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A novel vascular endothelial growth factor encoded by Orf virus, VEGF-E, mediates angiogenesis via signalling through VEGFR-2 (KDR) but not VEGFR-1 (Flt-1) receptor tyrosine kinases

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The different members of the vascular endothelial growth factor (VEGF) family act as key regulators of endothelial cell function controlling vasculogenesis, angiogenesis, vascular permeability and endothelial cell survival. In this study, we have functionally characterized a novel member of the VEGF family, designated VEGF-E. VEGF-E sequences are encoded by the parapoxvirus Orf virus (OV). They carry the characteristic cysteine knot motif present in all mammalian VEGFs, while forming a microheterogenic group distinct from previously described members of this family. VEGF-E was expressed as the native protein in mammalian cells or as a recombinant protein in *Escherichia coli* and was shown to act as a heat-stable, secreted dimer. VEGF-E and VEGF-A were found to possess similar bioactivities, i.e. both factors stimulate the release of tissue factor (TF), the proliferation, chemotaxis and sprouting of cultured vascular endothelial cells *in vitro* and angiogenesis *in vivo*. Like VEGF-A, VEGF-E was found to bind with high affinity to VEGF receptor-2 (KDR) resulting in receptor autophosphorylation and a biphasic rise in free intracellular Ca^{2+} concentration, whilst in contrast to VEGF-A, VEGF-E did not bind to VEGF receptor-1 (Flt-1). VEGF-E is thus a potent angiogenic factor selectively binding to VEGF receptor-2. These data strongly indicate that activation of VEGF receptor-2 alone can efficiently stimulate angiogenesis.
Keywords: angiogenesis/Flt-1/KDR/Orf virus/VEGF

Introduction

The development of blood vessels from *in situ* differentiating endothelial cells (angioblasts) in the early embryo is termed vasculogenesis (Risau and Lemmon, 1988), while the formation of the rest of the vascular tree is thought to occur as a result of vascular sprouting from pre-existing vessels by a process called angiogenesis (Folkman, 1995; Risau, 1997). In the newly formed vessels, vascular endothelial cells undergo tissue-specific changes to generate several types of functionally and morphologically distinct vessels (Risau and Flamme, 1995). Angiogenesis in the adult occurs under normal circumstances almost exclusively in the female reproductive system (Folkman, 1995). However, angiogenesis can be activated in response to tissue damage and is important in certain pathological conditions such as tumour growth and metastasis, rheumatoid arthritis, diabetic rentinopathy, psoriasis and cardiovascular diseases (Folkman, 1995).

Vascular endothelial growth factor (VEGF or VEGF-A) was shown to play a pivotal role in the regulation of normal and pathological angiogenesis (for review see Ferrara and Davis-Smyth, 1997). *In vitro*, VEGF-A is a potent mitogen for micro- and macrovascular endothelial cells, but is devoid of consistent and appreciable mitogenic activity for other cell types. *In vivo*, VEGF-A is involved in the development of blood vessels as the formation of vessels in mouse embryos heterozygous for a disrupted *vegf* gene is aberrant and results in embryonic lethality (Carmeliet *et al*., 1996; Ferrara *et al*., 1996). VEGF-A is also a potent inducer of vascular permeability (Senger *et al*., 1983).

VEGF-A is a dimeric glycoprotein which, as a result of alternative splicing of a single gene, primarily exists as homodimers of one of five different molecular species, comprising in humans 121, 145, 165, 189 and 206 amino acids (VEGF-A_{121}, VEGF-A_{145}, VEGF-A_{165}, VEGF-A_{189}, VEGF-A_{206}), respectively (Ferrara and Davis-Smyth, 1997; Poltorak *et al*., 1997). The various isoforms of VEGF-A differ in their affinity for heparin and extracellular matrix components, which primarily affects their bioavailability. The angiogenic activity of VEGF-A is thought to be mediated by the high-affinity receptor tyrosine kinases VEGFR-1 (*fms*-like tyrosine kinase, Flt-1) and VEGFR-2 (kinase insert-domain containing receptor, KDR, in humans and its murine homologue, fetal liver kinase-1, Flk-1). These are co-expressed on most endothelial cells (for review see Klagsbrun and D'Amore, 1996; Ferrara and Davis-Smyth, 1997; Poltorak *et al*., 1997), although VEGFR-2 is thought to dominate the angiogenic response (Waltenberger *et al*., 1994). Recently, VEGF-A_{165} was found to bind to an additional, unrelated receptor, neuropilin-1, which appears to modulate binding of VEGF-A_{165} to VEGFR-2 and thereby may modulate VEGF-A-induced angiogenesis (Soker *et al*., 1998).

Numerous proteins closely related in primary structure to VEGF-A have been reported and were grouped in the VEGF family. Placenta growth factor (PIGF) binds to

VEGFR-1 but not VEGFR-2 (Park *et al*., 1994). Although not mitogenic itself, it can potentiate the action of limiting concentrations of VEGF-A *in vitro* and *in vivo* (Maglione *et al*., 1991). VEGF-B also binds selectively to VEGFR-1 and may be involved in regulation of extracellular matrix degradation, cell adhesion and migration (Olofsson *et al*., 1996, 1998). VEGF-C and VEGF-D possess N- and C-terminal extensions that are not present in other VEGF family members. Their biosynthesis, however, involves proteolytic processing that gives rise to mature secreted proteins that consist essentially of the central VEGF homology domain (Joukov *et al*., 1996, 1997; Lee *et al*., 1996; Achen *et al*., 1998). VEGF-C and VEGF-D do not bind VEGFR-1, while both are ligands for VEGFR-2 and VEGFR-3 (Flt-4). VEGFR-3 is a receptor tyrosine kinase expressed on endothelial precursor cells and later in development of lymphatic endothelial cells (Kukk *et al*., 1996; Achen *et al*., 1998). Both VEGF-C and VEGF-D are thought to affect primarily the development of the lymphatic vasculature via activation of VEGFR-3 (Joukov *et al*., 1996; Kukk *et al*., 1996; Achen *et al*., 1998).

Based on sequence similarity to VEGF-A_{121}, a gene encoding a VEGF homologue has recently been discovered in the genome of Orf virus (OV) (Lyttle *et al*., 1994; Cottone *et al*., 1998; Rziha *et al*., 1998). OV is an epitheliotropic parapoxvirus with a world-wide distribution that induces proliferative lesions in the skin of sheep, goat and man (for review see Haig and Mercer, 1998). These lesions are histopathologically characterized by massive capillary proliferation and dilation (Groves *et al*., 1991). Although the viral VEGF homologue may be involved in mediating vascular remodelling in OV lesions, a putative vascular growth factor activity has not been reported for this protein.

The objective of the present study was to investigate the functional activity of the viral VEGF homologue, designated VEGF-E. To this end, the VEGF-E of OV strain D1701 was expressed as a native, secreted protein in mammalian cells and as a recombinant protein in *Escherichia coli* and was purified to homogeneity. VEGF-E was found to be a potent angiogenesis factor with a bioactivity similar to VEGF-A, i.e. it induced tissue-factor (TF) expression, the proliferation, migration and sprouting of cultured vascular endothelial cells and angiogenesis *in vivo*. Strikingly, VEGF-E was found to bind to and activate VEGFR-2, while it did not bind to VEGFR-1. The potent angiogenic activity of VEGF-E and its restricted receptor specificity thus allowed us to demonstrate dispensability of VEGFR-1 activation for VEGFR-2 mediated angiogenesis.

Results

Culture supernatants of epithelial cells infected with OV stimulate vascular endothelial cell proliferation in vitro

Although the gene encoding a VEGF homologue in the genome of OV is known to be expressed early in infection (Lyttle *et al*., 1994; Rziha *et al*., 1998), the function of the encoded putative growth factor has not been investigated. We have used the OV isolate D1701 to infect cells of the bovine epithelial cell line BKKL3A for 24 h and tested the virus-free filtered cell culture supernatant for mitogenic activity on cultured vascular endothelial cells. Supernatants from infected cells stimulate the proliferation of human umbilical vein endothelial cells (HUVECs), while supernatants from mock-infected cells or medium alone does not (Figure 1A). These data suggest that OV-infected cells secrete a vascular endothelial growth factor, which may correspond to the encoded VEGF homologue as previously suspected (Lyttle *et al*., 1994).

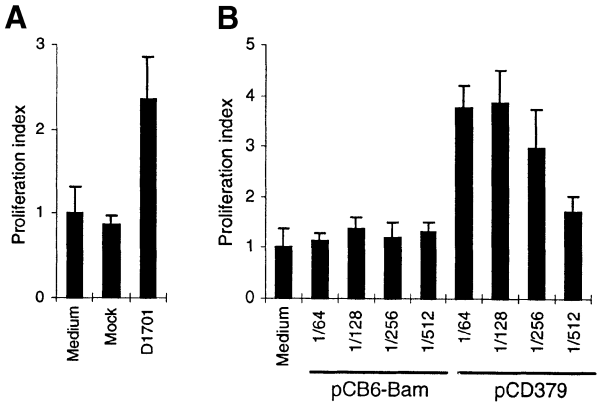

Fig. 1. Mitogenic activity of HUVECs after addition of culture supernatants from epithelial cells that were either infected with orf virus or transfected with an ovVEGF-E_{D1701} expression plasmid. (**A**) BKKL3A cells were infected with a MOI = 1 of OV D1701 or mock infected and medium was collected 24 h post-infection. (**B**) COS-7 cells were transfected with the VEGF-E expression plasmid pCD379 or the empty expression vector pCB6-Bam serving as control, and culture medium conditioned between 4–52 h post-transfection was collected. Conditioned media were cleared by centrifugation and filtration, and dilutions were used in a 72 h proliferation assays with HUVECs. The columns and bars represent mean ± SD of samples performed in triplicate. The data illustrated are representative for one out of at least three independently performed experiments. For experimental details see Materials and methods.

VEGF-E is a novel member of the VEGF family

Oligonucleotides designed according to the nucleotide sequence of OV strain D1701 (H.-J.Rziha, unpublished) were used as PCR primers to amplify the complete gene encoding the VEGF homologue, which we designate *vegf-e*, from genomic DNA of this strain, and the generated PCR product was cloned and sequenced. The deduced protein was designated ovVEGF-E_{D1701}, representing the founding member of the VEGF-E subfamily, and the deduced protein sequences of the homologous genes from the OV strains NZ2 and NZ7 (Lyttle *et al*., 1994) were designated here ovVEGF-E_{NZ2} and ovVEGF-E_{NZ7}, respectively. The amino acid sequence of ovVEGF-E_{D1701} was compared with that of ovVEGF-E_{NZ2} and ovVEGF-E_{NZ7} as well as with the human sequences of various members of the mammalian VEGF family of growth factors (Figure 2A). All eight cysteine residues of the central cysteine knot motif characteristic of members of the VEGF family (McDonald and Hendrickson, 1993) are conserved among other residues in the VEGF-E proteins. An average distance tree of the fully aligned cysteine knot motif of representative mammalian VEGF sequences indicated that VEGF-E is distinct from the previously described VEGFs (Figure 2B). Remarkably, VEGF-A sequences, which are most closely related to VEGF-E by BLAST similarity searches, form a uniform cluster which is separated from VEGF-E, even if sequences of species phylogenetically

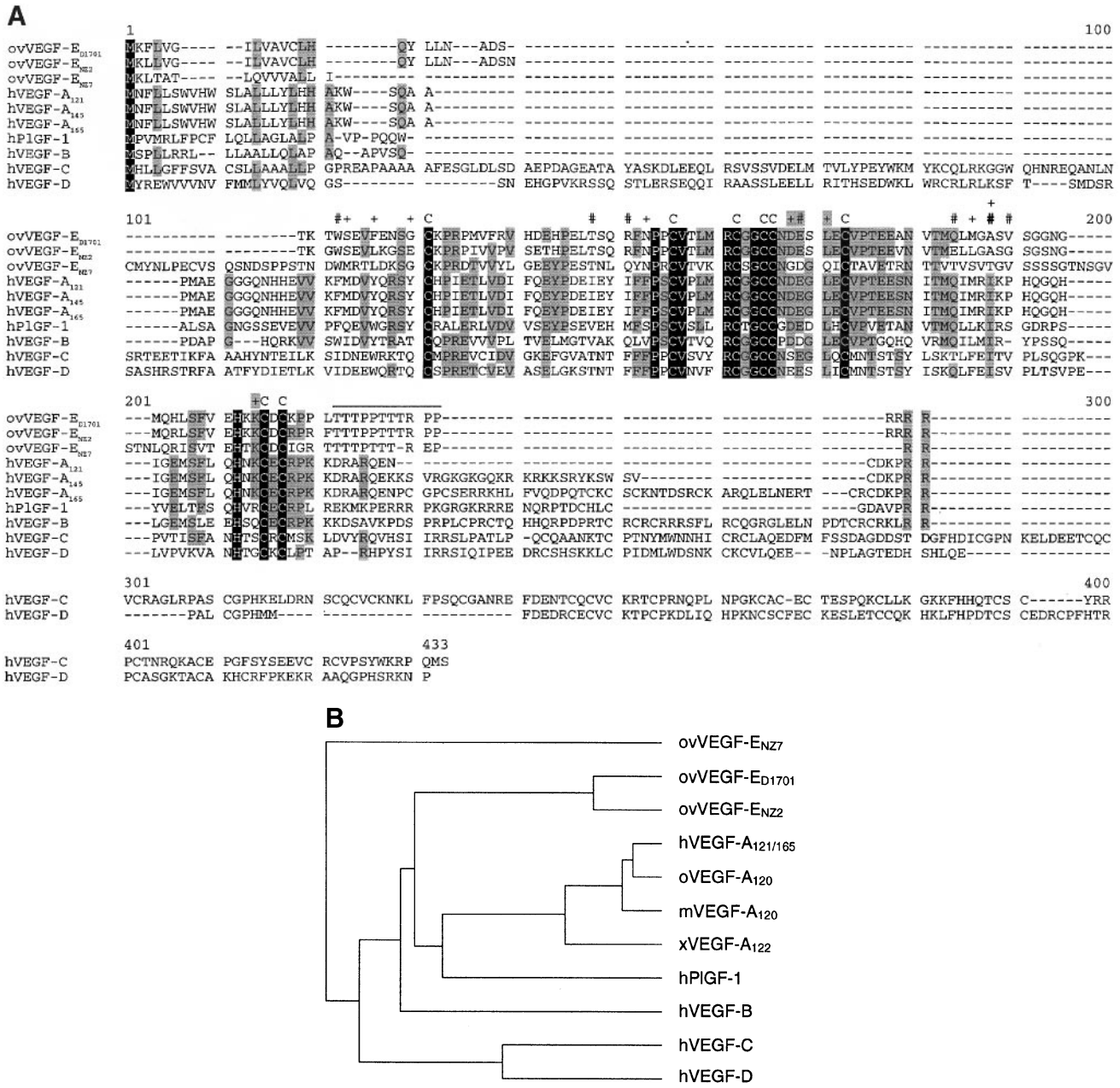

Fig. 2. Comparison of VEGF-E sequences with other members of the VEGF family. (**A**) Alignment of the deduced amino acid sequences of OV-encoded ovVEGF-E_{D1701} (DDBJ/EMBL/GenBank accession No. AF106020), ovVEGF-E_{NZ2} (DDBJ/EMBL/GenBank accession No. S67520) and ovVEGF-E_{NZ7} (DDBJ/EMBL/GenBank accession No. S67522) with various human VEGFs, i.e. hVEGF-A_{121} (PIR accession No. A41551), hVEGF-A_{145} (Poltorak *et al*., 1997), hVEGF-A_{165} (SWISS-PROT accession No. P15692), hVEGF-B_{167} (SWISS-PROT accession No. P49765), hVEGF-C (SWISS-PROT accession No. P49767), hVEGF-D (DDBJ/EMBL/GenBank accession No. D89630) and hPlGF-1 (SWISS-PROT accession No. P49763). The multiple sequence alignment was generated with the program Multalin (Corpet, 1988) and further optimized manually. Residues that are conserved in at least 50% of the aligned sequences are shaded in grey, those fully conserved are reversed out. The eight cysteine residues that are conserved in all members of the VEGF family are denoted above the compared VEGF sequences. Amino acids of the VEGFR-1 binding domain of VEGF-A, which are at least 50% buried in the receptor–ligand interface (Wiesmann *et al*., 1997), are denoted by '+' symbols above the ovVEGF-E_{D1701} sequence. '#' symbols denote amino acids of the putative VEGFR-2 binding domain of VEGF-A, which result in a >5-fold decrease in binding upon alanine substitution (Muller *et al*., 1997). The line above the compared VEGF sequences marks a unique motif-rich in threonine and proline which is found exclusively in VEGF-E sequences. (**B**) Average distance tree of the fully aligned cysteine knot domain of various VEGFs beginning with the first and ending with the last of the eight conserved cysteine residues. In addition to the human VEGFs and the VEGF-Es aligned in (A), this tree includes ovine VEGF-A_{120} (oVEGF-A_{120}; SWISS-PROT accession No. P50412), bovine VEGF-A_{120} (bVEGF-A_{120}, PIR accession No. A33787), mouse VEGF-A_{120} (mouse VEGF-A_{120}, DDBJ/EMBL/GenBank accession No. S38100) and *Xenopus* (frog) VEGF-A_{122} (xVEGF-A_{122}, DDBJ/EMBL/GenBank accession no. AF009538). The average distance tree using percentage identities (PID) was generated by the program Jalview (http://circinus.ebi.ac.uk:6543/~michele/java3/dev/contents.html) based on a multiple sequence alignment generated with the program ClustalW (Thompson *et al*., 1994).

distant from the natural mammalian hosts of OV are included (i.e. frog). Interestingly, ovVEGF-E_{D1701} and ovVEGF-E_{NZ2} sequences are highly related, clustering next to the branching points of VEGF-A/PIGF and VEGF-B, while ovVEGF-E_{NZ7} was found to be most distantly related among all compared VEGFs (Figure 2B).

All three VEGF-E sequences, however, have a conserved threonine- and proline-rich motif at the C-terminus that is not present in any mammalian VEGF (Figure 2A, marked by a line). Thus, according to sequence alignment the VEGF-E constitutes a novel subfamily of the VEGF family of growth factors, with ovVEGF-E_{NZ7} representing an exceptionally divergent sequence.

Culture supernatants of COS-7 cells transfected with a VEGF-E expression plasmid are mitogenic for vascular endothelial cells in vitro

In order to test if VEGF-E is a secreted vascular endothelial growth factor, the *vegf-e* gene was fused to the cytomegalovirus promoter and hGII polyadenylation signal in the mammalian expression vector pCB6-Bam, and the resulting expression plasmid pCD379 was transfected into COS-7 cells. Culture supernatants conditioned 4–52 h post-transfection showed a concentration-dependent stimulation of HUVEC proliferation, while corresponding supernatants of pCB6-Bam transfected cells or medium alone had no mitogenic effect in this assay (Figure 1B). These data indicate that VEGF-E is a secreted vascular endothelial growth factor and suggest that its activity accounts for the mitogenic activity for endothelial cells present in the supernatants of OV-infected cell cultures (compare with Figure 1A).

Expression of VEGF-E in E.coli and purification of the recombinant protein

To study further the biological and biochemical characteristics of VEGF-E, we have expressed ovVEGF-E_{D1701} as a fusion protein in *E.coli* and purified the recombinant protein, rovVEGF-E_{D1701}, to homogeneity. To this end, the expression plasmid pCD369 was constructed in the T7-polymerase-based expression vector pET15b. The encoded fusion protein is composed at its N-terminus of 21 vector-encoded amino acids including a hexa-histidine-tag to facilitate purification and a thrombin-cleavage site to allow removal of this tag, fused to amino acids 21–133 of the ovVEGF-E_{D1701} polypeptide. Following isopropyl-β-D-galactopyranoside (IPTG) induction, SDS–PAGE of bacterial lysates showed strong expression of rovVEGF-E_{D1701} that migrated as a monomeric protein both under reducing as well as non-reducing conditions (Figure 3A and 3B, respectively, lane 2). rovVEGF-E_{D1701} was found primarily as soluble protein in the cleared supernatant of ruptured cells and could be purified by affinity chromatography on a Ni-chelate column. Eluted protein was allowed to form disulfide bonds and dimers were subsequently purified by gel chromatography. The final protein preparation was essentially free of protein contamination as estimated by SDS–PAGE followed by Coomassie Blue staining (Figure 3A, lane 3) or silver staining (data not shown) and migrated predominately as a dimer under non-reducing conditions (Figure 3B, lane 3). The endotoxin content in the limulus amebocyten lysate assay was lower than 0.15 EU/μg protein.

When the hexa-histidine-tag was proteolytically removed, the resulting processed recombinant protein (provVEGF-E_{D1701}) was of a similar homogeneity and biological activity as the unprocessed recombinant protein rovVEGF-E_{D1701} (data not shown).

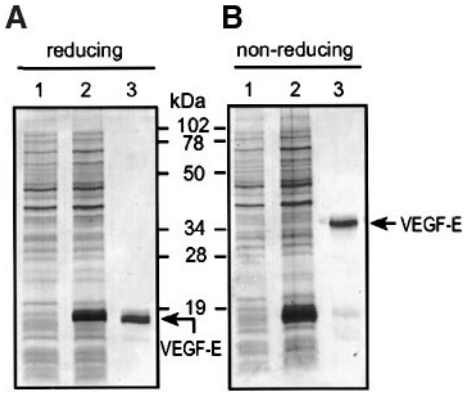

Fig. 3. Expression and purification of VEGF-E as an *E.coli* fusion protein. rovVEGF-E_{D1701} was expressed in *E.coli* and purified as described in Materials and methods. SDS–PAGE (15%) was performed under (**A**) reducing conditions and (**B**) non-reducing conditions and stained by Coomassie Blue. Lane 1, bacteria without IPTG-induction; lane 2, IPTG-induced bacteria; lane 3, purified rovVEGF-E_{D1701}.

VEGF-E is a vascular endothelial-specific growth factor stimulating the release of tissue factor

The mitogenic activity of rovVEGF-E_{D1701} for HUVECs was found to be heat stable as previously reported for VEGF-A (Ferrara *et al*., 1992) and sensitive to digestion by proteinase K (Figure 4A), and the dose reponse was similar to rhVEGF-A_{165} (Figure 4B; Myoken *et al*., 1991). Like rhVEGF-A_{165}, rovVEGF-E_{D1701} is a potent mitogen for human macrovascular endothelial cells (HUVECs) and human dermal microvascular endothelial cells (HDMVECs) but has no appreciable mitogenic effect on normal human dermal fibroblasts (NHDFs) or human smooth muscle cells (HSMCs) (Figure 4C). rovVEGF-E_{D1701} also induces the production of TF in HUVECs. Comparable to rhVEGF-A_{165} (Clauss *et al*., 1996b), incubation of endothelial cells with 6 ng/ml rovVEGF-E_{D1701} for 6 h results in a remarkable production of TF (60 pg/ml) which is not observed after incubation with medium alone (2 pg/ml). Thus VEGF-E and VEGF-A display a similar capacity for vascular endothelial cells to stimulate proliferation and production of tissue factor.

VEGF-E stimulates chemotactic migration and sprouting of endothelial cells in in vitro angiogenesis assays

Endothelial cells have previously been shown to migrate in response to a chemotactic gradient of VEGF-A (Waltenberger *et al*., 1994). In a modified Boyden-chamber assay both VEGF-A and VEGF-E promoted migration of porcine aortic endothelial cells (PAE) expressing KDR (PAE/KDR), but not of PAE/Flt-1 cells, suggesting that these growth factors mediate a chemotactic signal via VEGFR-2 rather than VEGFR-1 (Figure 5). These observations are strongly supported by the fact that monocytes, which only migrate in response to activation of VEGFR-1 (Clauss *et al*., 1996a), showed no chemotactic activity secondary to VEGF-E stimulation (J.Waltenberger, A.Bergler and C.Dehio, unpublished).

In order to assess further the biological activity of VEGF-E towards endothelial cells, rovVEGF-E_{D1701} was tested for its ability to stimulate *in vitro* angiogenesis in collagen gels. Without exogenous cytokine stimulation, only a few short capillary sprouts originated from HUVEC

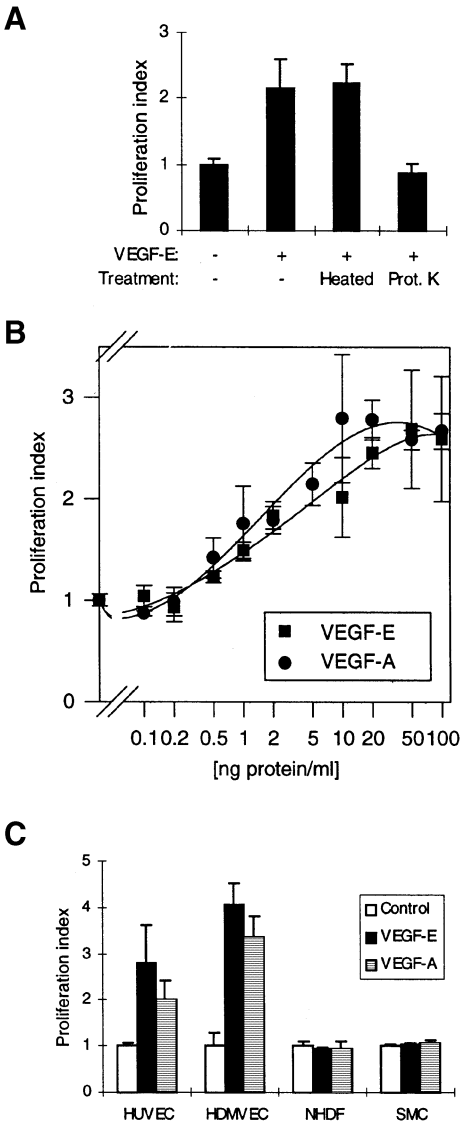

Fig. 4. VEGF-E is a vascular endothelial cell-specific mitogen. (**A**) rovVEGF-E_{D1701} in PBS was boiled for 25 min at 100°C or treated for 3 h at 37°C with 25 ng/ml proteinase K band samples were then diluted in M199–10% FCS to a final concentration of 50 ng/ml. (**B**) Dose–response curve of rovVEGF-E_{D1701} and rhVEGF-A_{165} for the stimulation of HUVEC proliferation. In (A) and (B), a 72 h proliferation assay with HUVECs was performed. (**C**) rovVEGF-E_{D1701} and rhVEGF-A_{165} (each 10 ng/ml final concentration) stimulate the proliferation of HUVECs and HDMVECs, but have no mitogenic effect on NHDFs or HSMCs. For experimental details of the proliferation assays see Materials and methods section. The columns and bars represent mean ± SD of samples performed in triplicate. The data illustrated are representative for one of at least three independently performed experiments.

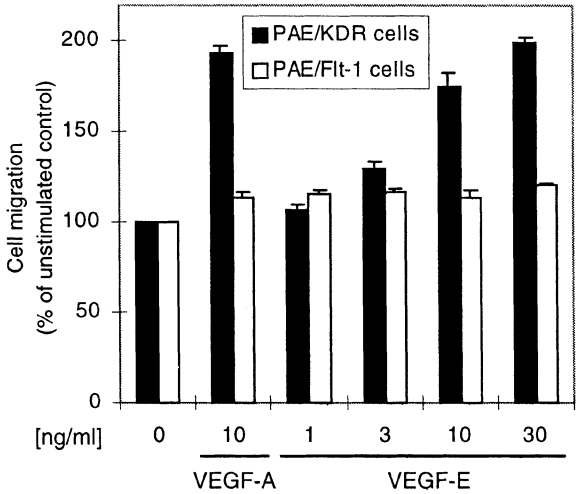

Fig. 5. Stimulation of VEGFR-2-dependent migration of endothelial cells by VEGF-E. PAE/KDR cells and PAE/Flt-1 cells were incubated for 4 h at 37°C in a modified Boyden chamber. The chemoattractant was either rovVEGF-E_{D1701} or rhVEGF-A_{165} at the concentrations indicated. Data are given as mean ± SD of samples performed in quadruplicate. The data illustrated are representative for one of at least three independently performed experiments.

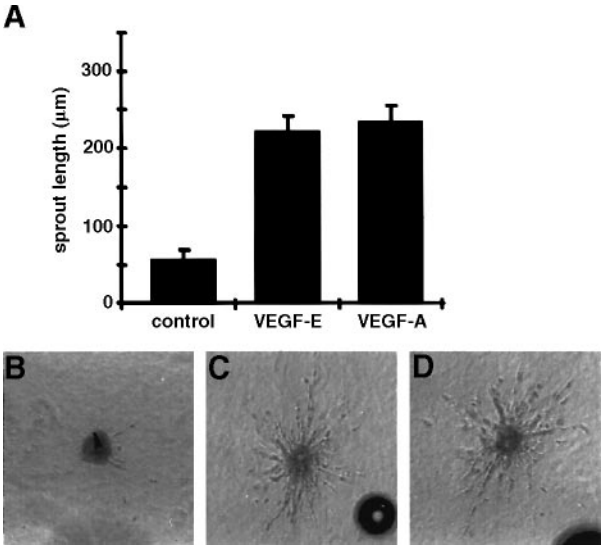

Fig. 6. Induction of *in vitro* angiogenesis in collagen gels by VEGF-E. (**A**) Quantitative analysis of *in vitro* angiogenic activity of 50 ng/ml rovVEGF-E_{D1701} or 50 ng/ml rhVEGF-A_{165}. Angiogenic capacity was assessed by quantitating the length of capillary sprouts originating from collagen-embedded endothelial cell spheroids (see Materials and methods). The data are expressed as mean ± SD of three independent experiments quantitating the length of capillary sprouts of at least 10 spheroids per experiment. rovVEGF-E_{D1701} exerts similar angiogenic potency as rhVEGF-A_{165} (all treatment groups $p < 0.001$ compared with control). (B–D) Representative phase contrast micrographic pictures of endothelial spheroids after 3 days of incubation in (**B**) normal medium or (**C**) in the presence of 50 ng/ml rovVEGF-E_{D1701} or (**D**) 50 ng/ml rhVEGF-A_{165}.

spheroids embedded in collagen gels in the presence of 20% fetal calf serum (FCS) (Figure 6A and B). Addition of 50 ng/ml rovVEGF-E_{D1701} potently stimulated capillary sprouting, increasing both the length and the number of capillary sprouts (Figure 6A and C). rovVEGF-E_{D1701} exerted a similar *in vitro* angiogenic potency as the same concentration of rhVEGF-A_{165} (Figure 6A and D).

Taken together these results demonstrate that, similar to VEGF-A, VEGF-E stimulates the migration and sprouting of vascular endothelial cells, thus showing angiogenic activity *in vitro*.

VEGF-E stimulates angiogenesis in vivo

The *in vivo* angiogenic activity of slow-release preparations of VEGF-E was assessed in the avascular rabbit cornea of albino rabbits. The effect of increasing concentrations of VEGF-E (100–400 ng/pellet) was monitored daily and its angiogenic activity was compared with VEGF-A (200 ng/pellet) and buffer alone. VEGF-E induced new vessel sprouts from pre-existing capillaries

in a concentration-dependent manner, and sustained their growth and elongation over time. During the first week the efficacy of 200 and 400 ng/pellet VEGF-E in inducing neovascular growth was the same (Figure 7A). At day 12, neovascularization induced by 400, 200 and 100 ng/pellet VEGF-E was observed in 100, 75 and 60% of the implants, respectively. The angiogenic response induced by 400 ng/pellet VEGF-E persisted over time with no sign of regression at day 14, whereas at 100 and 200 ng/pellet the newly formed vessels started to regress (Figure 7A). A comparable extent of corneal vascularization was induced by 200 ng/pellet of either VEGF-E or VEGF-A (Figure 7A, and compare B and C), although early after implantation VEGF-E tended to promote neovascularization more effectively than VEGF-A. The control buffer implants elicited growth of a few capillaries which appeared at 3 days but regressed over time (Figure 7A and D). No macroscopic signs of inflammatory reaction like corneal opacity was detected, suggesting that the angiogenic response induced by VEGF-E was direct.

Taken together, the *in vivo* results indicate a potent angiogenic activity for VEGF-E which is comparable with that of VEGF-A.

VEGF-E is a high-affinity ligand for VEGFR-2 and stimulates receptor autophosphorylation and subsequent signalling processes

The interaction of rovVEGF-E_{D1701} with the high-affinity receptors of VEGF-A, VEGFR-1 and VEGFR-2 was assessed by receptor binding and activation studies. In contrast to unlabelled rhVEGF-A_{165}, rovVEGF-E_{D1701} did not show any appreciable displacement of ^{125}I-labelled rhVEGF$_{165}$ bound to VEGFR-1-overexpressing cells (Figure 8A). Consistently, an ~30-fold molar excess of the soluble extracellular domain of Flt-1 (sFlt-1) (Roeckl *et al*., 1998) did abolish the stimulation of HUVEC proliferation by rhVEGF$_{165}$ but had no significant effect on the mitogenic activity of rovVEGF-E_{D1701} (Figure 8C). In contrast, rovVEGF-E_{D1701} was found to displace ^{125}I-labelled rhVEGF-A_{165} from PAE/KDR cells in a concentration-dependent manner (Figure 8B). Albeit ~3-fold higher concentrations were necessary to achieve a similar effect as observed with unlabelled rhVEGF-A_{165}, these data indicate that rovVEGF-E_{D1701} is a high-affinity ligand for VEGFR-2. VEGFR-2 activation by VEGF-A has been shown to activate a signalling cascade including autophosphorylation of tyrosine residues (Heldin, 1995), activation of phospholipase C-gamma (PLCγ) as well as of p42 MAP kinase (Waltenberger *et al*., 1994, 1996; Kroll and Waltenberger, 1997) and an increase in free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Quinn *et al*., 1993). Activa-

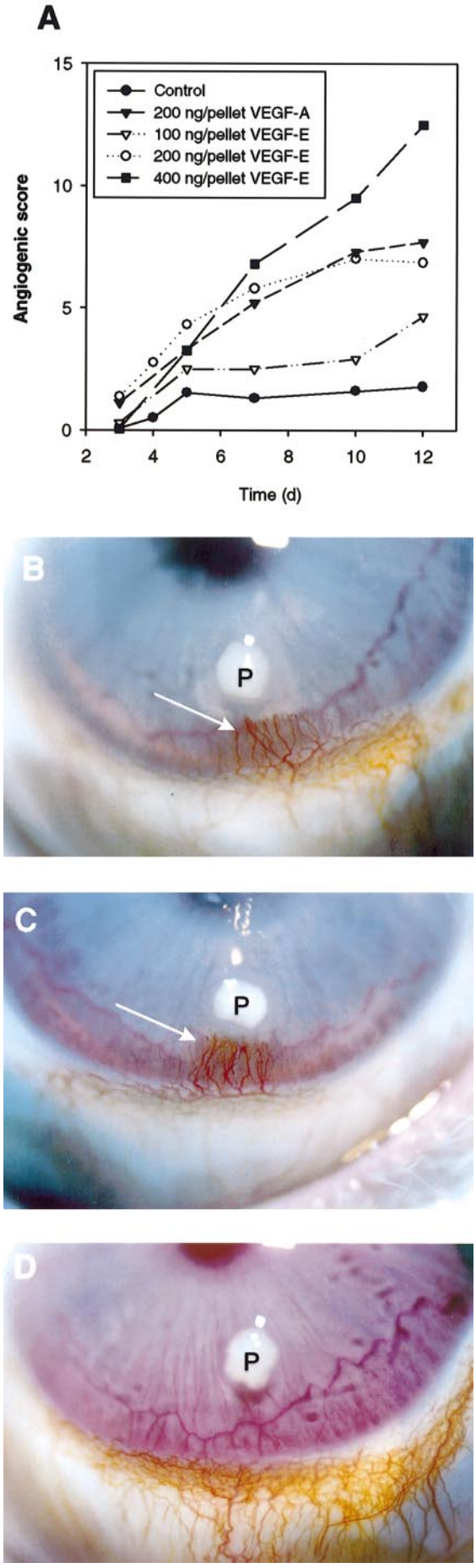

Fig. 7. VEGF-E stimulates angiogenesis *in vivo* dose-dependently. (**A**) The angiogenic activity of various concentrations of VEGF-E, VEGF-A or control buffer were tested as slow release preparations in the rabbit cornea assay. Angiogenic scores were calculated on the basis of the number of vessels and their growth rate and plotted versus time (for experimental details see Materials and methods). Angiogenic score data are the mean values obtained from the response scored in all animals in this study (n = 4–8). (B–D) Representative pictures of the angiogenic response elicited by (**B**) 200 ng/pellet VEGF-E, (**C**) 200 ng/pellet VEGF-A or (**D**) control buffer 6 days after the implant. VEGF-E and VEGF-A elicited the growth of vessels that progressed towards the implant. Arrows indicate the newly formed vessels. P, pellet implant. Magnification ×18.

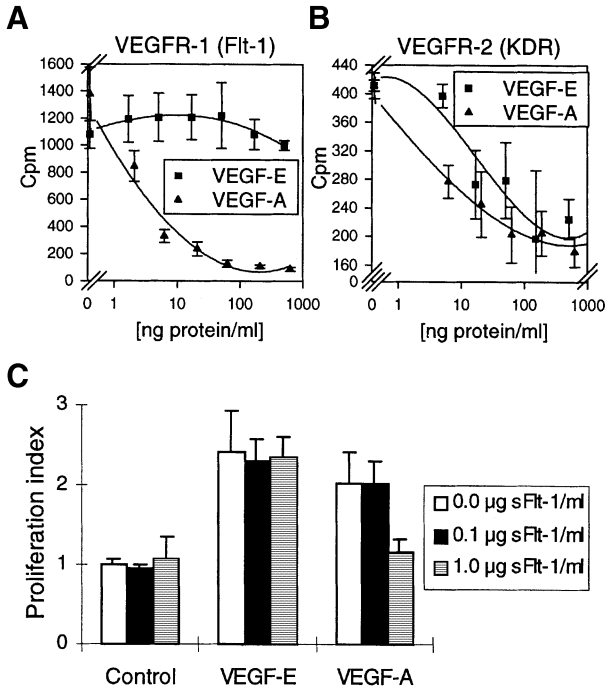

Fig. 8. Characterization of VEGF-E binding to VEGFR-1 and VEGFR-2. Competitive displacement of ^{125}I-labelled rhVEGF-A_{165} (10 ng/ml) bound to (**A**) Flt1- and (**B**) KDR-expressing cells by rovVEGF-E_{D1701} or rhVEGF-A_{165}, respectively. (**C**) Inhibition of HUVEC proliferation induced by rovVEGF-E_{D1701} or rhVEGF-A_{165}, respectively, by soluble Flt-1 (sFlt-1) in a 72 h proliferation assay. For experimental details see Materials and methods. The columns and bars represent mean ± SD of samples performed in triplicate. The data illustrated are representative for one of at least three independently performed experiments.

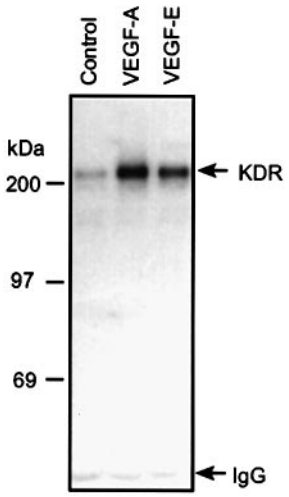

Fig. 9. VEGF-E stimulates autophosphorylation of VEGFR-2 (KDR). PAE/KDR cells were stimulated with 50 ng/ml of either rhVEGF-A_{165} (lane 2) or rovVEGF-E_{D1701} (lane 3) for 5 min at 37°C, respectively. Untreated cells served as control (lane 1). After cell lysis, KDR was immunoprecipitated followed by an *in vitro* kinase reaction for 7 min in the presence of of [γ-^{32}P]ATP, SDS–PAGE and autoradiography.

tion of VEGFR-2 by rovVEGF-E_{D1701} was demonstrated by the concomitant autophosphorylation of KDR in PAE/KDR cells (Figure 9). We could further demonstrate that rovVEGF-E_{D1701} stimulates a biphasic increase in $[Ca^{2+}]_i$ in HUVECs (Figure 10A) and HDMVECs, (Figure 10B). This rise in $[Ca^{2+}]_i$ was also observed in PAE/KDR cells,

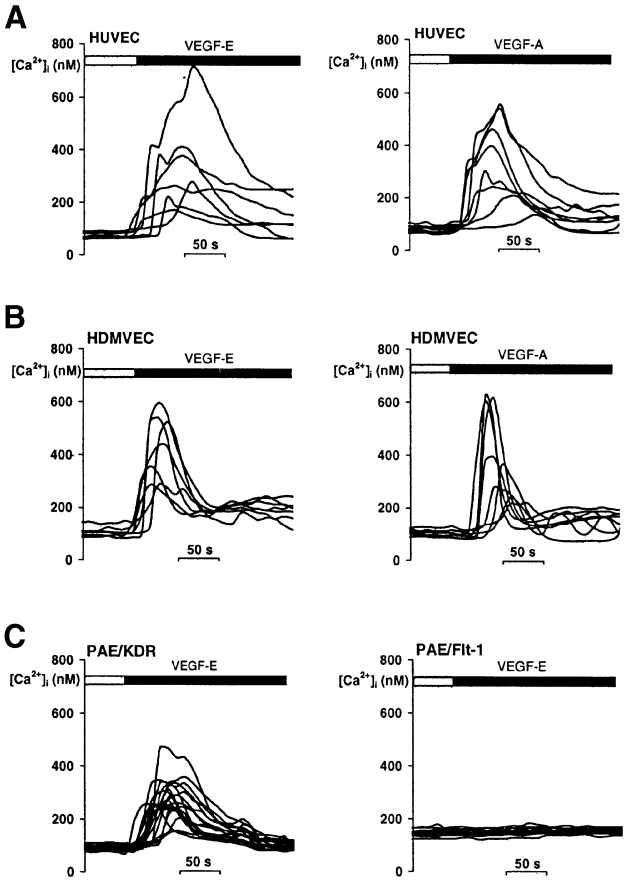

Fig. 10. VEGF-E stimulates a VEGFR-2-dependent biphasic increase of $[Ca^{2+}]_i$ in macrovascular and microvascular endothelial cells. Changes in $[Ca^{2+}]_i$ resulting from perfusion of (**A**) HUVECs, (**B**) HDMVECs, (**C**) PAE/KDR cells or PAE/Flt-1 cells by 50 ng/ml of either rovVEGF-E_{D1701} or rhVEGF-A_{165}, as indicated, were recorded for individual cells by digital ratiometric fluorescence microscopy of cells preloaded with fluo-2 AM. For experimental details see Materials and methods.

but not in PAE/Flt-1 cells (Figure 10C) or parental PAE cells (data not shown), demonstrating that rovVEGF-E_{D1701} stimulates a calcium signal specifically via VEGFR-2.

Taken together, these results demonstrate that, similar to VEGF-A, VEGF-E binds to VEGFR-2 with high affinity and stimulates receptor activation by autophosphorylation resulting in a subsequent rise in $[Ca^{2+}]_i$, while unlike VEGF-A, VEGF-E is not a ligand for VEGFR-1.

Discussion

In this study we report on the functional characterization of a homologue of mammalian vascular endothelial growth factor, VEGF, encoded by the parapoxvirus Orf virus, OV. Due to a similar bioactivity but different receptor specificity relative to mammalian VEGF-A, the viral VEGF homologue allowed us to dissect the role of the major VEGF-A receptors, VEGFR-1 and VEGFR-2, in stimulating angiogenesis. According to the unique structural, functional and biochemical characteristics, this novel vascular endothelial growth factor was classified as VEGF-E, a novel member of the family of VEGF-like growth factors.

VEGF-E encoded by OV strain D1701 (designated as

ovVEGF-E_{D1701}) was expressed as a native protein in mammalian cells (Figure 1B) or purified after recombinant expression in *E.coli* (Figure 3) and was found to be a heat-stable, secreted dimer of ~34 kDa, as previously reported for VEGF-A (Ferrara *et al*., 1992). VEGF-E and VEGF-A were tested in parallel in various bioassays and were found to possess a remarkably similar bioactivity, i.e. they both stimulate the release of tissue factor and the proliferation of vascular endothelial cells (Figure 4), their chemotactic migration (Figure 5) and their sprouting (Figure 6) as well as angiogenesis *in vivo* (Figure 7). Thus, VEGF-E is a novel angiogenic factor with an activity comparable to that of VEGF-A.

Based on a BLAST similarity search, VEGF-E is structurally closely related to VEGF-A. A multiple sequence alignment of full-length amino acid sequences of representative VEGFs (Figure 2A) and an average distance tree, based upon the fully aligned central cysteine knot motif present in all VEGFs, was generated (Figure 2B). Two VEGF-E sequences isolated from different OV strains, ovVEGF-E_{D1701} (this work) and ovVEGF-E_{NZ2} (Lyttle *et al*., 1994), are closely related clustering next to the branching point of a homogenous VEGF-A cluster and PlGF-1, while the third VEGF-E sequence, ovVEGF-E_{NZ7} (Lyttle *et al*., 1994), is highly divergent (Figure 2B). We have meanwhile cloned and sequenced 10 additional *vegf-e* alleles from independent OV isolates and the deduced microheterogenic VEGF-E sequences are very closely related to ovVEGF-E_{D1701} and ovVEGF-E_{NZ2} (M.Meyer, M.v.Rechenberg, H.Weich, T.Korff, L.Morbidelli, J.Krolls, T.Laun, M.Büttner, H.-J.Rziha and C.Dehio, in preparation). This result indicates that, for unknown reasons, ovVEGF-E_{NZ7} may have undergone genetic drift. The unique sequence characteristics of VEGF-E, i.e. a unique conserved threonine- and proline-rich sequence signature present at the C-terminus (Figure 2A), justifies the grouping of these viral vascular endothelial growth factors as a novel subfamily within the VEGF family.

Despite striking similarities in structure and bioactivity of VEGF-E and VEGF-A, we found important differences concerning their utilization of high-affinity receptors expressed on endothelial cells. VEGF-A binds to both VEGFR-1 and VEGFR-2 receptor tyrosine kinases (Klagsbrun and D'Amore, 1996; Poltorak *et al*., 1997), while ovVEGF-E_{D1701} was found to bind with high affinity only to VEGFR-2, but not to VEGFR-1. This was demonstrated by displacement experiments of ^{125}I-labelled VEGF-A interacting with cells overexpressing VEGFR-1 or VEGFR-2 (Figure 8A and B) and by the use of a soluble VEGFR-1 (sFlt-1) receptor as a specific competitor in bioactivity assays (Figure 8C). Preliminary experiments using a Biacore chip surface coated with sFlt-1 or VEGFR-2 (sKDR) further indicated the unique receptor specificity of rovVEGF$_{D1701}$, as well as of four other microheterogenic VEGF-E proteins (M.Meyer, M.v.Rechenberg, H.Weich, T.Korff, L.Morbidelli, J.Krolls, T.Laun, M.Büttner, H.-J.Rziha and C.Dehio, in preparation). Furthermore, binding of VEGF-E to VEGFR-2 was shown to result in receptor activation by autophosphorylation (Figure 9) and triggering of a VEGFR-2-dependent biphasic rise in the $[Ca^{2+}]_i$ (Figure 10), in accordance with the established signalling cascade of this receptor (Quinn *et al*., 1993; Waltenberger *et al*., 1994; Kroll and Waltenberger, 1997). Thus, VEGF-E activates VEGFR-2 selectively.

The receptor-binding determinants of VEGF-A_{165} for VEGFR-1 and VEGFR-2 have recently been mapped by structure determination of the VEGFR-1–VEGF-A complex (Wiesmann *et al*., 1997; residues that are at least 50% buried in the receptor–ligand interface are denoted in Figure 2A by '+' symbols) and by alanine scanning mutagenesis (Muller *et al*., 1997; residues that result in a >5-fold decrease in binding to VEGFR-2 upon alanine substitution are denoted in Figure 2A by '#' symbols), respectively. The receptor binding face of VEGF-A specified by these residues is similar for both receptors (Wiesmann *et al*., 1997). However, consistent with the low level of amino acid identity between VEGF-E and mammalian VEGFs, the residues of VEGF-A implicated in binding to both VEGFR-1 and VEGFR-2 are poorly conserved in VEGF-E, except for three binding determinants clustering between cysteines 5 and 6 of the cysteine knot motif (Figure 2A, symbols denoting conserved binding determinants are shaded). Interestingly, an alanine substitution of isoleucine 83 in VEGF-A results in a 100-fold drop in the binding affinity to VEGFR-2 (Muller *et al*., 1997), while alanine is found in the corresponding position of VEGF-E_{D1701} (Figure 2A, denoted by a **#** symbol in bold). As VEGF-E binds to VEGFR-2 with almost the same affinity as VEGF-A, the binding motifs for VEGFR-2 appear to be different for these two related growth factors.

In this study, VEGF-E was found to possess an angiogenic activity as potent as VEGF-A. Due to the selective binding of VEGF-E to VEGFR-2 but not VEGFR-1, our data strongly indicate that VEGFR-2 activation can mediate angiogenesis efficiently without concomitant VEGFR-1 activation. This finding is in accordance with previous studies suggesting that the angiogenic signal by VEGF-A itself may be predominately mediated by VEGFR-2 (Waltenberger *et al*., 1994; Keyt *et al*., 1996), at least regarding the selected aspects of *in vitro* angiogenesis (i.e. endothelial proliferation and chemotactic migration) investigated in these reports. Interestingly, a complete knock-out mutant of VEGFR-1 in mice gives rise to an embryo lethal phenotype grossly affected in angiogenesis (Fong *et al*., 1995), while mice expressing a mutant VEGFR-1, which lacks the tyrosine kinase domain, still undergo normal development and angiogenesis (Hiratsuka *et al*., 1998). Due to the ~10-fold higher affinity of VEGF-A for VEGFR-1 versus VEGFR-2 (de Vries *et al*., 1992; Terman *et al*., 1992) and the dispensability of the VEGFR-1-kinase function, the main biological rule of this receptor was suggested to be as a ligand sink (Hiratsuka *et al*., 1998) which may regulate the local availability of VEGF-A for stimulating endothelial proliferation and angiogenesis via VEGFR-2. As a consequence of the lack of VEGFR-1 binding by VEGF-E, its effective local concentration available for binding to VEGFR-2 may increase. Indeed, as we observed a similar concentration dependency for the bioactivity exerted by VEGF-E and VEGF-A, this effect may compensate for the ~3-fold reduced affinity of VEGF-E for VEGFR-2 compared with VEGF-A (Figure 8B). In conclusion, using VEGF-E, we have shown that VEGFR-2 can potently

mediate angiogenesis independently of VEGFR-1 activation.

Lesions resulting from OV infection in human and sheep display massive capillary proliferation and dilation (Groves *et al*., 1991). The potent angiogenic activity of VEGF-E characterized in this work may be directly responsible for the formation of these vasoproliferative tumours. Indeed, deletion of *vegf-e* by recombinant techniques does not affect virus growth in tissue culture, but results in substantially reduced lesion development in experimentally infected sheep when compared with the parental OV strain D1701 (M.Henkel, C.von Einem, M.Meyer, C.Dehio, M.Büttner and H.-J.Rziha, in preparation). Consistently, supernatants of cell cultures infected with the parental OV strain (Figure 1A), but not the respective *vegf-e* deletion mutant (M.Henkel, C.von Einem, M.Meyer, C.Dehio, M.Büttner and H.-J.Rziha, in preparation), are mitogenic for macrovascular endothelial cells *in vitro*. In conclusion, VEGF-E appears to represent a key regulator of the vascular remodelling typically observed in OV lesions.

Besides OV, other viruses may encode angiogenic factors like VEGF-E. The Tat protein of human immunodeficiency virus (HIV) was identified recently as an angiogenic factor associated with the appearance of Kaposi's sarcoma, a highly vascularized lesion (Adriana *et al*., 1996a). Interestingly, activation of VEGFR-2 appears to be the mandatory requirement for angiogenic activity of Tat (Adriana *et al*., 1996b), as shown here for the structurally unrelated VEGF-E. However, as Tat is also taken up into the cell nucleus where it acts as a transactivator or repressor of gene expression (Buonaguro *et al*., 1994; Weissman *et al*., 1998), VEGF-E might be more suitable for selectively studying VEGFR-2 dependent angiogenesis. The precise role of VEGF-E-mediated angiogenesis in OV pathogenicity remains to be determined. Moreover, considering the therapeutic impact of VEGF-A in treatment of coronary heart disease (Waltenberger, 1997; Mack *et al*., 1998) or critical limb ischemia (Baumgartner *et al*., 1998; Folkman, 1998), VEGF-E with its similar structure and bioactivity but altered receptor specificity represents a novel, potentially beneficial candidate for therapeutic intervention.

Materials and methods

Cell culture

HUVECs were isolated and cultured as described (Dehio *et al*., 1997). PAE cells transfected with either cDNA encoding VEGFR-2 (PAE/KDR cells) or VEGFR-1 (PAE/Flt-1 cells; Waltenberger *et al*., 1994) were cultured in Ham's F12 medium–10% FCS. HDMVECs, NHDFs and HSMCs (all obtained from Promocell, Heidelberg, Germany) were cultured in EGM-MV, FGM and SMGM medium (all from Promocell), respectively. COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)–10% FCS. The bovine kidney cell line BKKL3A was cultured in DMEM–10% fetal bovine serum (FBS).

Infection of epithelial cells with OV

BKKL3A cells cultured in the medium M199–10% FCS were infected with OV D1701 at a MOI (multiplicity of infection) of 1 or mock infected essentially as described (Cottone *et al*., 1998) and medium was collected 24 h post-infection, filtrated through 0.1 μm nitrocellulose to remove cell debris and virus particles and stored at –20°C until use. Filtered samples were tested for residual infectivity by inoculation of BKKL3A cells.

Cloning of vegf-e

Based on the nucleotide sequence of the *vegf-e* gene of OV strain D1701 (DDBJ/EMBL/GenBank accession No. AF106020), oligonucleotides were deduced which served to amplify this gene by PCR. The oligonucleotides 5′-GGG TGA GAA TTC ATG AAG TTT CTC GTC GGC-3′ and 5′-AAA GTT GGA TCC CTA GCG GCG TCT TCT GGG-3′ are homologous or complementary to the first and last six codons of the open reading frame, respectively. Furthermore, these primers introduce an *Eco*RI- or *Bam*HI-site next to the ATG start codon or TAG stop codon, respectively. Genomic DNA of OV strain D1701 and these primers were used to generate by PCR the complete coding sequence of *vegf-e*. This 418 bp PCR fragment was cloned by the introduced *Eco*RI- and *Bam*HI-sites into the corresponding sites of vector pSPT18 (Boehringer Mannheim, Mannheim, Germany), generating plasmid pVEGF, and sequenced on both strands to verify authentic DNA sequence. The *Eco*RI–*Bam*HI fragment was further subcloned into the correponding sites of vector pSK$^-$ (Stratagene, Heidelberg, Germany), generating plasmid pCD325.

Expression of ovVEGF-ED1701 in COS-7 cells

The *vegf-e* gene was introduced into the eukaryotic expression vector pCB6-Bam (Dehio *et al*., 1995). In order to improve expression, the 5′ untranslated leader sequence (22 bp) present in the viral genome were reintroduced into the cloned ov*vegf-eD1701* gene by PCR using the oligonucleotides 5′-GGG GAA GCT TAC TTT TAA GGG TGA GGC GCC ATG AAG TTT CTC GTC GGC ATA CTG-3′ and 5′-CTC GGA TCC CTA GCG GCG TCT TCT-3′, which also introduce a *Hin*dIII and a *Bam*HI site, respectively, as primers and pCD325 plasmid DNA as template. A PCR-fragment of the expected size was cloned via the *Hin*dIII and *Bam*HI sites into pCB6-Bam, generating pCD379. ovVEGF-E_{D1701} was transiently expressed in COS-7 cells by transfection of pCD379 using the calcium phosphate coprecipitation method (Sambrook *et al*., 1989), and transfection of the empty vector pCB6-Bam served as a control. Medium M199–10% FCS incubated with transfected cells from 4–52 h post-transfection was collected, cleared from cell debris by centrifugation and subsequently filtered through 0.22 μm nitrocellulose and stored at –70°C until use in proliferation assays.

Expression of a VEGF-E fusion protein in E.coli and purification of the dimeric recombinant protein rovVEGF-ED1701

For the recombinant expression of ovVEGF-E_{D1701} as a fusion protein in *E.coli*, a 345 bp *Mvn*I–*Bam*HI fragment of pCD325 was cloned into the expression vector pET-15b (Novagene, Madison, WI), which was cut by *Nde*I, filled-in, and recut by *Bam*HI. The fusion protein encoded by the resulting plasmid pCD369 consists of the vector encoded amino acids sequence MGSSHHHHHHSSGLVPRGSHT that contains an His-tag (aa 5–10) and a thrombin-cleavage site (aa 14–19, cleavage in between aa17 and aa18) followed by amino acids 21–133 of ovVEGF-E_{D1701}. This plasmid was introduced into the *E.coli* expression strain BL21(DE3) (Novagene). Plasmid pSB161 (Schenk *et al*., 1995), which directs overexpression of *argU* (^{arg}t-RNA) and thereby allows the overexpression of genes containing a critical number of rare AGG/AGA codons (like in *ovvegf-eD1701*), had also to be introduced to allow high level production of rovVEGF-E_{D1701} following IPTG induction. For purification of rovVEGF-E_{D1701}, bacterial cultures induced with IPTG for 2 h, as indicated in the instructions of the distributer (Novagene), were harvested, resuspended in sonication buffer (5 mM imidazol, 500 mM NaCl, 20 mM Tris–HCl pH 7.9) and cells were ruptured by sonication on ice. The lysate was cleared by centrifugation for 30 min at 16 000 r.p.m. at 4°C and used to purify recombinant protein via binding to a Ni-chelate column under non-denaturing conditions as outlined by the manufacturer (Novagene). Eluted protein was allowed to dimerize and form intramolecular and intermolecular disulfide bridges by incubation for 24 h at 4°C. After concentration and buffer exchange to phosphate-buffered saline (PBS) by ultrafiltration on Centricon 10 tubes according to the instructions of the manufacturer (Amicon, Beverly, MA), dimerized protein was purified by gel-filtration on Superose 12 (Pharmacia, Uppsala) in PBS. In some cases, the 15 N-terminal amino acids including the hexa-histidine-tag were cleaved by digestion with biotinylated thrombin (Novagene) before adsorption of the N-terminal cleavage peptide by passage through a Ni-chelate column as instructed by the manufacturer (Novagene, Madison, WI) and adsorption of the biotinylated thrombin by streptavidin–Sepharose (Sigma, Deisenhofen, Germany). The protein was then concentrated by ultrafiltration as before and dimeric protein was further purified by gel filtration on a fresh low-endotoxin Sephacryl S-200 column (Pharmacia, Uppsala, Sweden). The

eluted protein was again concentrated by ultrafiltration, aliquoted and stored at –70°C. Endotoxin levels were quantified by a commercially available test (Limulus Amebocyten Lysate Coatest® Endotoxin, Chromogenix, AB, Sweden), and this result was verified at the Fresenius Institute (Freiburg, Germany).

Cell proliferation assays

HUVECs or HDMVECs were seeded sparsely in 24-well culture dishes in M199–10% FCS. After 16 h, the cell numbers in a microscopic field in the centre of the dish were counted (initial cell number) and cells were stimulated with various concentrations of growth factors or dilutions of culture supernatants in M199–10% FCS as indicated. After 72 h, the cell number in the same microscopic field was determined again (final cell number). The proliferation index represents the quotient of the final cell number divided by the initial cell number and normalized by a factor calculated for the untreated control (medium M199–10% FCS alone) to result in a proliferation index = 1. For measurement of the proliferation of HSMCs and NHDFs, cells were seeded in 24-well culture dishes and starved for 48 h in M199–0.1% FCS. After adding the stimulus to the same medium the cells were incubated for a further 72 h (HSMCs) or 120 h (NHDFs). In each case, samples were performed in triplicate, and data illustrate mean ± SD of one representative of three independently performed experiments.

Chemotactic migration assay

Chemotactic migration assay of PAE/KDR cells in response to a gradient of stimulus was performed in a modified Boyden chamber as previously described (Waltenberger *et al*., 1994). In brief, the chemotactic response of PAE/KDR cells and PAE/Flt-1 cells was assayed using a 48-well modified Boyden chamber (Neuro Probe®, Inc.) and collagen-coated (collagen type A, Sigma) polycarbonate membrane filters with a pore diameter of 8 μm (Nucleopore®). Cell suspensions were loaded to the upper part of the chemotaxis chamber and stimulated for 4 h with VEGF-A (10 ng/ml) or increasing concentrations of VEGF-E, respectively. Migration was assessed by counting the number of cells that migrated to the lower side of the filter membrane. Migrated cells were stained with Giemsa dye. The migration of unstimulated cells (chemokinesis) served as a control and is referred to as 100% migration. The assay was performed in quadruplicate and five independent high-power fields per well were counted using a light microscope (Jenalab). Data illustrate mean ± SD of one representative of three independently performed experiments.

In vitro angiogenesis assay

In vitro angiogenesis in collagen gels was quantitated using spheroids of macrovascular endothelial cells in modification of the microcarrier-bead angiogenesis assay (Nehls and Drenckhahn, 1995). In brief, HUVEC spheroids containing ~750 cells were generated overnight (Korff and Augustin, 1998) after which they were embedded into collagen gels. A collagen stock solution was prepared prior to use by mixing 8 vol acidic collagen extract of rat tails (equilibrated to 3 mg/ml, 4°C) with 1 vol 10× EBSS (Gibco-BRL, Eggenstein, Germany) and ~1 vol 0.1 N NaOH to adjust the pH to 7.4. This stock solution (0.5 ml) was mixed with 0.5 ml room temperature (rt) ECGM basal medium (PromoCell, Heidelberg, Germany) containing 40% FCS (Biochrom, Berlin, Germany), 1.2% (v/w) carboxymethylcellulose (Sigma, Deisenhofen, Germany) to prevent sedimentation of spheroids prior to polymerization of the collagen gel, ~50 HUVEC spheroids and the corresponding test substance [rovVEGF-E_{D1701} in a final concentration of 50 ng/ml and rhVEGF-A_{165} (Upstate Biotechnology, Lake Placid, NY) in a final concentration of 50 ng/ml]. The spheroid-containing gel was rapidly transferred into 24 well plates and allowed to polymerize (1 min) after which 0.15 ml ECGM basal medium was pipetted on top of the gel. The gels were incubated at 37°C, 5% CO_2 and 100% humidity. After 3 days, *in vitro* angiogenesis was quantitated by measuring the length of the three longest capillary sprouts that had grown out of each spheroid (ocular grid at 100× magnification) analysing at least 10 spheroids per experimental group and experiment.

In vivo angiogenesis assay

The angiogenic activity was determined *in vivo* using the rabbit cornea assay (Ziche *et al*., 1989). Angiogenesis was studied in the cornea of albino rabbits since this is an avascular and transparent tissue where inflammatory reactions and growing capillaries can be easily monitored and changes quantitated by stereomicroscopic examination (Ziche *et al*., 1994). Slow-release pellets (1×1×0.5 mm) were prepared under sterile conditions incorporating the test substances into a casting solution of a ethynil-vinyl copolymer (Elvax-40), in 10% methylene chloride (10 μl/droplet). In the lower half of New Zealand white rabbit eye (Charles River, Calco, Como, Italy), anaesthetized by sodium pentothal (30 mg/kg), a micro pocket (1.5×3 mm) was surgically produced using a pliable iris spatula 1.5 mm wide. The pellets were implanted in the micropockets located into the transparent avascular corneal stroma. Subsequent daily observations of the implants were made with a slit lamp stereomicroscope without anaesthesia by two independent operators in blind. An angiogenic response was scored as positive when budding of vessels from the limbal plexus occurred after 3–4 days and capillaries progressed to reach the implanted pellet according to the scheme previously reported (Ziche *et al*., 1994; Ziche *et al*., 1997). Angiogenic activity was expressed as the number of implants exhibiting neovascularization over the total implants studied. Potency was scored by the number of newly formed vessels and by their growth rate and the data were expressed as angiogenic score as previously reported (Ziche *et al*., 1994). The efficiency in recruiting the quiescent capillary endothelium from pre-existing capillaries by the peptides was also scored on the basis of the kinetics of vascular morphogenesis for capillary elongation and density. The extent of elongation into the corneal stroma over time was scored by the use of an ocular grid and expressed in mm. A density value of 1 corresponded to 0–25 vessels per cornea, 2 from 25–50, 3 from 50–75, 4 from 75–100 and 5 for <100 vessels.

TF release

Experiments were carried out within 48 h of HUVEC cells achieving confluency. Expression of TF was assessed by incubating cultures in the absence/presence of rhVEGF-A_{165} or rovVEGF-E_{D1701} in MDCB 131 medium containing 10 mM HEPES pH 7.4, 8% FCS (PAA, Cölbe, Germany), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were further incubated for 6 h at 37°C. Assays were carried out with whole cells obtained in suspension following scraping and TF activity equivalents were determined as previously described (Clauss *et al*., 1990). Briefly, endothelial cells were removed from the dish by scraping with a rubber policeman in 100 μl of clotting buffer, consisting of 8 mM sodium acetate, 8 mM sodium diethyl-barbiturate and 141 mM NaCl. The resuspended cells were mixed with 100 μl of human citrated plasma and clotting times were measured after recalcification with 100 μl of a 20 mM $CaCl_2$-solution. Tissue factor equivalents were determined by using a standard curve of purified human TF (Loxo, Dossenheim, Germany).

VEGF labelling and binding studies

rhVEGF-A_{165} used for labelling and binding competition studies was expressed in baculovirus and purified as described (Clauss *et al*., 1996b). Iodination was performed using the chloramine T method as modified for VEGF (Plouet and Moukadiri, 1990) and specific activities of $3–4\times10^6$ c.p.m./ng were achieved. Alternatively, ^{125}I-rhVEGF-A_{165} was obtained from NEN (Boston, MA). For binding competition studies, ^{125}I-rhVEGF-A_{165} was mixed with various concentrations of either unlabelled rhVEGF-A_{165} or rovVEGF-E_{D1701} in RPMI containing 20 mM HEPES and 0.1% gelatine. Medium from 3T3-fibroblasts stably transfected with Flt-1, which were kindly provided by Dr Georg Breier (Bad Nauheim, Germany), or PAE-cells stably transfected with KDR (Waltenberger *et al*., 1994) was replaced by this mixture and incubated for 3 h on ice. Following four washes with RPMI medium containing 0.1% gelatine, cells were lysed with 0.1% SDS and the c.p.m. of the released ^{125}I-rhVEGF-A_{165} was measured in a gamma counter (Packard, Frankfurt, Germany).

Autophosphorylation

The assay for detection of autophosphorylation of KDR on tyrosine residues following growth factor stimulation of PAE/KDR cells was performed as described (Kroll and Waltenberger, 1997). Subconfluent PAE/KDR cells were starved overnight in serum-free medium containing 0.01 mg/ml bovine serum albumen (BSA) and preincubated for 5 min with 100 μM Na_3VO_4 to inhibit phosphatase activity. Cells were stimulated for 5 min at 37°C with 50 ng/ml VEGF-A or 50 ng/ml VEGF-E, respectively. After a wash with ice-cold PBS containing 100 μM Na_3VO_4, cells were solubilized in a lysis buffer [150 mM NaCl, 20 mM Tris–HCl, pH7.4, 1% CHAPS (Sigma), 10 mM EDTA, 10% glycerol, 100 μM Na_3VO_4, 1% Trasylol® (Bayer), 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The cell lysates were centrifuged at 10 000 *g* for 15 min. The supernatants were used for immunoprecipitation with the receptor-specific antiserum NEF (Kroll and Waltenberger, 1997). Immunoprecipitates immobilized on Protein A–Sepharose CL 4B (Pharmacia) were used for immune complex kinase assay, which was

carried out for 7 min at RT in 25 μl of 50 mM HEPES buffer pH 7.4, containing 10 mM $MnCl_2$, 1 mM dithiothreitol and 5 μCi of [γ-^{32}P]ATP (Amersham International, UK). Following electrophoresis, the gels were incubated for 30 min in 2.5% glutaraldehyde, washed two times for 15 min in 10% acetic acid, 40% methanol, treated for 1 h at 55°C in 1 M KOH, washed three times for 20 min in 10% acetic acid 40% methanol, dried and exposed to Hyperfilm MP (Amersham International, UK).

Measurements of $[Ca^{2+}]_i$

HUVECs or HDMVECs were seeded on glass cover slips coated with 0.2% gelatine and PAE, PAE/KDR and PAE/Flt-1 cells were seeded on uncoated glass cover slips in culture medium containing 2% FCS. After 16 h, cells were loaded with 1 μM fura 2-AM (Molecular Probes, Eugene, OR) for 30 min at 37°C. Changes in $[Ca^{2+}]_i$ were recorded by digital ratiometric fluorescence microscopy. Image ratios at 340/380 nm excitation and 510 nm emission wavelengths were obtained on a Zeiss Axiovert 135 fluorescence microscope at 34°C. Video images from an intensified CCD camera (Proxitronic, Göttingen, Germany) were recorded every 10 s and digitized with an Axon image lightning system (Axon Instr., Foster City, CA). The standard medium was Ringer solution with 1 mM Ca^{2+}, and growth factors were washed with constant perfusion via a multichannel pipette allowing rapid solution exchange. Calibration was performed *in situ* using 10 μM ionomycin and varying external Ca^{2+} concentrations and $[Ca^{2+}]_i$ was calculated as described (Grynkiewicz *et al*., 1985).

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Note added in proof

After acceptance of this work, a paper was published by Ogawa *et al*. (*J. Biol. Chem.*, 1998, **273**, 31273–31282) showing that VEGF-E_{NZ7}, the most divergent VEGF-E form, is an angiogenic factor preferentially utilizing VEGFR-2; this is consistent with our findings for ovVEGF-E_{D1701}.