



Molecular and biological characterization of vascular endothelial growth factor of parapoxviruses isolated from wild Japanese serows ()

Yasuo Inoshima, Naotaka Ishiguro

► To cite this version:

Yasuo Inoshima, Naotaka Ishiguro. Molecular and biological characterization of vascular endothelial growth factor of parapoxviruses isolated from wild Japanese serows (). *Veterinary Microbiology*, 2009, 140 (1-2), pp.63. 10.1016/j.vetmic.2009.07.024 . hal-00535919

HAL Id: hal-00535919

<https://hal.science/hal-00535919>

Submitted on 14 Nov 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: Molecular and biological characterization of vascular endothelial growth factor of parapoxviruses isolated from wild Japanese serows (*Capricornis crispus*)

Authors: Yasuo Inoshima, Naotaka Ishiguro



PII: S0378-1135(09)00346-0
DOI: doi:10.1016/j.vetmic.2009.07.024
Reference: VETMIC 4516

To appear in: *VETMIC*

Received date: 4-3-2009
Revised date: 18-7-2009
Accepted date: 31-7-2009

Please cite this article as: Inoshima, Y., Ishiguro, N., Molecular and biological characterization of vascular endothelial growth factor of parapoxviruses isolated from wild Japanese serows (*Capricornis crispus*), *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2009.07.024

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1 Molecular and biological characterization of vascular endothelial growth

2 factor of parapoxviruses isolated from wild Japanese serows (*Capricornis*

3 *crispus*)

4
5 Yasuo Inoshima*, Naotaka Ishiguro

6
7 Laboratory of Food and Environmental Hygiene, Department of Veterinary

8 Medicine, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

9
10 *Correspondence author: Yasuo Inoshima

11 Laboratory of Food and Environmental Hygiene, Department of Veterinary

12 Medicine, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

13 Tel.: +81 58 293 2863; Fax: +81 58 293 2840

14 *E-mail*: inoshima@gifu-u.ac.jp.

16 **Abstract**

17 Contagious dermatitis in domestic and wild ruminants caused by
18 parapoxvirus (PPV) occurs worldwide. Although PPV infections appear in
19 cattle, sheep and goats, the papular, nodular, pustular and ulcerated skin
20 lesions of wild Japanese serows (*Capricornis crispus*) are significantly more
21 severe than those of other animals. To determine the factors involved in the
22 severity of these skin lesions, we compared the molecular characteristics of 4
23 PPV isolates from Japanese serows and 2 isolates from sheep in Japan, and
24 also investigated the biological properties of primary endothelial cells from
25 different host species. All of the 6 Japanese isolates harbored a vascular
26 endothelial growth factor (VEGF) gene, which is known as one of the
27 virulence factors of PPVs. Three different amino acid sequences of viral
28 VEGF were identified and the sequence was identical among the 4 isolates
29 from the Japanese serows. The temporal expression pattern of viral VEGF
30 mRNA was almost the same among 3 isolates encoding the 3 different
31 VEGFs in infected cells from different host species. Recombinant forms of
32 the 3 different VEGFs showed the ability to induce vascular permeability
33 and endothelial cell-proliferation. Primary endothelial cells from Japanese
34 serows were most responsive to recombinant viral VEGF compared to cells

35 from cattle, sheep, and goats. These results suggest that not only the
36 biological activity of viral VEGF but also the high responsiveness to viral
37 VEGF of endothelial cells might be involved in the severe proliferative skin
38 lesions induced by PPV infection in Japanese serows.

39

40 Keywords:

41 Dermatitis

42 Endothelial cells

43 Japanese serow

44 Parapoxvirus

45 VEGF

46

1. Introduction

Parapoxviruses (PPVs) are the etiological agents of papular stomatitis and pseudocowpox in cattle, and contagious pustular dermatitis in sheep and goats (Damon, 2007; Robinson and Lyttle, 1992). PPV infections in sheep have been known as scabby disease or “orf” among shepherds since the 1780s (Robinson and Lyttle, 1992) and the infections are now widespread in both domestic and wild ruminants worldwide. PPV infection of wild Japanese serows (*Capricornis crispus*) has been common knowledge in Japan since the first outbreak was observed in 1976 (Kumagai et al., 1979). The histopathological features are similar to those of domestic animals (Okada et al., 1984a, b; Suzuki et al., 1986); however, the skin lesions tend to be much more severe. Weakened or dead Japanese serows are frequently found in their natural territories, as they were unable to eat due to such lesions. Generally, the level of severity of the skin lesions is greatest in Japanese serows, moderate in sheep and goats, and low in cattle (Fig. 1). Although PPVs from Japanese serows experimentally infect sheep, goats and cattle, the highly proliferative skin lesions seen in Japanese serows is not reproduced (Masegi et al., 2002; Ogino et al., 1996; Okada et al., 1986). The

65 factors involved in these clinical differences are still unspecified for both the
66 pathogen and host.

67 Previous studies revealed that orf virus, one of the members of PPV,
68 encodes an apparent homologue of the vascular endothelial growth factor
69 (VEGF) gene (Lyttle et al., 1994; Mercer et al., 2002; Rziha et al., 1999), and
70 recent studies have shown that other members of PPV, including
71 pseudocowpoxvirus, bovine papular stomatitis virus and parapoxvirus of red
72 deer in New Zealand, also encode a VEGF gene (Delhon et al., 2004; Ueda et
73 al., 2003, 2007). Cellular VEGF is a potent mitogen for vascular endothelial
74 cells and essential for embryonic vasculogenesis and angiogenesis. VEGF
75 also activates vascular permeability and plays a role in pathological
76 angiogenesis including tumor growth and inflammation (Ferrara, 1999,
77 2005; Ferrara and Davis-Smyth, 1997; Robinson and Stringer, 2001; Stacker
78 and Achen, 1999). The histological characteristics of the lesions of PPV
79 infection, such as extensive vascularization, cell proliferation and vacuolar
80 degeneration of epithelial cells, are thought to be the result of viral VEGF
81 activity. Recent studies have demonstrated that PPV lacking functional
82 VEGF causes relatively reduced skin lesions and histopathological changes
83 (Savory et al., 2000; Wise et al., 2007).

The aim of this study was to determine the factors involved in the severe proliferative skin lesions of PPV infection in Japanese serows. Here, we described the molecular and biological characterization of the VEGF of PPVs from Japanese serows and the responsiveness to VEGF of serow endothelial cells compared with Japanese PPVs from sheep.

2. Materials and methods

2.1. Viruses and cells

Four PPV isolates from Japanese serows and 2 isolates from sheep in Japan were used in this study (Table 1). All isolates were propagated in primary fetal bovine muscle (FBM) cells. Primary FBM, fetal lamb lung (FLL), and vascular smooth muscle cells of Japanese serow (SeSM) were maintained in Eagle's minimum essential medium (Nissui, Tokyo, Japan) supplemented with 0.3% tryptose phosphate broth (Difco, Detroit, MI, USA), 10% fetal bovine serum (FBS), 100 µg/ml of streptomycin, and 100 U/ml of penicillin. Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Walkersville, MO, USA) or Cell Applications (San Diego, CA, USA). Primary aortic endothelial cells of cattle (BAEC), sheep (OAEC), goats

103 (CAEC) and Japanese serows (SeAEC) were isolated from aortic vessels.
 104 Endothelial cells were maintained in endothelial cell growth medium-2
 105 (EGM-2, Lonza) containing supplements as per the manufacturer's
 106 instructions and 10% FBS. Primary skeletal muscle cells of Japanese serow
 107 were kindly provided by Dr. T. Ishida (Graduate School of Science, The
 108 University of Tokyo, Tokyo, Japan) and were maintained in RPMI 1640
 109 (Wako, Osaka, Japan) supplemented with 10% FBS, 100 µg/ml of
 110 streptomycin, and 100 U/ml of penicillin.

111

112 *2.2. Polymerase chain reaction (PCR) and sequencing*

113 For PCR, DNA was extracted from FBM cells infected with each of
 114 the isolates using a DNeasy Tissue Kit (Qiagen, Hilden, Germany). PCR for
 115 the viral envelope gene using the primers PPP-1 and PPP-4 (Supplementary
 116 Table S1) was carried out for identification of PPV species as described
 117 previously (Inoshima et al., 2000, 2001a). PCR primers (D27F, 11R and D2R;
 118 Supplementary Table S1) were designed for the flanking regions of the viral
 119 VEGF gene of the orf virus strains, NZ2 (DDBJ/EMBL/GenBank accession
 120 no. S67520) (Lyttle et al., 1994), NZ7 (S67522) (Lyttle et al., 1994), D1701
 121 (AY186732) (Rziha et al., 2003), and orf-11 (AY236150). PCR consisted of 9

122 min at 95°C followed by 40 cycles of 94°C for 1 min, 40°C for 1 min and 72°C
 123 for 1 min, with a final extension at 72°C for 5 min. Nucleotide sequences of
 124 the viral VEGF gene were determined by direct sequencing of the PCR
 125 products. Sequences were obtained from both strands for verification.

126

127 *2.3. Reverse transcription-PCR (RT-PCR)*

128 FBM, FLL and SeSM cells were infected with virus at a titer of 10^3
 129 TCID₅₀/ml. Total RNA was extracted from cells at various intervals after
 130 virus infection (0, 1, 2, 4, 8, 12, 24, and 36 h) using an RNeasy Mini Kit
 131 (Qiagen), and the temporal expression pattern of viral VEGF mRNAs was
 132 determined by RT-PCR using the primers VEGF orf-11F, VEGF cons340R,
 133 VEGF HISF, and VEGF HISR (Supplementary Table S1). After treatment of
 134 RNA samples with DNase I (Invitrogen, Carlsbad, CA, USA), RT-PCR was
 135 carried out using 0.5 µg of DNase I-treated RNA and a Titan One Tube
 136 RT-PCR Kit (Roche Applied Science, Mannheim, Germany) according to the
 137 manufacturer's instructions.

138

139 *2.4. Cloning and expression of viral VEGF*

140 For the recombinant expression of viral VEGF, the VEGF gene was

141 amplified from DNA extracted from virus-infected cells by PCR using
 142 primers BamConsSigF, EcoConsR and PstConsR (Supplementary Table S1).
 143 PCR products were digested with *Bam*HI and *Eco*RI, or with *Bam*HI and
 144 *Pst*I, and then cloned into the corresponding sites of expression vector
 145 pRSET (Invitrogen). Recombinant VEGFs with a hexa-His-tag were
 146 expressed in *Escherichia coli* BL21(DE3)pLysS (Invitrogen) and purified by
 147 affinity chromatography on a Ni-chelating column according to the
 148 manufacturer's instructions and previous studies (Meyer et al., 1999;
 149 Yamamoto et al., 2001). Purified recombinant VEGFs were analyzed by
 150 SDS-PAGE followed by Coomassie brilliant blue staining and western blot
 151 analysis using anti-His G antibody (R941-25, Invitrogen). The amount of
 152 purified recombinant protein was quantified by Lowry's method with DC
 153 protein assay reagents (Bio-Rad Laboratories, Hercules, CA, USA).

154

155 *2.5. Miles assay (vascular permeability assay)*

156 The vascular permeability activity of recombinant VEGF was
 157 detected using guinea pigs according to the Miles assay (Miles and Miles,
 158 1952; Ogawa et al., 1998; Wise et al., 1999). Three guinea pigs were used for
 159 each recombinant VEGF. Handling of guinea pigs was reviewed and

160 monitored according to the Guidelines for Animal Handling of Gifu
 161 University.

162

163 *2.6. Indirect immunofluorescence assay (IFA)*

164 Isolated primary cells of cattle, sheep, goats, and Japanese serows
 165 (BAEC, OAEC, CAEC, and SeAEC) were confirmed as endothelial cells by
 166 IFA and flow cytometry. After fixation by cold acetone, primary cells
 167 cultivated on a coverslip were incubated with anti-human von Willebrand
 168 factor (vWF) antibody (F 3520, Sigma-Aldrich, St. Louis, MO, USA), washed
 169 with phosphate buffered saline (PBS), and then incubated with fluorescein
 170 isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody (62-6111,
 171 Zymed Laboratories, South San Francisco, CA, USA). The same procedure
 172 with omission of the primary antibody served as the negative control.

173

174 *2.7. Flow cytometry*

175 Primary endothelial cells were incubated with FACS permeabilizing
 176 solution 2 (BD Biosciences, San Jose, CA, USA), washed in sorter buffer (2%
 177 FBS, 5 mM EDTA and 0.1% sodium azide in PBS), incubated with or without
 178 anti-human vWF antibody, washed, and then incubated with

179 FITC-conjugated goat anti-rabbit IgG antibody. After washing, the cells were
180 analyzed using a FACSCalibur flow cytometer (BD Biosciences).

181

182 *2.8. Proliferation assay*

183 Cell proliferation was determined with a colorimetric assay using the
184 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfonyl)-2H-tetr
185 a-zolium, monosodium salt (WST-8), which measures the number of viable
186 cells. HUVEC and primary endothelial cells were seeded in 96-well plates at
187 1,000 cells/well. After 24 h, the supernatant was removed and the cells
188 rinsed in PBS. To starve the cells of supplemental VEGF in the culture
189 medium, the cells were cultivated with human endothelial cell serum-free
190 basal medium (Invitrogen) containing 2% FBS overnight. After changing to
191 fresh medium with or without recombinant VEGF, the cells were incubated
192 at 37°C for 2 days. The supernatant was removed and 100 µl/well of fresh
193 medium containing 10% WST-8 solution (Cell Counting Kit-8, Dojindo,
194 Kumamoto, Japan) was added. The cells were incubated for 2 h at 37°C and
195 the absorbance of each well was measured at 450 nm by a plate reader,
196 Multiskan MS-UV (Labsystems, Helsinki, Finland). The proliferation index
197 was determined as the ratio of the absorbance at 450 nm of cells treated with

198 VEGF against the absorbance at 450 nm of untreated cells. Primary skeletal
199 muscle cells of Japanese serow were also treated with recombinant VEGF
200 and used as the control.

201

202 *2.9. Statistical analysis*

203 The data were analyzed for statistical significance by parametric
204 non-repeated measures ANOVA and the post hoc test using Dunnett's or
205 SNK tests, and also parametric unpaired Student's *t*-test.

206

207 **3. Results**

208

209 *3.1. Identification and sequences of viral VEGF gene*

210 All of the 6 PPV isolates were classified as orf virus by sequence
211 analysis of the viral envelope gene (data not shown). Several PCR primers
212 were first designed to bind outside the viral VEGF gene. However, as already
213 reported by Mercer et al. (2002), these primers were unable to amplify a
214 specific viral VEGF gene (data not shown), indicating sequence variation in
215 the flanking regions of the VEGF gene. The VEGF gene was amplified from
216 all of the 6 PPV isolates using primers D27F and 11R, except for isolate HIS,

217 for which primers D27F and D2R were used. The complete nucleotide
218 sequences of the viral VEGFs were successfully determined by direct
219 sequencing. The deduced amino acid sequences were aligned with each other
220 and with those of the NZ2, D1701 and orf-11 strains by Genetyx ver. 8.2.2.
221 software (Genetyx, Tokyo, Japan) (Fig. 2). Interestingly, among the 4 isolates
222 from the Japanese serows, not only the amino acid sequences but also the
223 nucleotide sequences showed 100% identity. Among the 6 Japanese isolates,
224 the amino acid sequences of VEGF were able to be classified into 3 different
225 types, designated Iwate, HIS and S-1 (S-1 was found in all serow isolates).
226 Sequence identity was also determined using the Genetyx software
227 (Supplementary Table S2). The Iwate and S-1 VEGFs were composed of 137
228 amino acids, with 1 amino acid substitution between them (99.3% identity),
229 whereas HIS was shorter at 132 amino acids and included many amino acid
230 substitutions in the sequence. The identity of the amino acid sequence
231 between Iwate and HIS was 69.8%, and between HIS and S-1 was 70.5%.
232 The Iwate and S-1 VEGFs showed high identity with orf-11 VEGF,
233 conversely HIS VEGF showed higher identity with the NZ2 and D1701
234 VEGFs than Iwate and S-1 (Supplementary Table S2). Eight cysteine
235 residues that are conserved in all members of the VEGF family are also

236 conserved in the sequences of the Iwate, HIS and S-1 VEGFs. The orf virus
 237 VEGF utilizes the VEGF receptor 2 and neuropilin-1 (Ogawa et al., 1998,
 238 Meyer et al., 1999, Wise et al., 1999). An asparagine residue at the 73
 239 position (Iwate VEGF) is conserved in Iwate, HIS and S-1 VEGF; this is an
 240 accessible residue on the receptor-binding face of VEGF-A that correlates
 241 strongly with binding of the VEGF receptor 2 and is also conserved in PPV
 242 VEGFs (Mercer et al., 2002, Ueda et al., 2003, 2007, Inder et al., 2007). The
 243 specific mutation corresponding to the binding ability to VEGF receptor 1
 244 described by Mercer et al. (Mercer et al., 2002) was not observed at any
 245 positions among our 3 VEGF sequences. These results suggested that the
 246 Iwate, HIS and S-1 VEGFs bind to VEGF receptor 2 but not to receptor 1, as
 247 seen in the other orf viruses. The DNA sequences determined in this study
 248 were submitted to DDBJ/EMBL/GenBank under accession numbers
 249 AB436805 to AB436810.

250

251 *3.2. Expression of viral VEGF mRNA*

252 Expression of VEGF mRNA was detected in the early stage of
 253 infection (Fig. 3). No amplicons were found in PCR products without RT
 254 (data not shown). In all cells infected with the Iwate and S-1 VEGFs,

255 expression was detected as early as 4 h post-infection. Expression of HIS
256 VEGF mRNA occurred slightly later, appearing first at 8 h after infection.
257 However, no significant difference was observed among the 3 different types
258 of cells and the 3 isolates.

259

260 *3.3. Expression of recombinant VEGF*

261 Viral VEGFs were expressed in *E.coli* as a fusion protein with a
262 hexa-His-tag. The recombinant VEGFs were purified and estimated by
263 SDS-PAGE followed by Coomassie brilliant blue staining and Western blot
264 analysis. As expected from the sequence analysis, recombinant HIS VEGF is
265 smaller in molecular weight than Iwate and S-1 VEGFs. Under reducing
266 conditions with 2-mercaptoethanol, and as estimated using a pre-stained
267 protein marker (P7708, New England Biolabs, Ipswich, MA, USA), the
268 apparent molecular weight of HIS VEGF is about 18 kDa while that of Iwate
269 and S-1 VEGF is about 22 kDa.; under non-reducing conditions the apparent
270 molecular weight of HIS VEGF is about 44 kDa while that of Iwate and S-1
271 VEGF is about 48 kDa (data not shown). These results indicate that
272 recombinant VEGF forms a homodimer, which is consistent with previous
273 studies (Meyer et al., 1999).

274

275 *3.4. Vascular permeability activity*

276 The purified recombinant VEGFs were next investigated for their
277 biological activities. Vascular permeability activity was evaluated using the
278 Miles assay. Dose-dependent permeabilization was induced by all of the viral
279 VEGFs, which was statistically different from the PBS control by parametric
280 non-repeated measures ANOVA and Dunnett's test (Fig. 4a). However,
281 among the 3 VEGFs, no statistically significant difference was observed in
282 each dilution by parametric non-repeated measures ANOVA and the SNK
283 test.

284

285 *3.5. HUVEC-proliferative activity*

286 HUVEC were treated with recombinant VEGF for 2 days. Each
287 VEGF showed mitogenic activity on HUVEC proliferation (Fig. 4b). The
288 proliferation index was determined as the ratio of the absorbance at 450 nm
289 of cells treated with VEGF against the absorbance at 450 nm of untreated
290 cells. When compared at 50 ng/ml of VEGF dilution, the HIS and S-1 VEGFs
291 showed significantly higher activity ($P < 0.01$) on HUVEC proliferation than
292 the Iwate VEGF ($P < 0.05$), but no significant difference was observed

293 between the HIS and S-1 VEGFs by parametric non-repeated measures
 294 ANOVA and the SNK test (Fig. 4b). Statistically identical results were
 295 obtained using 10 ng/ml of VEGF.

296

297 *3.6. Isolation and identification of primary endothelial cells*

298 Primary endothelial cells were isolated from the aortas of cattle,
 299 sheep, goats, and Japanese serows. Isolated endothelial cells were
 300 sometimes retrieved with smooth muscle cells. To purify endothelial cells,
 301 several possible antibodies for surface markers to endothelial cells, ovine and
 302 human CD31, VEGF receptors and plasmalemmal vesicle associated
 303 protein-1 were used in a magnetic activated cell sorting (MACS) assay.
 304 However, no antibody was successful in the purification of endothelial cells
 305 by MACS (data not shown), suggesting that cross-reactivity of commercially
 306 available antibodies seems to be weak against animal endothelial cells, or
 307 that the expression patterns are different in ruminants or changed by
 308 trypsinization. Thus the purity of the primary endothelial cells was
 309 estimated using an antibody to vWF by IFA and flow cytometry. Primary
 310 endothelial cells were positive for the presence of vWF in the cytoplasm by
 311 IFA, whereas serow skeletal muscle cells were negative (Fig. 5a). Endothelial

312 cells showing over 80% purity by flow cytometry (Fig. 5a) were used for the
313 proliferation assay.

314

315 *3.7. Comparison of responsiveness to VEGF*

316 Primary endothelial cells from different host species (BAEC, OAEC,
317 CAEC, and SeAEC) were treated with recombinant S-1 VEGF (S-1 was
318 found in all serow isolates) for 2 days. The responsiveness to VEGF was
319 estimated by cell proliferation and was significantly different among the cell
320 types (Fig. 5b). The proliferation index was the lowest in BAECs and the
321 greatest in SeAECs, suggesting that the SeAECs are the most responsive to
322 S-1 VEGF.

323

324 **4. Discussion**

325

326 Japanese serows are a protected ruminant species in Japan. PPV
327 infection among wild Japanese serows has been continuously observed in
328 various areas since the 1970s (Inoshima et al., 1999, 2001b; Kumagai et al.,
329 1979). Proliferative severe skin lesions by PPV infection in Japanese serows
330 are frequently observed in serows in their natural territories, and most of the

331 affected animals die from debilitation (Okada et al., 1984b; Suzuki et al.,
332 1986, 1997). In cattle, the mortality due to PPV infection is significantly
333 lower and the lesions are considerably milder. Previously we isolated several
334 PPVs from affected Japanese serows and revealed that the PPVs circulating
335 among wild Japanese serows are mainly orf virus and accidentally bovine
336 papular stomatitis virus (Inoshima et al., 2001a, 2002). To define the factors
337 responsible for the severity of PPV-induced lesions in Japanese serows, we
338 characterized the molecular and biological characteristics of viral VEGF
339 using 6 Japanese PPV isolates.

340 At first, we determined the sequence of the VEGF gene from the 6
341 Japanese PPV isolates. Surprisingly, the amino acid sequences of VEGF
342 were identical among the 4 isolates (S-1 type) from the Japanese serows.
343 Next, mRNA expression was compared temporally among the 3 VEGF types
344 (Iwate, HIS and S-1). No significant temporal difference in mRNA expression
345 was observed among the 3 PPVs. Our results are consistent with previous
346 observations. Lyttle et al. (1994) demonstrated that RNA transcripts from
347 the viral VEGF gene can be detected as early as 6 h post-infection in cells.
348 Moreover, Scagliarini et al. (2006) used 4 different PPVs, including an isolate
349 IT 01 from a severe fatal outbreak in sheep, and showed that the expression

350 of VEGF mRNA in cells did not differ chronologically, as estimated by
351 real-time PCR. The current findings revealed that the expression of S-1
352 VEGF mRNA is temporally similar to that of the other types of VEGF, not
353 only in cells from Japanese serows but also in cells from cattle and sheep.

354 Next, the biological activities of the 3 viral VEGFs were compared.
355 Although all 3 VEGFs induced vascular permeabilization, no significant
356 difference was observed at each dilution. In HUVEC-proliferative activity, a
357 significant difference was observed between the S-1 and Iwate VEGFs.
358 Unexpectedly, no significant difference either in vascular permeability or
359 HUVEC-proliferative activity was observed between the S-1 and HIS VEGFs.
360 Although we are unsure of the exact mechanisms involved, there are some
361 possible explanations for these findings. 1) A proline residue at amino acid
362 position 128 of Iwate VEGF is involved in the decrease in
363 HUVEC-proliferation, since the HIS and S-1 VEGFs and previously reported
364 VEGFs from 21 isolates of orf virus except orf-11 (Mercer et al., 2002) have a
365 threonine residue at this position. Amino acid 128 is located in the
366 C-terminal region, which has a threonine/proline-rich unique sequence
367 contributing to the binding of VEGF to cellular co-receptors, neuropilin-1
368 and heparin (Tokunaga et al., 2006). 2) The sequence of the loop 1 region is

369 conserved among the 3 VEGFs, whereas loop 3 of HIS differs from that of the
370 Iwate and S-1 VEGFs. Kiba et al. (2003) demonstrated that the set of the
371 loop 1 and 3 regions is crucial for the formation of the three-dimensional
372 structure important for the high-affinity binding to VEGF receptor 2.
373 Moreover, an arginine residue at amino acid position 115 in the C-terminal
374 region of NZ2 VEGF, which correlates with binding of VEGF to neuropilin-1
375 and heparin (Tokunaga et al., 2006), is glutamine in the Iwate and S-1
376 VEGFs but lysine in the HIS VEGF (Fig. 2). It is conceivable that these
377 differences at loop 3 and the arginine residue, together with other sequence
378 difference between the Iwate and HIS VEGFs, influence the receptor-binding
379 ability and biological activity of HUVEC-proliferation. However, the
380 combined effect of the complex of differences in the loop region, residue and
381 other sequences should be that HUVEC-proliferative activity is not
382 significantly different between the Iwate and HIS VEGFs. Further
383 comparative analyses using other viral and mutated VEGFs are required to
384 clarify the situation. Wise et al. (2003) reported that PPV VEGFs varied in
385 receptor-binding ability and vascular permeability. VEGF has angiogenic
386 activity and Scagliarini et al. (2006) demonstrated that an isolate, IT 01,
387 from a severe fatal outbreak in sheep, produced a higher number of vessels.

388 Moreover it is reported that, despite extreme variation in sequence, the
389 biological activities of viral VEGF including vascular permeability,
390 proliferation of endothelial cells, and angiogenicity, are conserved (Mercer et
391 al., 2002, 2006; Scagliarini et al., 2006; Wise et al., 2003, 2007). These results
392 suggest that the biological activity to stimulate vascular permeability and
393 HUVEC proliferation should be conserved among the Iwate, HIS and S-1
394 VEGFs despite sequence variations.

395 Next, we speculated that Japanese serows are more sensitive to PPV
396 or viral VEGF than the other host species and may be involved in the
397 pathogenesis. Since it was difficult to obtain microvascular endothelial cells
398 from each species involved under the natural conditions of infection, aortic
399 endothelial cells were used in our experiments. A proliferation assay using
400 primary endothelial cells from cattle, sheep, goats and Japanese serows
401 indicated different proliferation properties, in which endothelial cells from
402 Japanese serows showed the greatest response to S-1 VEGF. These results
403 suggest that the high responsiveness of endothelial cells to the viral VEGF
404 may be one of the factors in the severity of skin lesions in Japanese serows.
405 However, the effects on serow cells were not much greater than that on ovine
406 cells. Thus, the severe proliferative skin lesions in Japanese serow most

likely result from the combined effects of viral VEGF, other virulence/immune modulator genes such as the genes for viral IL-10 (Fleming et al., 2007), viral interferon resistance, and granulocyte/monocytes-colony stimulating factor inhibitory factor (Haig and McInnes, 2002), and host factors such as immune conditions and species-specific factors. It is possible that the orf viruses infecting Japanese serows are much more adapted to Japanese serows than to sheep and cattle, that Japanese serows may have a poorer defense against the infection than sheep and cattle, or that some cellular proteins of serows may interact more efficiently with the viral VEGF promoter or the production of VEGF protein.

Taken together, our findings suggest that the biological activities of the viral VEGFs and the responsiveness of endothelial cells to the VEGFs, appear to be factors involved in the severe proliferative skin lesions seen in infected Japanese serows. In addition, other viral and host factors may be involved. Further analyses are required to determine other associated factors, including receptor/co-receptor usage, genetic mutation of immune modulator genes (Rziha et al., 2000), and behavior in, and environmental/physiological conditions of, Japanese serows. Moreover, both *in vitro* and *in vivo* analyses using VEGF-deleted recombinant viruses or

426 VEGF-exchanged viruses are required for further interpretation of the role
427 of viral VEGF.

428

429 **Acknowledgements**

430

431 We thank Dr. N. Minamoto (Gifu University, Gifu, Japan), National
432 Institute of Animal Health (Ibaraki, Japan), Dr. T. Ishida (The University of
433 Tokyo), and Wadayama Livestock Hygiene Service Center (Hyogo, Japan) for
434 providing S-1 and the other isolates, for the primary serow skeletal muscle
435 cells and for the photograph of cattle, respectively. We are grateful to Drs. Y.
436 Ohba (Gifu University, Gifu, Japan), Y. Higuchi, M. Noike, M. Yasuda, I.
437 Hosono, K. Koshi, N. Tamura (Seki Public Health Center, Gifu, Japan), H.
438 Nakajima (Northern Meat Sanitary Inspection Center, Okinawa, Japan), M.
439 Fujita (National Livestock Breeding Center, Nagano Branch, Nagano,
440 Japan), Y. Mizuhashi (Takayama City Government, Gifu, Japan), H. Kawabe,
441 H. Okishima, and K. Yagai (Gifu Hunting Association, Japan) for providing
442 aortas of cattle, sheep, goats and Japanese serows. We are also thankful to
443 Drs. K. Murakami (National Institute of Animal Health, Ibaraki, Japan) and
444 H. Hara (Gifu Pharmaceutical University, Gifu, Japan) for their technical

advice regarding the isolation of primary endothelial cells and cell proliferation assay, respectively. This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

449

450 **References**

451

Damon, I. K., 2007. Poxviruses. In: Knipe, D.M., Howley, P.M. (Eds.), *Fields Virology*, 5th edn. Lippincott Williams & Wilkins, Philadelphia, pp. 2947-2975.

Delhon, G., Tulman, E.R., Afonso, C.L., Lu, Z., de la Concha-Bermejillo, A., Lehmkuhl, H.D., Piccone, M.E., Kutish, G.F., Rock, D.L., 2004. Genomes of the parapoxviruses ORF virus and bovine papular stomatitis virus. *J. Virol.* 78, 168-177.

Ferrara, N., 1999. Molecular and biological properties of vascular endothelial growth factor. *J. Mol. Med.* 77, 527-543.

Ferrara, N., 2005. The role of VEGF in the regulation of physiological and pathological angiogenesis. *EXS* 94, 209-231.

Ferrara, N., Davis-Smyth, T., 1997. The biology of vascular endothelial

- 464 growth factor. *Endocr. Rev.* 18, 4-25.
- 465 Fleming, S.B., Anderson, I.E., Thomson, J., Deane, D.L., McInnes, C.J.,
 466 McCaughan, C.A., Mercer, A.A., Haig, D.M., 2007. Infection with
 467 recombinant orf viruses demonstrates that the viral interleukin-10 is a
 468 virulence factor. *J. Gen. Virol.* 88, 1922-1927.
- 469 Haig, D.M., McInnes, C.J., 2002. Immunity and counter-immunity during
 470 infection with the parapoxvirus orf virus. *Virus Res.* 88, 3-16.
- 471 Inder, M.K., Ueda, N., Mercer, A.A., Fleming, S.B., Wise, L.M., 2007. *Bovine*
 472 *papular stomatitis virus* encodes a functionally distinct VEGF that
 473 binds both VEGFR-1 and VEGFR-2. *J. Gen. Virol.* 88, 781-791.
- 474 Inoshima, Y., Morooka, A., Sentsui, H. 2000. Detection and diagnosis of
 475 parapoxvirus by the polymerase chain reaction. *J. Virol. Methods* 84,
 476 201-208.
- 477 Inoshima, Y., Murakami, K., Wu, D., Sentsui, H., 2002. Characterization of
 478 parapoxviruses circulating among wild Japanese serows (*Capricornis*
 479 *crispus*). *Microbiol. Immunol.* 46, 583-587.
- 480 Inoshima, Y., Murakami, K., Yokoyama, T., Sentsui, H., 2001a. Genetic
 481 heterogeneity among parapoxviruses isolated from sheep, cattle and
 482 Japanese serows (*Capricornis crispus*). *J. Gen. Virol.* 82, 1215-1220.

- 483 Inoshima, Y., Shimizu, S., Minamoto, N., Hirai, K., Sentsui, H., 1999. Use of
484 protein AG in an enzyme-linked immunosorbent assay for screening for
485 antibodies against parapoxvirus in wild animals in Japan. Clin. Diagn.
486 Lab. Immunol. 6, 388-391.
- 487 Inoshima, Y., Yamamoto, Y., Takahashi, T., Shino, M., Katsumi, A., Shimizu,
488 S., Sentsui, H., 2001b. Serological survey of parapoxvirus infection in
489 wild ruminants in Japan in 1996-9. Epidemiol. Infect. 126, 153-156.
- 490 Kanou, Y., Inoshima, Y., Shibahara, T., Ishikawa, Y., Kadota, K., Ohashi, S.,
491 Morioka, K., Yoshida, K., Yamada, S., 2005. Isolation and
492 characterization of a parapoxvirus from sheep with papular stomatitis.
493 JARQ 39, 197-203.
- 494 Kiba, A., Yabana, N., Shibuya, M., 2003. A set of Loop-1 and -3 structures in
495 the novel vascular endothelial growth factor (VEGF) family member,
496 VEGF-ENZ-7, is essential for the activation of VEGFR-2 signaling. J. Biol.
497 Chem. 278, 13453-13461.
- 498 Kumagai, T., Shimizu, M., Ito, Y., Kanno, S., Nakagawa, M., Sato, K.,
499 Mukainakano, K., Ohta, H., 1971. Contagious papular dermatitis of
500 sheep. In: Abstracts of the 71st Meeting of the Japanese Society of
501 Veterinary Science, p. 15. (in Japanese).

- 502 Kumagai, T., Shimizu, M., Ito, Y., Yamamoto, S., Ito, T., Ohyama, S., Toshibe,
503 M., 1979. Contagious pustular dermatitis in Japanese serows in Akita
504 Prefecture. In: Abstracts of the 87th Meeting of the Japanese Society of
505 Veterinary Science, p. 121. (in Japanese).
- 506 Lyttle, D.J., Fraser, K.M., Fleming, S.B., Mercer, A.A., Robinson, A.J., 1994.
507 Homologs of vascular endothelial growth factor are encoded by the
508 poxvirus orf virus. *J. Virol.* 68, 84-92.
- 509 Masegi, T., Hori, M., Sakai, H., Inoshima, Y., Yanai, T., 2002. Parapoxvirus
510 infection in wild Japanese serow: a possibility of infection from wild
511 serow to sheep. *Jpn. J. Zoo Wildl. Med.* 7, 39-43.
- 512 Mercer, A.A., Ueda, N., Friederichs, S.M., Hofmann, K., Fraser, K.M.,
513 Bateman, T., Fleming, S.B., 2006. Comparative analysis of genome
514 sequences of three isolates of Orf virus reveals unexpected sequence
515 variation. *Virus Res.* 116, 146-158.
- 516 Mercer, A.A., Wise, L.M., Scagliarini, A., McInnes, C.J., Buttner, M., Rziha,
517 H.J., McCaughan, C.A., Fleming, S.B., Ueda, N., Nettleton, P.F., 2002.
518 Vascular endothelial growth factors encoded by Orf virus show
519 surprising sequence variation but have a conserved, functionally
520 relevant structure. *J. Gen. Virol.* 83, 2845-2855.

- 521 Meyer, M., Clauss, M., Lepple-Wienhues, A., Waltenberger, J., Augustin, H.
 522 G., Ziche, M., Lanz, C., Buttner, M., Rziha, H.J., Dehio, C., 1999. A novel
 523 vascular endothelial growth factor encoded by Orf virus, VEGF-E,
 524 mediates angiogenesis via signalling through VEGFR-2 (KDR) but not
 525 VEGFR-1 (Flt-1) receptor tyrosine kinases. *EMBO J.* 18, 363-374.
- 526 Miles, A.A., Miles, E.M., 1952. Vascular reactions to histamine,
 527 histamine-liberator and leukotaxine in the skin of guinea-pigs. *J.*
 528 *Physiol.* 118, 228-257.
- 529 Mohan, M., Malayer, J.R., Geisert, R.D., Morgan, G.L., 2001. Expression of
 530 retinol-binding protein messenger RNA and retinoic acid receptors in
 531 preattachment bovine embryos. *Mol. Reprod. Dev.* 60, 289-296.
- 532 Ogawa, S., Oku, A., Sawano, A., Yamaguchi, S., Yazaki, Y., Shibuya, M., 1998.
 533 A novel type of vascular endothelial growth factor, VEGF-E (NZ-7
 534 VEGF), preferentially utilizes KDR/Flk-1 receptor and carries a potent
 535 mitotic activity without heparin-binding domain. *J. Biol. Chem.* 273,
 536 31273-31282.
- 537 Ogino, H., Nakabayashi, D., Nabeya, M., Hoshi, K., Okazawa, T., 1996.
 538 Contagious papular dermatitis of Japanese serows in Niigata
 539 Prefecture. *J. Jpn. Vet. Med. Assoc.* 49, 615-618.

- 540 Okada, H.M., Matsukawa, K., Chihaya, Y., 1986. Experimental transmission
541 of contagious pustular dermatitis from a Japanese serow, *Capricornis*
542 *crispus*, to a calf and goats. J. Jpn. Vet. Med. Assoc. 39, 578-581.
- 543 Okada, H.M., Okada, K., Numakunai, S., Ohshima, K., 1984a. Electron
544 microscopy on mucosal and cutaneous lesions in contagious papular
545 dermatitis of Japanese Serow (*Capricornis crispus*). Jpn. J. Vet. Sci. 46,
546 297-302.
- 547 Okada, H.M., Okada, K., Numakunai, S., Ohshima, K., 1984b.
548 Histopathologic studies on mucosal and cutaneous lesions in contagious
549 papular dermatitis of Japanese serow (*Capricornis crispus*). Jpn. J. Vet.
550 Sci. 46, 257-264.
- 551 Palmarini, M., Holland, M.J., Cousens, C., Dalziel, R.G., Sharp, J.M., 1996.
552 Jaagsiekte retrovirus establishes a disseminated infection of the
553 lymphoid tissues of sheep affected by pulmonary adenomatosis. J. Gen.
554 Virol. 77, 2991-2998.
- 555 Robinson, A.J., Lyttle, D.J., 1992. Parapoxviruses: their biology and
556 potential as recombinant vaccines. In: Binns, M., Smith, G.L. (Eds.),
557 Recombinant poxviruses, CRC Press, Boca Raton, pp. 285-327.
- 558 Robinson, C.J., Stringer, S.E., 2001. The splice variants of vascular

- endothelial growth factor (VEGF) and their receptors. *J. Cell Sci.* 114, 853-865.
- Rziha, H.J., Bauer, B., Adam, K.H., Rottgen, M., Cottone, R., Henkel, M., Dehio, C., Buttner, M., 2003. Relatedness and heterogeneity at the near-terminal end of the genome of a parapoxvirus bovis 1 strain (B177) compared with parapoxvirus ovis (Orf virus). *J. Gen. Virol.* 84, 1111-1116.
- Rziha, H.J., Henkel, M., Cottone, R., Bauer, B., Auge, U., Götz, F., Pfaff, E., Röttgen, M., Dehio, C., Büttner, M., 2000. Generation of recombinant parapoxviruses: non-essential genes suitable for insertion and expression of foreign genes. *J. Biotechnol.* 83, 137-145.
- Rziha, H.J., Henkel, M., Cottone, R., Meyer, M., Dehio, C., Buttner, M., 1999. Parapoxviruses: potential alternative vectors for directing the immune response in permissive and non-permissive hosts. *J. Biotechnol.* 73, 235-242.
- Savory, L.J., Stacker, S.A., Fleming, S.B., Niven, B.E., Mercer, A.A., 2000. Viral vascular endothelial growth factor plays a critical role in orf virus infection. *J. Virol.* 74, 10699-10706.
- Scagliarini, A., Dal Pozzo, F., Gallina, L., Guercio, A., Vaccari, F., Battilani,

- 578 M., Ciulli, S., Prosperi, S., 2006. In vitro activity of VEGF-E produced by
579 orf virus strains isolated from classical and severe persistent contagious
580 ecthyma. Vet. Microbiol. 114, 142-147.
- 581 Stacker, S.A., Achen, M.G., 1999. The vascular endothelial growth factor
582 family: signalling for vascular development. Growth Factors 17, 1-11.
- 583 Suzuki, T., Minamoto, N., Sugiyama, M., Kinjo, T., Suzuki, Y., Sugimura, M.,
584 Atoji, Y., 1993. Isolation and antibody prevalence of a parapoxvirus in
585 wild Japanese serows (*Capricornis crispus*). J. Wildl. Dis. 29, 384-389.
- 586 Suzuki, Y., Komatsu, T., Yamamoto, Y., Atoji, Y., 1997. Pathology of
587 interdigital glands in a wild Japanese serow (*Capricornis crispus*)
588 infected with parapoxvirus. J. Vet. Med. Sci. 59, 1063-1065.
- 589 Suzuki, Y., Sugimura, M., Atoji, Y., Minamoto, N., Kinjo, T., 1986.
590 Widespread of parapox infection in wild Japanese serows, *Capricornis*
591 *crispus*. Jpn. J. Vet. Sci. 48, 1279-1282.
- 592 Tokunaga, Y., Yamazaki, Y., Morita, T., 2006. Localization of heparin- and
593 neuropilin-1-recognition sites of viral VEGFs. Biochem. Biophys. Res.
594 Commun. 348, 957-962.
- 595 Ueda, N., Inder, M.K., Wise, L.M, Fleming, S.B., Mercer, A.A., 2007.
596 Parapoxvirus of red deer in New Zealand encodes a variant of viral

- 597 vascular endothelial growth factor. *Virus Res.* 124, 50-58.
- 598 Ueda, N., Wise, L.M., Stacker, S.A., Fleming, S.B., Mercer, A.A., 2003.
- 599 Pseudocowpox virus encodes a homolog of vascular endothelial growth
- 600 factor. *Virology* 305, 298-309.
- 601 Wise, L.M., Savory, L.J., Dryden, N.H., Whelan, E.M., Fleming, S.B., Mercer,
- 602 A.A., 2007. Major amino acid sequence variants of viral vascular
- 603 endothelial growth factor are functionally equivalent during Orf virus
- 604 infection of sheep skin. *Virus Res.* 128, 115-125.
- 605 Wise, L.M., Ueda, N., Dryden, N.H., Fleming, S.B., Caesar, C., Roufail, S.,
- 606 Achen, M.G., Stacker, S.A., Mercer, A.A., 2003. Viral vascular
- 607 endothelial growth factors vary extensively in amino acid sequence,
- 608 receptor-binding specificities, and the ability to induce vascular
- 609 permeability yet are uniformly active mitogens. *J. Biol. Chem.* 278,
- 610 38004-38014.
- 611 Wise, L.M., Veikkola, T., Mercer, A.A., Savory, L.J., Fleming, S.B., Caesar, C.,
- 612 Vitali, A., Makinen, T., Alitalo, K., Stacker, S.A., 1999. Vascular
- 613 endothelial growth factor (VEGF)-like protein from orf virus NZ2 binds
- 614 to VEGFR2 and neuropilin-1. *Proc. Natl. Acad. Sci. USA* 96, 3071-3076.
- 615 Yamamoto, M., Horiuchi, M., Ishiguro, N., Shinagawa, M., Matsuo, T.,

616 Kaneko, K., 2001. Glycidol degrades scrapie mouse prion protein. J. Vet.
 617 Med. Sci. 63, 983-990.

618

619 **Figure legends**

620 **Fig. 1.** Representative lesions of naturally infected PPV animals. (a) Papules
 621 and nodules in the gingiva and lip of an affected cow. (b) Scabby papules and
 622 nodules around the mouth of an affected goat. (c) Highly proliferative scabby
 623 papules, nodules, pustules and ulcers on the lips, muzzle and eye regions of
 624 an affected Japanese serow.

625 **Fig. 2.** Alignment of the deduced amino acid sequences of the viral VEGFs.
 626 The sequences of isolates Iwate and HIS from sheep in Japan and isolate S-1
 627 from a Japanese serow were aligned with the published sequences as follows:
 628 NZ2 (DDBJ/EMBL/GenBank accession nos. BD320268 and S67520), D1701
 629 (AF106020) and orf-11 (AY236150). The sequences of the 4 PPV isolates from
 630 Japanese serows were identical and denoted as S-1. The eight cysteine
 631 residues that are conserved in all members of the VEGF family are indicated
 632 above the Iwate sequence as C. The asterisk indicates the accessible
 633 asparagine residue on the receptor-binding face of VEGF-A, which correlates
 634 strongly with the binding of VEGF receptor 2 and is conserved in the

635 sequences of PPV VEGFs (Mercer et al., 2002, Ueda et al., 2003, 2007, Inder
 636 et al., 2007). The arginine residues that correlate with the binding of
 637 co-receptors, neuropilin-1 and heparin, in NZ2 VEGF (Tokunaga et al., 2006),
 638 are indicated as R. The bars above the Iwate sequence indicate the loop 1
 639 and loop 3 regions of the conformational structure of VEGF; these loops are
 640 essential for the binding and activation of VEGF receptor 2 (Kiba et al.,
 641 2003).

642 **Fig. 3.** Comparison of the temporal pattern of mRNA expression *in vitro*.
 643 FBM (fetal bovine muscle), FLL (fetal lamb lung) and SeSM (serow smooth
 644 muscle) cells were infected with PPV isolates Iwate, HIS, or S-1. RNA
 645 samples were collected 0, 1, 2, 4, 8, 12, 24 and 36 h after virus-inoculation. As
 646 a control for the reaction, mRNA of the housekeeping gene cellular
 647 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also amplified
 648 using primers as described previously (Mohan et al., 2001; Palmarini et al.,
 649 1996). Distilled water (DW) was used as the negative control.

650 **Fig. 4.** Comparison of the biological activities of the viral VEGFs. (a)
 651 Vascular permeability activity of the viral VEGFs by the Miles assay. VEGF
 652 was serially diluted in PBS and intradermally injected into the shaved backs
 653 of guinea pigs. Dose-dependent vascular permeabilization was observed for

all 3 viral VEGFs. No significant difference was observed among the 3 VEGFs at each dilution, as determined by parametric non-repeated measures ANOVA and the SNK test. Data represents the mean \pm SD of 3 guinea pigs. (b) HUVEC-proliferative activity of viral VEGFs. HUVECs were treated with the viral VEGFs. The proliferation index was determined as the ratio of the absorbance at 450 nm of cells treated with VEGF against the absorbance at 450 nm of untreated cells. Data represents the mean \pm SD of 6 evaluations. The HIS and S-1 VEGFs showed significantly higher activity on HUVEC proliferation than the Iwate VEGF at a concentration of 50 ng/ml ($*P < 0.05$, $**P < 0.01$). No significant difference was observed between the HIS and S-1 VEGFs by parametric non-repeated measures ANOVA and the SNK test. No statistically significant differences were observed at a VEGF concentration of 10 ng/ml.

Fig. 5. Comparison of the responsiveness of endothelial cells to viral VEGF among different host species. (a) Isolation of primary endothelial cells. Endothelial cells from animals were stained positively for vWF by IFA. Over 80% of cells were positive when counted by flow cytometry; these are shown as a dotted line in the flow cytometry panels. BAEC, bovine aortic endothelial cells; OAEC, ovine aortic endothelial cells; CAEC, caprine aortic

endothelial cells; SeAEC, Japanese serow aortic endothelial cells; SeMC, Japanese serow skeletal muscle cells. SeMC were used as control non-endothelial cells. (b) Comparison of the responsiveness to viral VEGF of primary endothelial cells from different host species. Primary endothelial cells were treated with 50 ng/ml of S-1 VEGF for 2 days. The proliferation index was determined as the ratio of the absorbance at 450 nm of cells treated with VEGF against the absorbance at 450 nm of untreated cells. Although no significant difference between OAEC and SeAEC among the 4 cells was observed by parametric non-repeated measures ANOVA and the SNK test, a significant difference ($*P < 0.05$) was observed between OAEC and SeAEC analyzed by parametric unpaired Student's *t*-test. Data represents the mean \pm SD of 6 evaluations. BAEC, bovine aortic endothelial cells; OAEC, ovine aortic endothelial cells; CAEC, caprine aortic endothelial cells; SeAEC, Japanese serow aortic endothelial cells; SeMC, Japanese serow skeletal muscle cells. $**P < 0.01$.

Table 1. Parapoxvirus isolates used in this study

Isolate	Original host	Prefecture	Year	of	isolation
---------	---------------	------------	------	----	-----------

692 References

693	Iwate	sheep	Iwate	1970
694		Kumagai et al., 1971		
695	HIS	sheep	Hokkaido	2004
696		Kanou et al., 2005		
697	S-1	Japanese serow	Gifu	1985
698		Suzuki et al., 1993		
699	GHF	Japanese serow	Gifu	1999
700		Inoshima et al., 2002		
701	R-1	Japanese serow	Toyama	1999
702		Inoshima et al., 2002		
703	Aichi	Japanese serow	Aichi	2000

704 Inoshima et al., 2002

705

706

707

708

709 Supplementary data

710

711 Table S1. Primers used in this study

712	Primers	Sequence (5' to 3')*
713	PPP-1	GTCGTCCACGATGAGCAGCT
714	PPP-4	TACGTGGGAAGCGCCTCGCT
715	D27F	AATGTAAATWMTAACGCC
716	11R	AACCCAGAAACGTCCCGCTAC
717	D2R	CGTTTGGATMTGCGGTCC
718	VEGF orf-11F	ATGAGGTTGCTCGTCTGC
719	VEGF cons340R	CGCACTTTGTATGCTCCACGA
720	VEGF HISF	ATGAGGTTGCTCGTCGGCATACTG
721	VEGF HISR	CTAGCGGCGTCTTCTGGGCGGCCT
722	BamConsSigF	CTGCTGGGATCCGACAGCAGCACAAAAAAA
723	EcoConsR	CAAAAAGAATTCCTAATAAGCCCTTCGGCG
724	PstConsR	CAAAAACCTGCAGCTAATAAGCCCTTCGGCG

725 * W, A/T. M, A/C. Underlines show restriction enzyme site.

726

727

728

729 Supplementary data

730

731 Table S2. Identity of VEGF amino acid sequence (%)*

732	Iwate	HIS	S-1	NZ2(BD)
733	NZ2(S)	D1701	orf-11	
734	Iwate	—	70.8	99.3
735	70.1	69.3	99.3	
736	HIS	—	70.8	84.2
737	83.5	89.4	71.3	
738	S-1		—	70.8
739	70.1	69.3	98.5	
740	NZ2(BD)			—
741	99.2	83.5	71.3	
742	NZ2(S)			
743	—	82.7	70.6	
744	D1701			
745	—	69.9		
746	orf-11			
747		—		

748 * NZ2(BD), data from accession no. BD320268. NZ2(S), data from accession
 749 no. S67520.

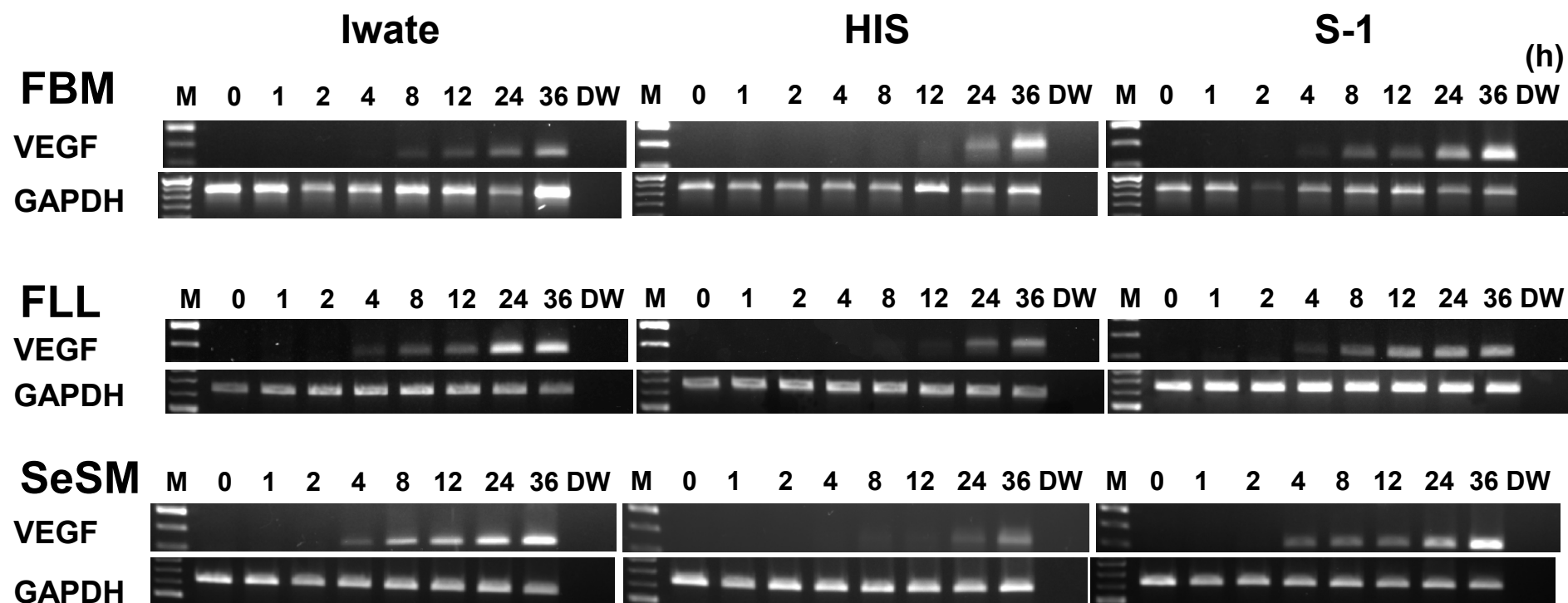
750



Fig. 1. Inoshima et al.

		C	Loop 1	C	C		
Iwate	1:	MRL	LLVCILVVVCLLHQHLLNAD	SSTKKWPEVLE	GSKCKPRPTVLSVNGEHPELTSQRFNPPCVTL	MRCGG 70	
HIS	1:G...	A...-...Y.....-..	T.S..F.S.....	VP.GEA.....Q...V.....	68	
S-1	1:				70	
NZ2 (BD320268)	1:	.K...	G...A...-...Y.....	N..G.S...K..E.....	I.VP.SET.....	69	
NZ2 (S67520)	1:	.K...	G...A...-...Y.....	N..G.S...K..E.....	I.VP.SET.....	69	
D1701	1:	.KF..	G...A...-...Y.....	-..T.S..F.N.G.....	M.FR.HD.....	68	
orf-11	1:	-.....			69	
		CC*	C	Loop 3	C CR	RR	
Iwate	71:	CCN	DESLE	CVPT	EEANVTMEF	MGVGVSSSTGSSVSTQHLEFVEHTKCD	CQPR--GGQQTTPPPRRRRRAY 137
HIS	69:QL..---	A.VSGGNGM...	I....K....K..LTTTPP..TR.P....--	132	
S-1	71:--.....T.....	137	
NZ2 (BD320268)	70:V.....LL.---	A.GSG.NGM.R.S....K....R..FTTTPP..TR.P....--	133		
NZ2 (S67520)	70:V..S..LL.---	A.GSG.NGM.R.S....K....R..FTTTPP..TR.P....--	133		
D1701	69:QL..---	A.VSGGNGM...	S....K....K.PLTTTPP..TR.P....--	132	
orf-11	70:--.....	136	

Fig. 2. Inoshima et al.



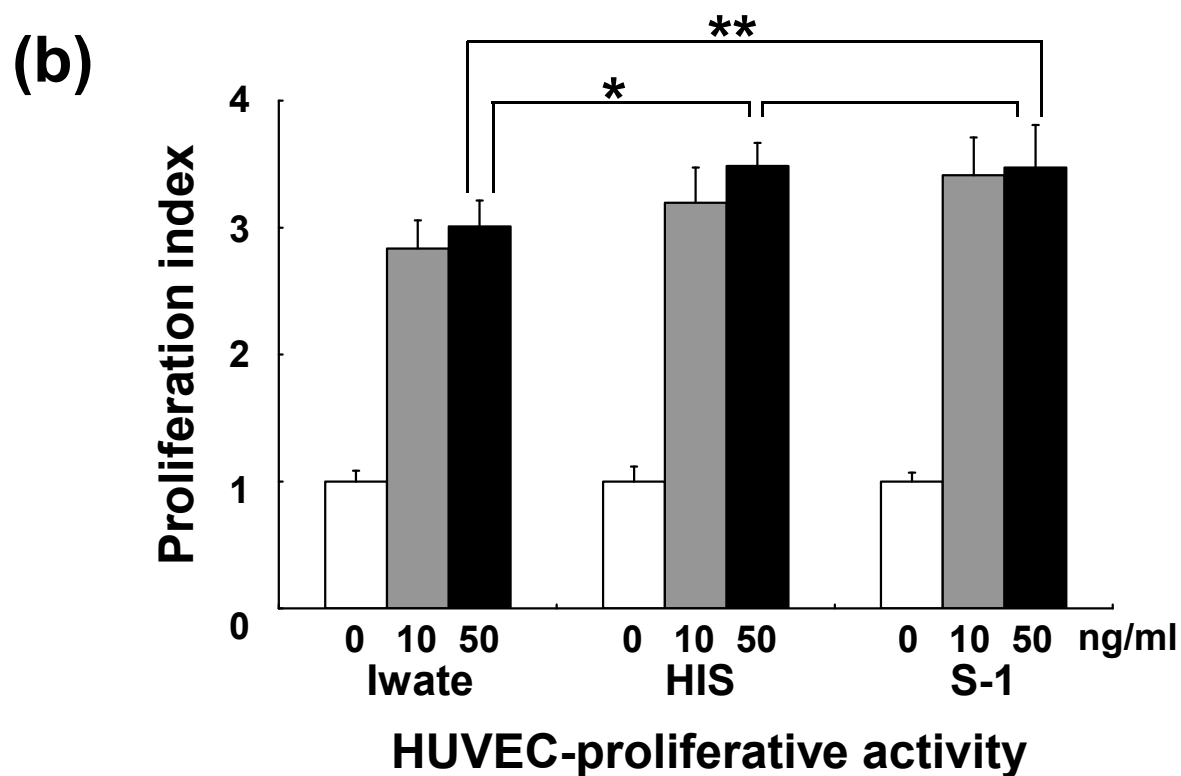
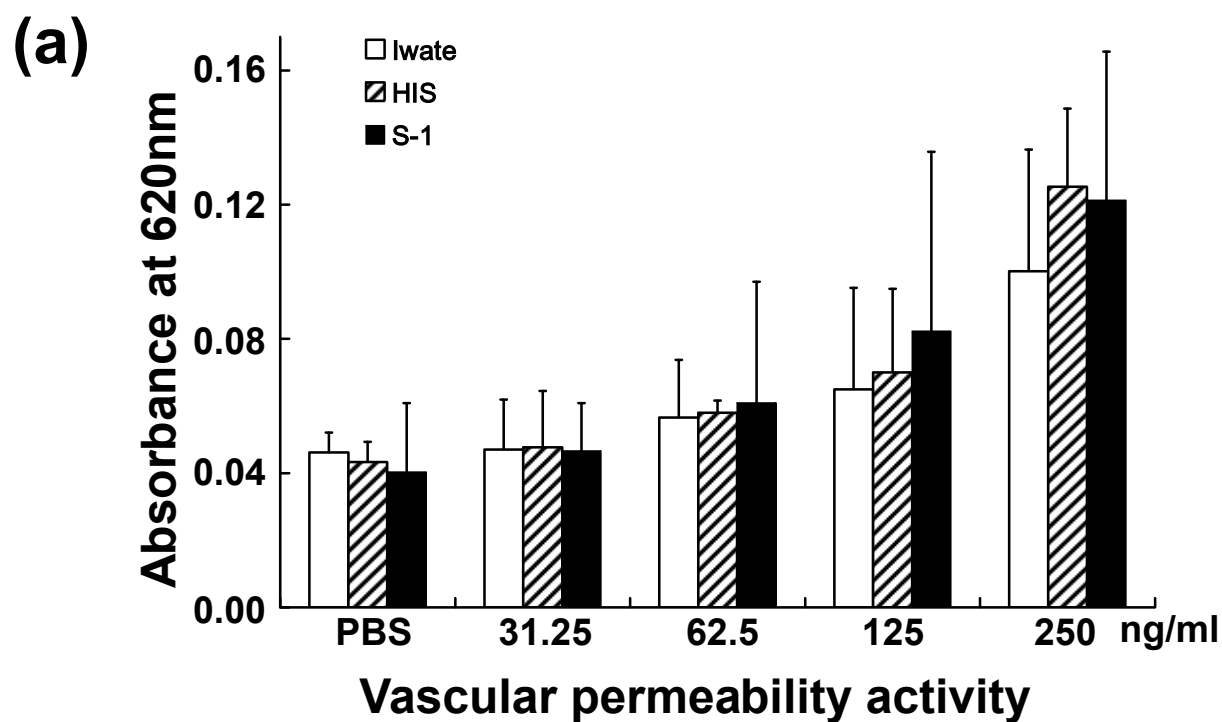


Fig. 4. Inoshima et al.

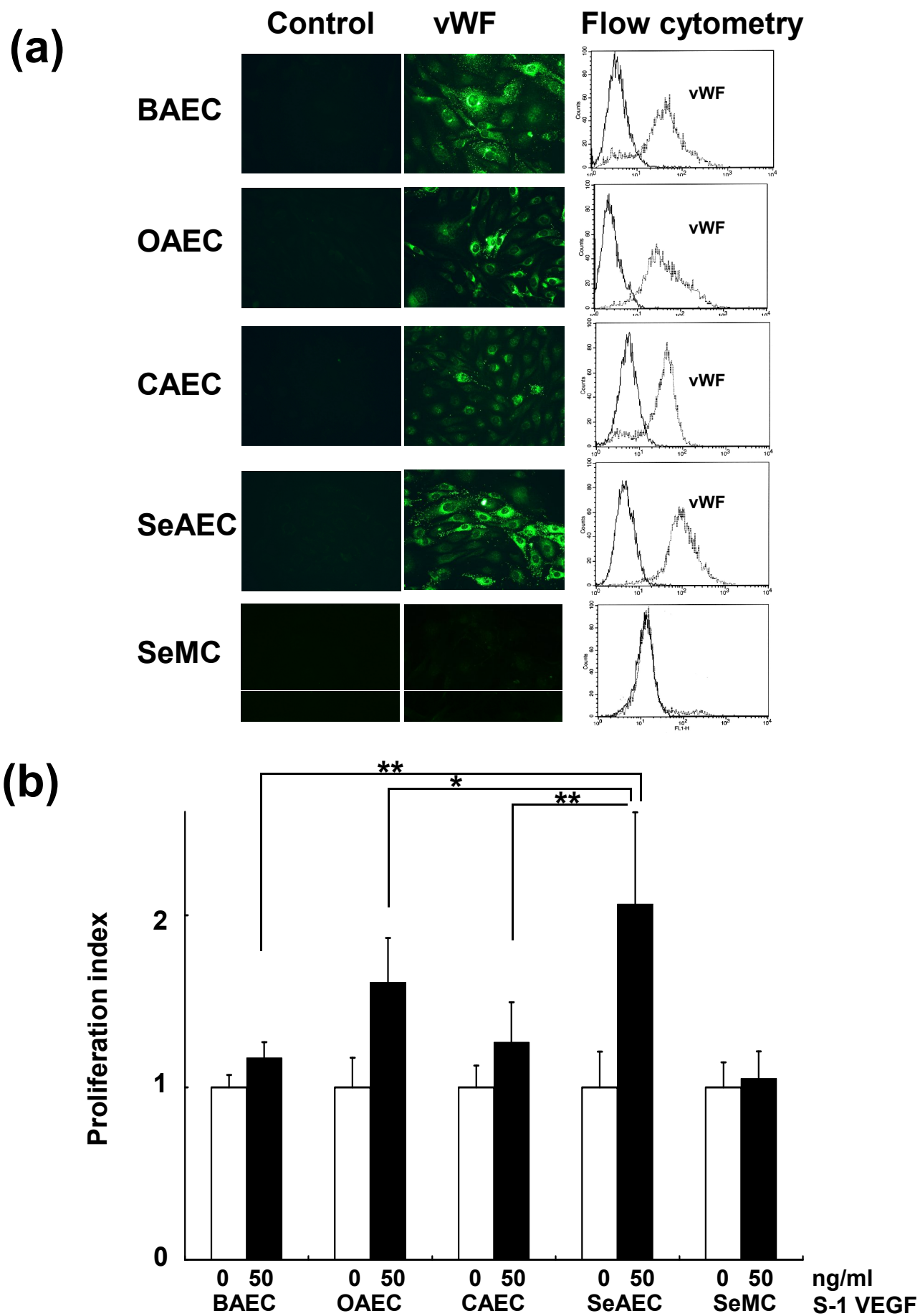


Fig. 5. Inoshima et al.