luo Y. Endostatin inhibits tumour lymphangiogenesis and lymphatic metastasis via cell surface nucleolin on lymphangiogenic endothelial cells. *J Pathol*. 2010;222(3):249-60.
- [19] Chaplin DJ, Olive PL, Durand RE. Intermittent blood flow in a murine tumor:

- radiobiological effects. *Cancer Res.* 1987;47(2):597-601.
- [20] Matsumoto G, Ueda T, Shimoyama J, Ijiri H, Omi Y, Yube H, et al. Bone regeneration by polyhedral microcrystals from silkworm virus. *Sci Rep.* 2012;2:935.
- [21] Sharapova NE, Kotnova AP, Galushkina ZM, Lavrova NV, Poletaeva NN, Tukhvatulin AE, et al. [Production of the recombinant human bone morphogenetic protein-2 in *Escherichia coli* and testing of its biological activity in vitro and in vivo]. *Mol Biol (Mosk).* 2010;44(6):1036-44.
- [22] Heiring C, Muller YA. Folding screening assayed by proteolysis: application to various cystine deletion mutants of vascular endothelial growth factor. *Protein Eng.* 2001;14(3):183-8.
- [23] Seyedarabi A, Cheng L, Zachary I, Djordjevic S. Production of soluble human vascular endothelial growth factor VEGF-A165-heparin binding domain in *Escherichia coli*. *PLoS One.* 2013;8(2):e55690.
- [24] Ramirez F, Rifkin DB. Cell signaling events: a view from the matrix. *Matrix Biol.* 2003;22(2):101-7.
- [25] Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol.* 2001;17:463-516.
- [26] Bashkin P, Doctrow S, Klagsbrun M, Svahn CM, Folkman J, Vlodavsky I. Basic fibroblast growth factor binds to subendothelial extracellular matrix and is released by heparitinase and heparin-like molecules. *Biochemistry.* 1989;28(4):1737-43.
- [27] Fairbrother WJ, Champe MA, Christinger HW, Keyt BA, Starovasnik MA. Solution structure of the heparin-binding domain of vascular endothelial growth factor. *Structure.* 1998;6(5):637-48.
- [28] Javaherian K, Park SY, Pickl WF, LaMontagne KR, Sjin RT, Gillies S, et al. Laminin

modulates morphogenic properties of the collagen XVIII endostatin domain. J Biol Chem. 2002;277(47):45211-8.

[29] Sasaki T, Larsson H, Kreuger J, Salmivirta M, Claesson-Welsh L, Lindahl U, et al. Structural basis and potential role of heparin/heparan sulfate binding to the angiogenesis inhibitor endostatin. EMBO J. 1999;18(22):6240-8.

[30] Fu Y, Tang H, Huang Y, Song N, Luo Y. Unraveling the mysteries of endostatin. IUBMB Life. 2009;61(6):613-26.

[31] Sun X, Krissansen GW, Fung PW, Xu S, Shi J, Man K, et al. Anti-angiogenic therapy subsequent to adeno-associated-virus-mediated immunotherapy eradicates lymphomas that disseminate to the liver. Int J Cancer. 2005;113(4):670-7.

[32] Noro T, Miyake K, Suzuki-Miyake N, Igarashi T, Uchida E, Misawa T, et al. Adeno-associated viral vector-mediated expression of endostatin inhibits tumor growth and metastasis in an orthotropic pancreatic cancer model in hamsters. Cancer Res. 2004;64(20):7486-90.

[33] Swartz MA. The physiology of the lymphatic system. Adv Drug Deliv Rev. 2001;50(1-2):3-20.

[34] Cao Y. Opinion: emerging mechanisms of tumour lymphangiogenesis and lymphatic metastasis. Nat Rev Cancer. 2005;5(9):735-43.

[35] Gao P, Zhou GY, Zhang QH, Su ZX, Zhang TG, Xiang L, et al. Lymphangiogenesis in gastric carcinoma correlates with prognosis. J Pathol. 2009;218(2):192-200.

[36] Fukumoto S, Morifuji M, Katakura Y, Ohishi M, Nakamura S. Endostatin inhibits lymph node metastasis by a down-regulation of the vascular endothelial growth factor C expression in tumor cells. Clin Exp Metastasis. 2005;22(1):31-8.

[37] Brideau G, Makinen MJ, Elamaa H, Tu H, Nilsson G, Alitalo K, et al. Endostatin

1
2
3 overexpression inhibits lymphangiogenesis and lymph node metastasis in mice. *Cancer*
4
5 *Res.* 2007;67(24):11528-35.
6

7 [38] Matsumoto G, Ohmi Y, Shindo J. Angiostatin gene therapy inhibits the growth of murine
8
9 squamous cell carcinoma in vivo. *Oral Oncol.* 2001;37(4):369-78.
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Legends to figures

Fig. 1 Phosphorylation of p44/p42 MAP kinase and proliferation of HUVECs by polyhedra encapsulating VEGF-A. (A) HUVECs were plated into a 6-well plate at a density of 1×10^5 cells per well and cultured to confluence in the growth medium. Cells were starved in the starvation medium for 6 hours and then incubated with rhVEGF (5 ng/ml) and empty or VEGF polyhedra (2.5×10^5 numbers/ml) for 30 min. VP3/VEGF and VEGF/VP3 show VEGF polyhedra in which VEGF was encapsulated into polyhedra by VP3 tag at N- or C-terminus with or without PDI, respectively. Empty is CP-H polyhedra. The phosphorylated p44 (phospho-p44) and p42 (phospho-p42) MAP kinase were immunoblotted with Phospho-p44/p42 MAP Kinase Antibody and total amounts of p44/p42 were detected by p44/p42 MAP Kinase Antibody. (B) HUVECs were plated into a 96-well plate at a density of 3×10^3 cells per well and starved in the starvation medium for 6 hours. Each number (0.2, 0.5, 1, 2×10^5) of VEGF polyhedra, 2×10^5 numbers of empty polyhedra and 2 ng of rhVEGF in 200 μ l of the assay medium were added into wells. After 3 days, the proliferation of HUVECs was measured by Cell Counting Kit-8. The graph shows mean $\pm$ S.E.

Fig. 2 Stimulation and inhibition of migration of HUVECs by VEGF and endostatin polyhedra. HUVECs were plated into a 24-well plate at a density of 8×10^3 cells per well and cultured to confluence in the growth medium. The cells were scraped away vertically by pipette tip and then incubated for 18 hours in 500 μ l of serum-free EGM-2 medium with 5 ng of rhVEGF or 4×10^5 numbers of empty and VEGF polyhedra. For endostatin polyhedra (H1/Endo polyhedra and Endo/VP3), 4×10^5 numbers of polyhedra were added to 500 μ l of serum-free EGM-2 medium containing rhVEGF (10 ng/ml).

Fig. 3 Stimulation and inhibition of network and tube formation of HUVECs by VEGF and endostatin polyhedra. (A) Subconfluent culture of HUVECs was suspended in the starvation

medium to synchronize cell cycles. After 2 ng of rhVEGF or 1.6×10^5 numbers of empty and VEGF polyhedra were mixed with ECMatrix solution, aliquots were added to each well of the 96-well plate and incubated at 37 °C to solidify the matrix solution. For endostatin polyhedra (H1/Endo and Endo/VP3), 1.6×10^5 numbers of polyhedra were added to ECMatrix solution containing rhVEGF (10 ng/ml). HUVECs (1×10^4 /well) were seeded onto presolidified ECMatrix in each well. Calcein AM solution was added to each well. a, control; b, empty polyhedra; c, rhVEGF; d, VEGF polyhedra; e, H1/Endo polyhedra; f, Endo/VP3 polyhedra. (B) Network formation was photographed using an inverted microscope and tube length of each cell was measured using an image analyzer (ESPEC TECHNO CORP). The graph shows mean $\pm$ S.E. (C) Normal human dermal fibroblast cells were plated into a collagen-coated 24-well plate at a density of 2×10^4 cells per well and incubated with 500 μ l of Medium 106 supplemented with Low Serum Growth Supplement. The medium was replaced with 500 μ l of the starvation medium and then 5 ng of rhVEGF or 4×10^5 numbers of empty and VEGF polyhedra were added to each well. For endostatin polyhedra (H1/Endo and Endo/VP3), 4×10^5 numbers of polyhedra were added to 500 μ l of the starvation medium containing rhVEGF (10 ng/ml). HUVECs (2×10^4 /well) were seeded into each well and incubated with the assay medium for 11 days at 37°C. The assay medium with or without rhVEGF (10 ng/ml) was used for wells of rhVEGF and polyhedra, respectively, at each 2 days medium change. To detect tube formation of HUVECs, mouse anti-human CD31 antibody was employed followed by goat anti-mouse IgG AP conjugate antibody. Tubes were observed by addition of substrate BCIP/NBT. a, control; b, empty polyhedra; c, rhVEGF; d, VEGF polyhedra; e, H1/Endo polyhedra; f, Endo/VP3 polyhedra.

Fig. 4 Inhibition of growth of subcutaneous SCC-VII tumors by intratumoral injection of endostatin polyhedra. Four treatment groups were established: H1/Endo polyhedra, Endo/VP3 polyhedra, empty polyhedra and no treatment as control. Each polyhedron was administrated at 2 and 4 weeks after SCC-VII tumor-cell inoculation and tumor volume was measured at 6 weeks after the

tumor-cell inoculation. The first quartile and third quartile were indicated by horizontal bars at the bottom and top of the box, respectively. Median is indicated as the center line inside the box. Outliers are indicated by dots.

Fig. 5 Endostatin polyhedra inhibit angiogenesis but not lymphangiogenesis in SCC-VII tumors.

(A) Representative double immunofluorescent staining image for CD31, the marker of vascular endothelial cells (green), and LYVE-1, the marker of lymphatic endothelial cells (red). a, control; b, empty polyhedra; c, H1/Endo polyhedra; d, Endo/VP3 polyhedra. (B) CD31-positive endothelial cells were randomly counted at 100-power magnification from five different fields. CD31-positive vessels in treated tumors treated with endostatin polyhedra (H1/Endo polyhedra and Endo/VP3 polyhedra) were markedly lower than for control and empty polyhedra groups (* $P < 0.05$, ** $P < 0.01$). Vessel numbers were compared using Student's *t* test. The graph shows mean $\pm$ S.E. (C) Quantification of LYVE-1-positive lymphatic vessels in the tumor tissues. There was no significant difference between the control, empty polyhedron, and endostatin polyhedra groups (H1/Endo polyhedra and Endo/VP3 polyhedra). Vessel numbers were compared using Student's *t* test. The graph shows mean $\pm$ S.E.

Fig.1

Fig.2

Fig.4

Fig.5