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A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases

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Angiogenesis, the sprouting of new blood vessels from pre-existing ones, and the permeability of blood vessels are regulated by vascular endothelial growth factor (VEGF) via its two known receptors Flt1 (VEGFR-1) and KDR/Flk-1 (VEGFR-2). The Flt4 receptor tyrosine kinase is related to the VEGF receptors, but does not bind VEGF and its expression becomes restricted mainly to lymphatic endothelia during development. In this study, we have purified the Flt4 ligand, VEGF-C, and cloned its cDNA from human prostatic carcinoma cells. While VEGF-C is homologous to other members of the VEGF/platelet derived growth factor (PDGF) family, its C-terminal half contains extra cysteine-rich motifs characteristic of a protein component of silk produced by the larval salivary glands of the midge, *Chironomus tentans*. VEGF-C is proteolytically processed, binds Flt4, which we rename as VEGFR-3 and induces tyrosine autophosphorylation of VEGFR-3 and VEGFR-2. In addition, VEGF-C stimulated the migration of bovine capillary endothelial cells in collagen gel. VEGF-C is thus a novel regulator of endothelia, and its effects may extend beyond the lymphatic system, where Flt4 is expressed.

Keywords: angiogenesis/endothelium/growth factor/lymphatic system/VEGF

Introduction

The development of blood vessels from early (*in situ*) differentiating endothelial cells is termed vasculogenesis (Risau and Lemmon, 1988). The formation of the rest of the vascular tree is thought to occur as a result of vascular sprouting from pre-existing vessels, a process called angiogenesis (Folkman, 1995). Vascular endothelial cells can give rise to several types of functionally and morphologically distinct vessels and when organs differentiate and begin to perform their specific functions, the phenotypic heterogeneity of endothelial cells increases (Risau, 1995). Angiogenesis also plays a major role in pathological conditions such as diabetic retinopathy, rheumatoid arthritis, psoriasis, cardiovascular diseases and tumour growth and metastasis (Folkman, 1995).

Angiogenesis is regulated by a balance between angiogenic factors and inhibitors which bind to specific receptors on target cells. Five endothelial cell-specific receptor tyrosine kinases, Flt-1 (VEGFR-1), KDR/Flk-1 (VEGFR-2), Flt4, Tie and Tek/Tie-2, have so far been described, which possess the intrinsic tyrosine kinase activity essential for signal transduction (for reviews, see Mustonen and Alitalo, 1995; Shibuya, 1995). Targeted mutations inactivating Flt-1, Flk-1, Tie and Tek in mouse embryos have indicated their essential and specific roles in vasculogenesis and angiogenesis at the molecular level (Dumont *et al.*, 1994; Millauer *et al.*, 1994; Fong *et al.*, 1995; Puri *et al.*, 1995; Sato *et al.*, 1995; Shalaby *et al.*, 1995). VEGFR-1 and VEGFR-2 bind VEGF with high affinity (K_d 16 pM and 760 pM, respectively) (de Vries *et al.*, 1992; Terman *et al.*, 1992; Millauer *et al.*, 1993; Waltenberger *et al.*, 1994) and VEGFR-1 also binds the related placenta growth factor (PlGF; K_d ~200 pM) (Maglione *et al.*, 1993; Park *et al.*, 1994), while the ligands for Tie, Tek and Flt4 have not yet been reported.

We report isolation of a novel vascular endothelial growth factor and its cloning from a cDNA library prepared from the human prostatic adenocarcinoma cell line PC-3. We show that the isolated cDNA encodes a protein which is proteolytically processed, secreted to cell culture medium, binds to the extracellular domain of Flt4 and induces tyrosine autophosphorylation of Flt4 and VEGFR-2. VEGF-C also stimulates the migration of endothelial cells in collagen gels.

Results

Identification, purification and N-terminal sequencing of the Flt4 ligand

In order to find a source for the Flt4 ligand, we screened conditioned media (CM) from human tumour cell cultures for their ability to stimulate the Flt4 receptor. Serum-free medium conditioned for 5 days with PC-3 prostatic adenocarcinoma cells was found to stimulate tyrosine phosphorylation of Flt4 expressed in transfected NIH 3T3 cells (Figure 1, lanes 1–3). The stimulating activity was increased upon concentration of CM by ultrafiltration through a 10 kDa cut-off membrane (lanes 2, 3 and 6). Pretreatment with the extracellular domain of Flt4 (Flt4EC) covalently bound to Sepharose completely abolished the ability of CM to stimulate tyrosine phosphorylation of Flt4 (lanes 3–5). No autophosphorylation of Flt4 was detected when transfected cells were treated with purified VEGF or PlGF (Pajusola *et al.*, 1994 and data not shown). These data indicated that the PC-3 cells produce a soluble ligand which binds to the extracellular domain of Flt4 and activates this receptor.

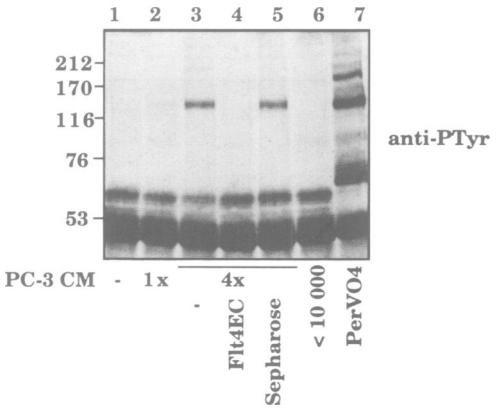

Fig. 1. Identification of the Flt4 ligand from PC-3 cell CM. Flt4-expressing NIH 3T3 cells were incubated with PC-3 cell CM, lysed and the lysates were immunoprecipitated with Flt4-specific antiserum followed by SDS–PAGE, Western blotting and detection using anti-phosphotyrosine (anti-PTyr) antibodies. Lane 1, unconditioned medium. Lane 2 shows weak phosphorylation of a band of 125 kDa upon stimulation with unconcentrated PC-3 CM. The 125 kDa band comigrated with the tyrosine phosphorylated, processed form of the mature Flt4 from pervanadate-treated cells (compare lanes 2 and 7). Lane 3, stimulation with PC-3 CM concentrated 4-fold using Centricon-10 device (Amicon). Lanes 4 and 5, stimulation after treatment of the concentrated PC-3 CM with 30 µl of the recombinant Flt4EC coupled to Sepharose or with unsubstituted Sepharose respectively. Lane 6, Centricon 10 flow-through containing proteins of <10 kDa molecular mass.

The Flt4-stimulating activity was concentrated from PC-3 CM (Figure 2A, lanes 1–3) and used to purify the ligand by affinity chromatography on Flt4EC (lanes 4–11). The Flt4-stimulating material was eluted at pH 2.4 (lanes 8 and 9). Aliquots of the chromatographic fractions were concentrated and subjected to SDS–PAGE under reducing conditions with subsequent silver staining of the gel. As shown in Figure 2B, a major polypeptide having a molecular mass of ~23 kDa (asterisk, lane 6) and a minor one of 32 kDa were detected only in the fractions containing Flt4-stimulating activity, whereas the other polypeptide bands were major components of the starting material. N-terminal amino acid sequence analysis of the 23 kDa band gave the sequence NH_2-XEETIKFAAAHYN-TEILK-COOH.

Cloning of the Flt4 ligand from a PC-3 cDNA library

Degenerate oligonucleotides designed on the basis of the N-terminal sequence of the isolated Flt4 ligand were used as primers in PCR to amplify cDNA encoding the N-terminal peptide from a PC-3 cell cDNA library (see Figure 3A and Materials and methods for details). The product of the expected size was cloned and sequenced and new primers were designed for amplification of the entire 5′ cDNA. The resulting PCR fragment was used as a probe to screen the PC-3 cell cDNA library. The two longest clones of 2.0 and 1.8 kb contained an open reading frame (ORF) of 350 residues shown in Figure 3B, having two possible methionine codons (marked in bold) for translational initiation and a putative secretory signal peptide (underlined) followed by the N-terminal sequence of the purified Flt4 ligand (marked in bold).

Flt4 ligand is a novel member of the PDGF family, VEGF-C

Comparison with the amino acid sequences of growth factors of the VEGF/PDGF family shows that all eight cysteine residues typical for members of this family (Heldin *et al.*, 1993), as well as several other residues are conserved in Flt4 ligand (Figure 3B). Thus, the Flt4 ligand is a novel member of the VEGF family of growth factors, which we have designated VEGF-C. Homologous portions of VEGF-C are ~30% identical to $VEGF_{165}$ (Leung *et al.*, 1989), ~27% to $VEGF\text{-}B_{167}$ (Olofsson *et al.*, 1996), ~25% to PlGF-1 (Maglione *et al.*, 1991) and ~22–24% to PDGF-A and PDGF-B (Betsholtz *et al.*, 1986). However, the VEGF-C polypeptide continues with sequences rich in cysteine residues, some of which can be aligned with the C-terminus of $VEGF_{165}$ as shown in Figure 3B. Interestingly, the C-terminal cysteine residues of VEGF-C occur in repeat units typical for the Balbiani ring 3 protein (BR3P), a major cysteine-rich protein of the larval saliva of the the midge, *Chironomus tentans* (Dignam and Case, 1990; Paulsson *et al.*, 1990). Three repeats, of 24 residues each, are followed by a shorter repeat of 19 residues (Figure 3C), all conforming to the most common type of repeat in BR3P (~40% identity with amino acid sequence 1244–1371) (Paulsson *et al.*, 1990).

Recombinant VEGF-C is proteolytically processed and activates the Flt4 receptor tyrosine kinase

The predicted molecular mass of the secreted polypeptide deduced from the VEGF-C ORF, 35.881 kDa suggests that VEGF-C mRNA may be first translated into a precursor, from which the mature ligand of 23 kDa is derived by proteolytic cleavage. Indeed, a putative precursor polypeptide with an apparent molecular mass of 32 kDa was bound to the Flt4EC affinity matrix from the CM of metabolically labelled cells transfected with a VEGF-C expression vector (Figure 4A). Increased amounts of a 23 kDa receptor binding polypeptide accumulated in the culture medium during a subsequent chase period of 3 h, but not thereafter (lanes 2–4 and data not shown), suggesting that the 23 kDa form is produced by proteolytic processing, which is cell-associated and incomplete, at least in the transiently transfected cells. In non-reducing conditions, higher molecular mass forms were seen, suggesting that the VEGF-C polypeptides can form disulfide-linked dimers (arrows in Figure 4B). The CM of the transfected cells also stimulated Flt4 autophosphorylation (Figure 4C, lanes 1 and 2), but when the CM was pre-absorbed with the Flt4EC, no phosphorylation was obtained (lane 3). On the basis of these results and the above nomenclature, we have renamed Flt4 as VEGFR-3.

Stimulation of VEGFR-2 autophosphorylation by VEGF-C

CM from 293 EBNA cells transfected with the VEGF-C vector was also used to stimulate porcine aortic endothelial (PAE) cells expressing VEGFR-2 (Pajusola *et al.*, 1994; Waltenberger *et al.*, 1994). The cells were lysed and immunoprecipitated using VEGFR-2-specific antiserum (Waltenberger *et al.*, 1994).

The results of the experiment are presented in Figure 5A. A basal level of tyrosine phosphorylation of VEGFR-2 was detected in cells stimulated by CM from the mock-

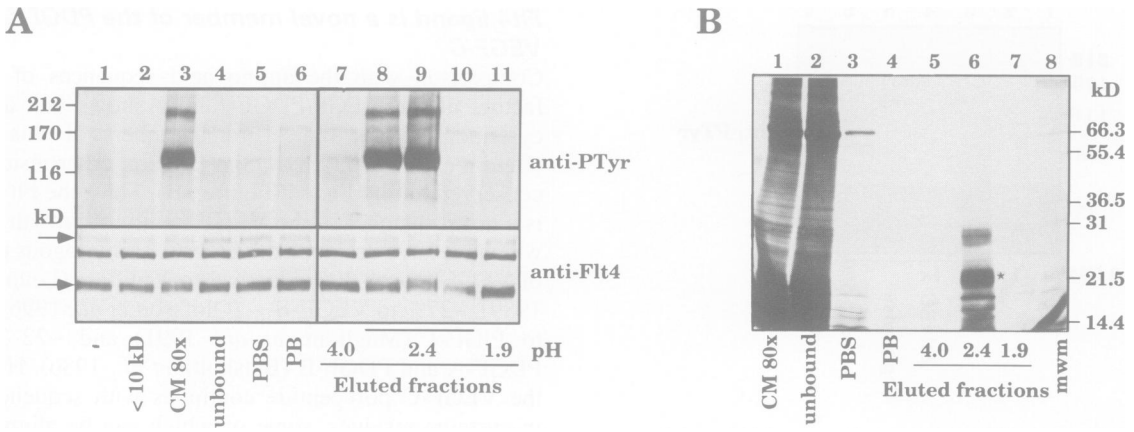

Fig. 2. Purification of the Flt4 ligand. (**A**) Flt4-expressing cells were treated with non-conditioned medium (lane 1), PC-3 cell CM or with different chromatographic fractions and Flt4 was immunoprecipitated and analysed in SDS–PAGE followed by Western blotting and detection with PTyr antibodies or Flt4-specific antiserum and the ECL method. The phosphorylated unprocessed 195 kDa and proteolytically processed 125 kDa forms of Flt4 (Pajusola *et al.*, 1994) are marked by arrows. Note that the (presumably intracellular) 175 kDa precursor of Flt4 is not phosphorylated upon stimulation. (**B**) Aliquots of the chromatographic fractions were concentrated and subjected to SDS–PAGE under reducing conditions with subsequent silver staining of the gel. The designations of the lanes are as follows: <10 kDa and CM 80×, filtrate and retained fractions, respectively, obtained after concentration of CM by ultrafiltration through a 10 kDa cut-off membrane; unbound, CM after absorption with FLT4EC; PBS and PB, washes of the affinity matrix with phosphate buffered saline and phosphate buffer pH 6.8, respectively; 4.0, 2.4 and 1.9, fractions eluted from the affinity matrix at indicated pHs; mwm, molecular mass markers.

transfected cells. A further concentration of this medium resulted in only a slight enhancement of VEGFR-2 phosphorylation (lanes 1 and 2). CM containing recombinant VEGF-C stimulated tyrosine autophosphorylation of VEGFR-2 and the intensity of the autophosphorylated polypeptide band was increased upon concentration of the VEGF-C CM (lanes 3–5). Furthermore, the stimulating effect was abolished after pretreatment of the medium with the Flt4EC affinity matrix (compare lanes 1, 5 and 6). The maximal effect of VEGF-C in this assay was comparable with the effect of recombinant VEGF added to the unconditioned medium at a concentration of 50 ng/ml (lane 8). Pretreatment of the medium containing VEGF with Flt4EC did not abolish its stimulating effect on VEGFR-2 (compare lanes 7 and 8). These results suggest that the VEGF-C expression vector encodes a ligand not only for VEGFR-3, but also for VEGFR-2.

In order to further confirm that the stimulating effect of VEGF-C on tyrosine phosphorylation of VEGFR-3 and VEGFR-2 was receptor-specific, we analysed the effect of VEGF-C on tyrosine phosphorylation of PDGF receptor β (PDGFR-β) which is abundantly expressed on fibroblastic cells. As can be seen from Figure 5B, a weak tyrosine phosphorylation of PDGFR-β was detected upon stimulation of Flt4-expressing NIH 3T3 cells with CM from the mock-transfected cells (compare lanes 1 and 2). A similar low level of PDGFR-β phosphorylation was observed when the cells were incubated with CM from the VEGF-C-transfected cells, with or without prior treatment with Flt4EC (lanes 3 and 4). In contrast, the addition of 50 ng/ml of PDGF-BB induced a prominent tyrosine autophosphorylation of PDGFR-β (lane 5).

VEGF-C stimulates endothelial cell migration in collagen gels

CM from cell cultures transfected with the VEGF-C expression vector was placed in a well which was made in collagen gel and used to stimulate the migration of bovine capillary endothelial (BCE) cells in the three-dimensional collagen gel assay described in Materials and methods. After 6 days of treatment, the cultures were stained and cells at different distances outside of the original ring of attachment were counted using fluorescent nuclear staining and detection with a fluorescence microscope equipped with a grid. A comparison of the numbers of migrating cells in successive 0.5×0.5 mm areas is shown in Figure 6A. As can be seen from the columns, VEGF-C-containing CM stimulated cell migration more than medium conditioned by the non-transfected or mock-transfected cells but less than medium from cells transfected with a VEGF expression vector. An example of typical phase contrast and fluorescent microscopic fields of cultures stimulated with medium from mock-transfected or VEGF-C-transfected cells is shown in Figure 6B. Daily addition of 1 ng of FGF2 into the wells resulted in the migration of approximately twice the number of cells when compared with the stimulation by CM from VEGF-transfected cells (data not shown).

VEGF-C is expressed in multiple tissues

Northern blotting and hybridization analysis showed that a 2.4 VEGF-C mRNA is present in the HT-1080 fibrosarcoma and PC-3 prostatic adenocarcinoma cell lines (Figure 7A). The 2.4 kb RNA and smaller amounts of a 2.0 kb mRNA were seen in multiple human tissues, most prominently in the heart, placenta, muscle, ovary and small intestine (Figure 7B). Very little VEGF-C RNA was seen in the brain, liver or thymus and peripheral blood leukocytes (pbl) appeared negative.

Discussion

Our results show that VEGFR-3 transmits signals for a novel growth factor. This conclusion is based on the specific binding of VEGF-C to recombinant Flt4EC protein and the induction of VEGFR-3 autophosphorylation by medium from VEGF-C-transfected cells. In contrast,

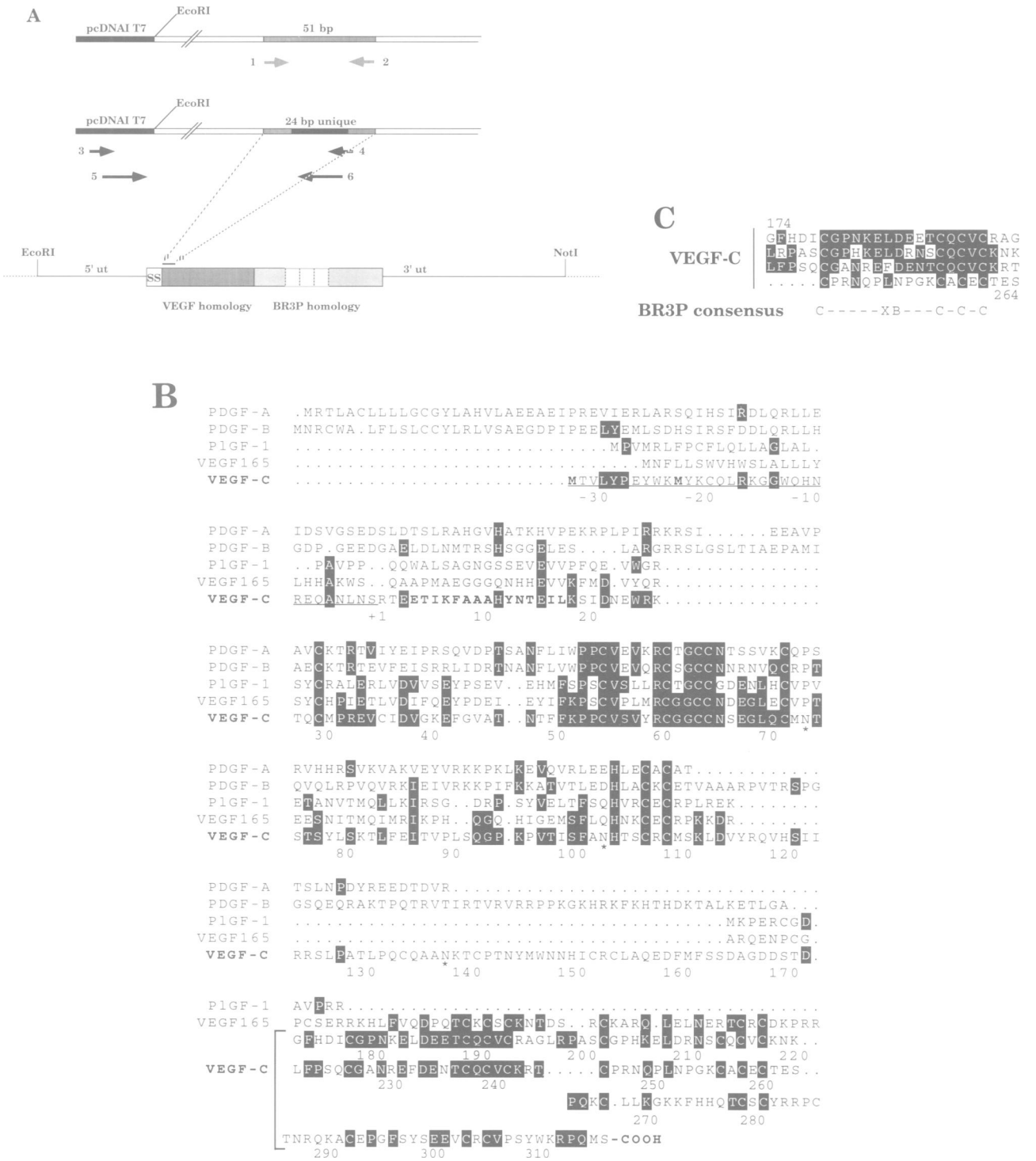

Fig. 3. Cloning and analysis of the Flt4 ligand (VEGF-C). (**A**) Schematic illustration of the PCR cloning of Flt4 ligand cDNA from PC-3 cDNA library. The primers and conditions used are detailed in Materials and methods. (**B**) Comparison of the amino acid sequence of VEGF-C with other growth factors of the VEGF/PDGF family (Betsholtz *et al.*, 1986; Leung *et al.*, 1989; Maglione *et al.*, 1991). VEGF-C amino acid residues are numbered beginning from the N-terminus after cleavage of the signal sequence. The PileUp program of Genetics Computer Group was used for alignment of the VEGF-homologous domains. The C-terminal motifs were aligned on basis of the pattern of cysteine residues. Three putative N-linked glycosylation sites (N-X-S/T) have been marked with asterisks. (**C**) Alignment of the repeated C-terminal motifs of VEGF-C with the consensus sequence of BR3P. B = D or N residue, X = non-polar or tyrosyl residue.

VEGF or PlGF did not show specific binding to VEGFR-3 or induce its autophosphorylation (Pajusola *et al.*, 1994).

Interestingly, the VEGF-C ORF is 350 amino acid residues long and our N-terminal sequence analysis confirmed that its putative signal sequence is removed before secretion. Glutamic acid was the second residue obtained in the N-terminal sequence analysis of the isolated protein, while the first residue could not be determined. According

to the deduced amino acid sequence of the VEGF-C cDNA this first residue is threonine. However, on the basis of the consensus residues surrounding signal sequence cleavage sites (von Heijne, 1986), the first residue following the signal sequence would be arginine, which may have been removed from the polypeptide after an additional proteolytic cleavage between arginine and threonine residues (see Figure 3B).

A major part of the difference in the observed molecular mass of the purified and recombinant VEGF-C and the deduced molecular mass of the VEGF-C encoded by the ORF may be due to proteolytic removal of sequences in the C-terminal region of the latter. Proteolytic processing of the VEGF-C precursor may occur at more than one cleavage site because the molecular mass of the recombinant secreted ligand, 32 kDa, was also less than the deduced molecular mass of the VEGF-C ORF without the signal peptide (see Figure 4A). By extrapolation from studies of the structure of PDGF (Heldin *et al.*, 1993), one can speculate that the region critical for receptor binding and activation by VEGF-C is contained within the first 180 or so amino acid residues of VEGF-C. Thus, the 23 kDa polypeptide binding VEGFR-3 is likely to represent the VEGF-homologous domain, which may be glycosylated at three putative N-linked glycosylation sites identified in the deduced VEGF-C amino acid sequence.

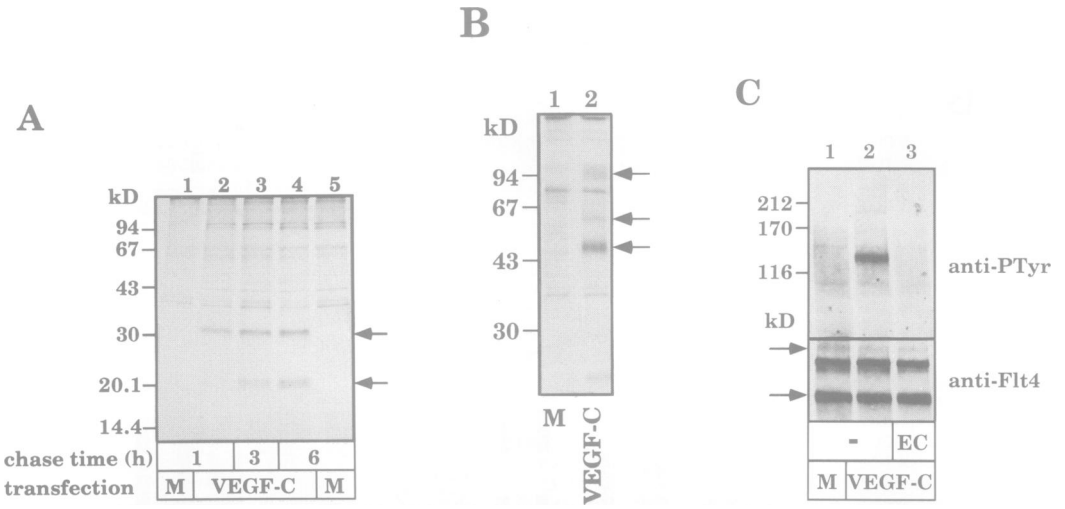

Fig. 4. Identification, dimerization and activity of recombinant VEGF-C. (**A**) Pulse–chase analysis of VEGF-C secreted by transfected cells. 293 EBNA cells were metabolically labelled using ^{35}S-labelled methionine and cysteine mixture for 2 h and then chased in non-radioactive medium for the indicated periods of time. The medium was collected and VEGF-C was bound to Flt4EC–Sepharose followed by alkylation, SDS–PAGE and autoradiography. M = mock-transfected cells. Arrows indicate the 32 kDa and 23 kDa polypeptides of secreted VEGF-C. (**B**) VEGF-C isolated using Flt4EC–Sepharose after a 4 h continuous metabolic labelling was analysed under non-reducing conditions. The arrows indicate putative dimeric forms. (**C**) Stimulation of VEGFR-3 autophosphorylation by VEGF-C. NIH 3T3 cells expressing VEGFR-3 were stimulated with medium conditioned by cells transfected with VEGF-C cDNA. The medium was either untreated (lane 2) or treated with Flt4 EC (lane 3). Arrows indicate the phosphorylated forms of Flt4 (see the legend of Figure 2A).

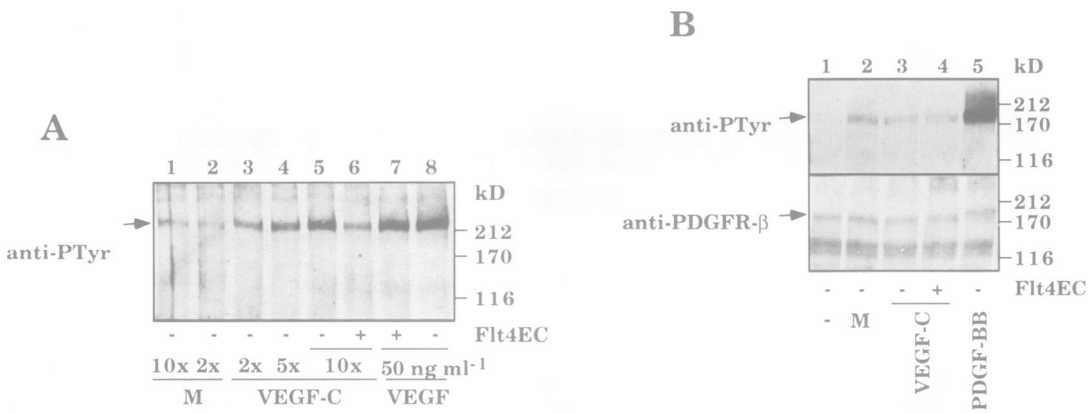

Fig. 5. VEGF-C stimulates autophosphorylation of VEGFR-2 (KDR) but has no effect on PDGFR-β phosphorylation. (**A**) PAE cells expressing VEGFR-2 were stimulated with 10- or 2-fold concentrated medium from mock-transfected 293 EBNA cells (lanes 1 and 2), or with 2-, 5- or 10-fold concentrated medium from 293-EBNA cell cultures expressing the recombinant VEGF-C (lanes 3–6). VEGFR-2 was immunoprecipitated with specific antibodies and analysed by SDS–PAGE and Western blotting using phosphotyrosine antibodies. For comparison, the stimulations were carried out with non-conditioned medium containing 50 ng/ml of purified recombinant VEGF (lanes 7 and 8). Lanes 6 and 7 show stimulation with VEGF-C or VEGF containing media pretreated with Flt4EC. (**B**) Flt4-expressing NIH 3T3 cells were stimulated with non-conditioned medium (lane 1), 5-fold concentrated CM from mock-transfected (lane 2) or VEGF-C-transfected (lanes 3 and 4) cells, or with non-conditioned medium containing 50 ng/ml of recombinant human PDGF-BB (lane 5). Medium containing VEGF-C was also pretreated with recombinant Flt4EC (lane 4). PDGFR-β was immunoprecipitated with specific antibodies and analysed by SDS–PAGE and Western blotting using phosphotyrosine antibodies with subsequent stripping and reprobing of the membrane with antibodies specific for PDGFR-β.

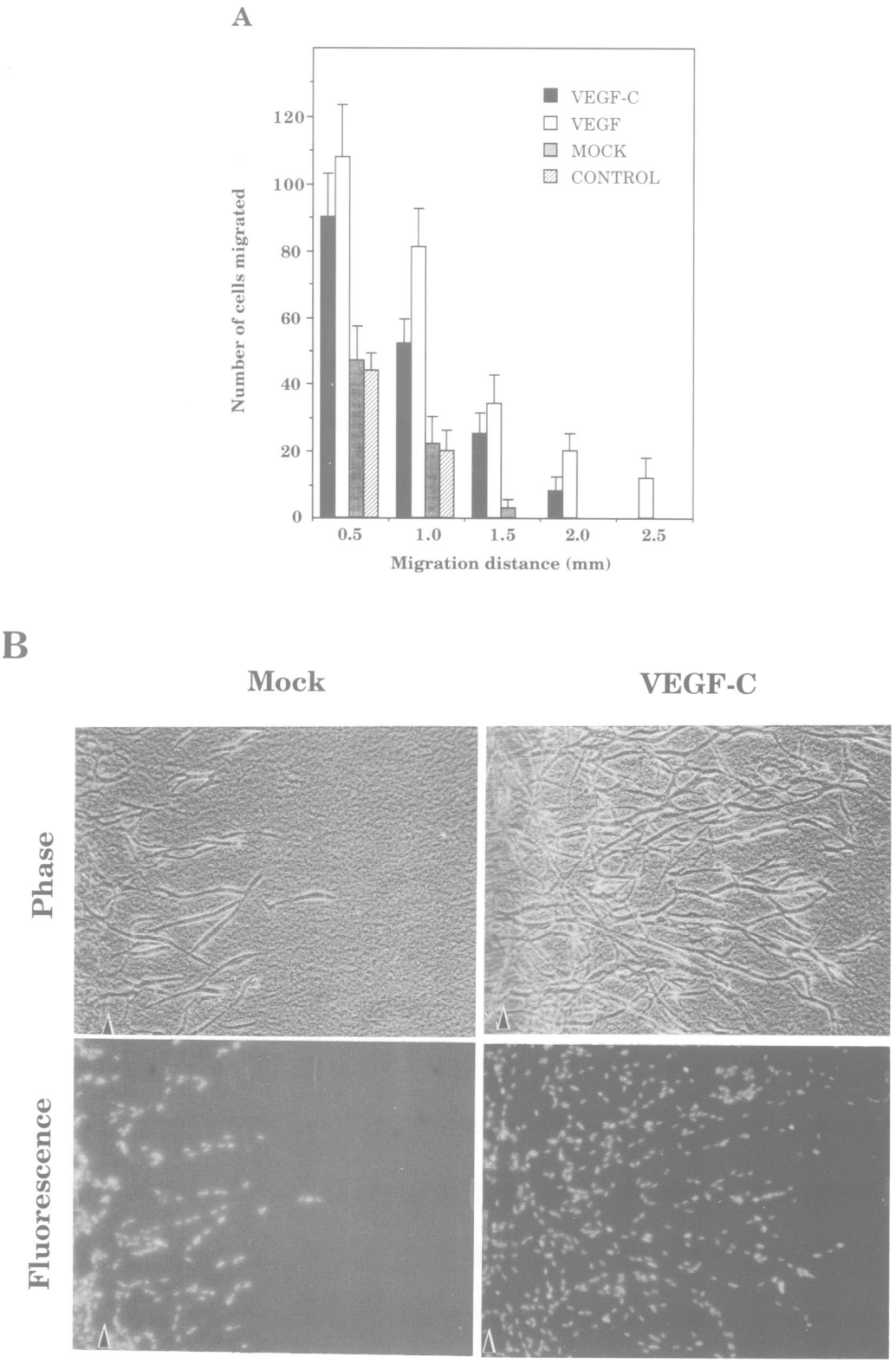

Fig. 6. VEGF-C stimulates endothelial cell migration in a three-dimensional collagen gel assay. (**A**) The diagram shows a comparison of the number of cells migrating at different distances from the original area of attachment towards wells containing media conditioned by the non-transfected (control) or transfected (mock; VEGF-C; VEGF) cells, 6 days after addition of the media. The number of cells migrating out from the original ring of attachment was counted in five adjacent 0.5×0.5 mm squares using a microscope ocular lens grid and 10× magnification. Cells migrating further than 0.5 mm were counted in a similar way by moving the grid in 0.5 mm steps. The experiments were carried out twice in duplicate with similar results, and medium values from the one of the experiments are presented with the standard error bars. (**B**) Phase-contrast microscopy and fluorescent microscopy of the nuclear staining of BCE cells migrating towards the wells containing media conditioned by the mock-transfected cells or by VEGF-C-transfected cells. The areas shown are ~1×1.5 mm and arrows indicate the borders of the original ring of attachment.

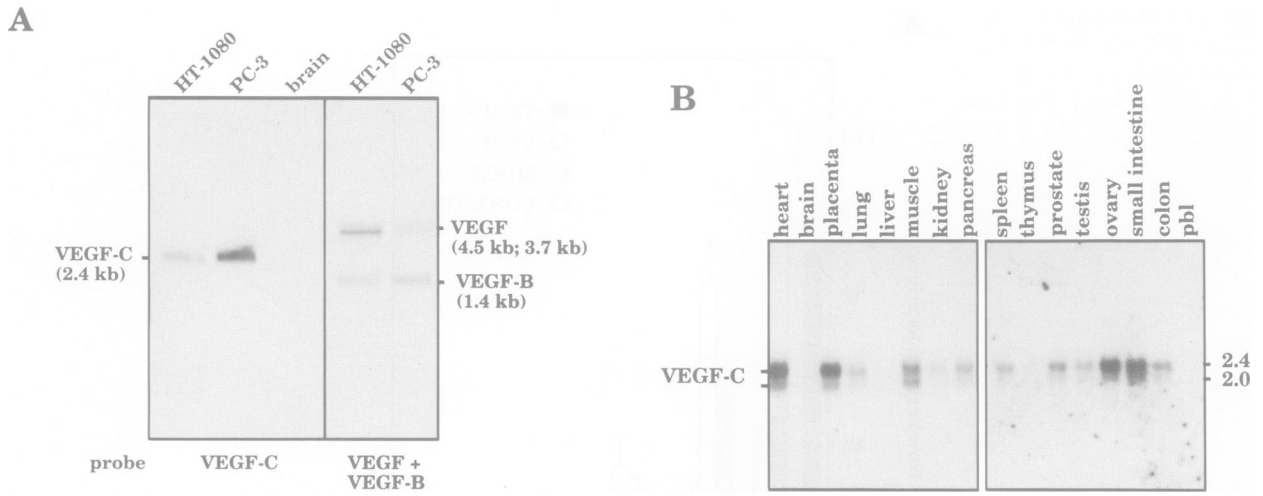

Fig. 7. Expression of VEGF-C mRNA in tumour cell lines and in human adult tissues. Northern blots containing 8 μg of isolated poly(A)$^+$ RNA from HT-1080 and PC-3 human tumour cells (**A**) and multiple human tissues (**B**, blot from Clontech) were probed with radioactively labelled insert of the 2.0 kb VEGF-C cDNA clone. Shown in (A) are also the 4.5 kb and 3.7 kb mRNA signals for VEGF and the 1.4 kb signal for VEGF-B (Olofsson *et al.*, 1996) in the same samples. Note that the tumour cell lines contain mainly mRNA of the 2.4 kb form.

The C-terminal amino acid sequences, which increase the length of the VEGF-C polypeptide in comparison with other ligands of this family show a pattern of spacing of cysteine residues reminiscent of the BR3P sequence (Dignam and Case, 1990; Paulsson *et al.*, 1990). This novel C-terminal silk-protein-like structural motif of VEGF-C may fold into an independent domain, which, on the basis of the considerations above, is at least partially cleaved off after biosynthesis. Interestingly, at least one cysteine motif of the BR3P type is also found in the C-terminus of VEGF. In our experiments both the putative precursor and cleaved ligand were detected in the cell culture medium, although processing was apparently cell-associated on the basis of the pulse–chase experiments. The determination of the N-terminal sequence of the isolated C-terminal fragment will allow the identification of the proteolytic processing site. On the other hand, the generation of antibodies against different parts of the VEGF-C molecule will allow the exact determination of the precursor–product relationship and ratio, their cellular distribution and the kinetics of processing and secretion.

We have recently cloned another factor structurally homologous to VEGF, designated accordingly as VEGF-B (Olofsson *et al.*, 1996). Both of these factors share a conserved pattern of eight cysteine residues, which may participate in the formation of intra- and interchain disulfide bonds creating an antiparallel dimeric biologically active molecule, similar to PDGF (Andersson *et al.*, 1992; Oefner *et al.*, 1992). Mutational analysis of the cysteine residues involved in the interchain disulfide bridges have shown that, in contrast to PDGF, VEGF dimers need to be held together by these covalent interactions in order to maintain biological activity (Pötgens *et al.*, 1994). Putative dimers were evident in the analysis of VEGF-C under non-reducing conditions. It will be interesting to analyse these possible dimerization patterns of VEGF-C, but this is made technically difficult by the presence of precursor and processed forms and the high cysteine content of VEGF-C, which causes anomalous migration in gel electrophoresis under non-reducing conditions.

VEGFR-3, which thus distinguishes between VEGF and VEGF-C, is closely related in structure to VEGFR-1 and VEGFR-2 (Pajusola *et al.*, 1992; Finnerty *et al.*, 1993; Galland *et al.*, 1993). However, the mature form of VEGFR-3 differs from the two other VEGFRs in that it is proteolytically cleaved in the extracellular domain into two disulfide-linked polypeptides (Pajusola *et al.*, 1994). Another difference is that the 4.5 and 5.8 kb VEGFR-3 mRNAs encode polypeptides differing in their C-termini and apparently in their signalling properties due to the use of alternative 3′ exons (Pajusola *et al.*, 1993; Borg *et al.*, 1995).

Besides VEGFR-3, VEGFR-2 tyrosine kinase also was shown to be activated in response to VEGF-C. VEGFR-2-mediated signals cause striking changes in the morphology, actin reorganization and membrane ruffling of PAE cells overexpressing this receptor. In these cells, VEGFR-2 also mediated ligand-induced chemotaxis and mitogenicity (Waltenberger *et al.*, 1994). Similarly, the receptor chimera CSF-1R/VEGFR-3 was mitogenic when ectopically expressed in NIH 3T3 fibroblastic cells, but not in PAE cells (Pajusola *et al.*, 1994). Consistent with such results, the bovine capillary endothelial cells, which express VEGFR-2 mRNA but very little or no VEGFR-1 or VEGFR-3 mRNAs (our unpublished data), showed enhanced migration when stimulated with VEGF-C. Light microscopy of the BCE cell cultures in collagen gel also suggested that VEGF-C stimulated the proliferation of these cells, but such data must be confirmed by more detailed analyses of cell proliferation and survival in the presence and absence of specific factors. The existing data thus indicate that the VEGF ligands and receptors show a great specificity in their signalling, which may be cell type dependent.

The expression pattern of the VEGFR-3 (Kaipainen *et al.*, 1995) suggests that VEGF-C may function in the

formation of the venous and lymphatic vascular systems during embryogenesis. Constitutive expression of VEGF-C in adult tissues further suggests that this gene product is also involved in the maintenance of the differentiated functions of the lymphatic endothelium where VEGFR-3 is expressed (Kaipainen *et al.*, 1995). Lymphatic capillaries do not have well formed basal laminae and an interesting possibility remains that the silk-like BR3P motif is involved in producing a supramolecular structure which could regulate the availability of VEGF-C in tissues. However, VEGF-C also activates VEGFR-2, which is abundant in proliferating endothelial cells of vascular sprouts and branching vessels of embryonic tissues, but decreased in adult tissues (Millauer *et al.*, 1993). These data have suggested that VEGFR-2 is a major regulator of vasculogenesis and angiogenesis. VEGF-C may thus have a unique effect in lymphatic endothelium and a more redundant function shared with VEGF in angiogenesis and possibly permeability regulation of several types of endothelia.

Taken together these results show an increased complexity of signalling in the vascular endothelium. They reinforce the concept that when organs differentiate and begin to perform their specific functions, the phenotypic heterogeneity of endothelial cells increases in several types of functionally and morphologically distinct vessels. However, upon suitable angiogenic stimuli, endothelial cells can re-enter the cell cycle, migrate, withdraw from the cell cycle and subsequently differentiate again to form new vessels which are functionally adapted to their tissue environment. This process of angiogenesis, concurrent with tissue development and regeneration, depends on the tightly controlled balance between positive and negative signals for endothelial cell proliferation, migration, differentiation and survival. Previously identified growth factors promoting angiogenesis include the fibroblast growth factors, hepatocyte growth factor/scatter factor, PDGF and TGF-α (for references, see Folkman, 1995; Friesel and Maciag, 1995; Mustonen and Alitalo, 1995). However, VEGF, which was identified ~10 years ago (Senger *et al.*, 1983), has been the only growth factor relatively specific for endothelial cells. Thus the newly identified factors VEGF-B (Olofsson *et al.*, 1996) and VEGF-C (the present data) increase our understanding of the complexity of the specific and redundant positive signals for endothelial cells involved in vasculogenesis, angiogenesis, permeability and perhaps other endothelial functions.

Materials and methods

Cell culture

Human prostatic adenocarcinoma PC-3 cells (American Type Culture Collection CRL 1435) were cultured in Ham's F12 medium supplemented with 7% fetal calf serum (FCS); 293 EBNA cells (Invitrogen) and NIH 3T3-Flt4 cells (Pajusola *et al.*, 1993) in DMEM–10% FCS; PAE-KDR cells (Waltenberger *et al.*, 1994) in Ham's F12 medium–10% FCS. BCE cells (Folkman *et al.*, 1979) were cultured as described in Pertovaara *et al.* (1994). After reaching confluence the monolayers of PC-3 cells were cultured for 5 days in Ham's F12 medium without FCS. CM was then collected, clarified by centrifugation at 10 000 *g* and used for purification of VEGF-C.

Analysis of stimulation of the receptors

Confluent NIH 3T3-Flt4 cells or PAE-KDR cells were starved overnight in DMEM or Ham's F12 media, respectively, supplemented with 0.2% BSA and then incubated for 5 min with the analysed media. Recombinant human VEGF (R&D Systems) and PDGF-BB were used as control stimulating agents. The cells were washed twice with ice-cold Tris-buffered saline (TBS) containing 100 μM sodium orthovanadate and lysed in RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 U/ml aprotinin and 1 mM sodium orthovanadate. The lysates were sonicated, clarified by centrifugation at 16 000 *g* for 20 min and incubated for 3–6 h on ice with 3–5 μl of antisera specific for Flt4 (Pajusola *et al.*, 1993), KDR or PDGFR-β (Claesson-Welsh *et al.*, 1989; Waltenberger *et al.*, 1994). Recombinant human PDGF-BB as well as antisera specific for KDR and PDGFR-β were kindly provided by Dr Lena Claesson-Welsh. Immunoprecipitates were bound to protein A–Sepharose, washed three times with TBS containing 1 mM PMSF and 1 mM sodium orthovanadate, twice with 10 mM Tris–HCl pH 7.4 and subjected to SDS–PAGE in a 7% gel (Laemmli, 1970). Polypeptides were transferred to nitrocellulose by Western blotting and analysed using PY20 phosphotyrosine-specific monoclonal antibodies (Transduction Laboratories) or receptor-specific antiserum and the ECL method (Amersham).

Production and purification of baculoviral Flt4EC protein

The segment of Flt4 cDNA (GenBank Accession Number X68203) encoding EC was amplified in PCR using primers which encoded six additional C-terminal His residues followed by a stop codon, and added *Bam*HI sites at both ends. The amplified fragment was then cloned into the *Bam*HI site in the pVTBac plasmid (Tessier *et al.*, 1991), which was used to generate a Flt4EC baculovirus. The Flt4EC protein was purified from the culture medium of baculovirus-infected High-Five cells (Invitrogen) by Ni-NTA affinity chromatography (Qiagen) and coupled to CNBr-activated Sepharose 4B (Pharmacia; 5 mg of Flt4 EC/ml Sepharose resin).

Isolation and N-terminal sequence analysis of VEGF-C

Eight litres of PC-3 CM was concentrated 80-fold using a 10 kDa cut-off ultrafiltration membrane (Filtron Technology Corporation) and incubated with the recombinant Flt4EC–Sepharose affinity matrix. The affinity matrix was washed successively with PBS and 10 mM PB (pH 6.8) and the bound material was eluted step-wise with 100 mM glycine–HCl, successive eluates having pHs of 4.0, 2.4 and 1.9. Eluates were collected in tubes containing 1/4 volume of 1 M Na-phosphate pH 8.0, dialysed against 1 mM Tris–HCl pH 7.5 and the aliquots were analysed for their ability to stimulate tyrosine phosphorylation of VEGFR-3.

Two fractions eluted from the affinity matrix at pH 2.4 were combined, vacuum dried and subjected to SDS–PAGE in a 12.5% gel. The proteins from the gel were then electroblotted to Immibilon-P transfer membrane (Millipore) and visualized by staining of the blot with Coomassie blue R-250. The region containing only the stained 23 kDa band was cut from the blot and subjected to N-terminal amino acid sequence analysis in a Prosite Protein Sequencing System (Applied Biosystems).

Cloning of VEGF-C from a PC-3 cell expression library

Six micrograms of poly(A)$^+$ RNA derived from PC-3 cells was used to prepare an oligo(dT)-primed cDNA library using the Librarian kit of Invitrogen. The first PCR was carried out using 1 μg of DNA from the library and the primers marked in the Figure 3A: 1. 5′-GCAGARG-ARACNATHAA-3′ (wherein R is A or G, N is A, G, C or T and H is A, C or T) and 2. 5′-GCAYTTNARDATYTCNGT-3′ (wherein Y is C or T and D is A, G or T). Two successive PCRs were carried out using 1 U per reaction of DynaZyme (Finnzymes), at an extension temperature of 72°C for 43 cycles, the first three cycles at annealing temperature 33°C for 2 min and the remaining ones at 42°C for 1 min. A band of the expected size (57 bp) was re-amplified for 30 cycles in the latter conditions, cloned into a pCRII vector (Invitrogen) and sequenced. All six clones analysed contained the sequence encoding the expected N-terminal peptide (although they were later found also to have mismatches with the final sequence of the cloned cDNA). Based on the unique nucleotide sequence obtained two pairs of nested primers were designed to amplify the complete 5′-end of the cDNA. The primers were 3. 5′-TAATACGACTCACTATAGGG-3′ and 4. 5′-TCNGTGTTGTAGTGTG-CTG-3′, the former corresponding to the pcDNAI vector used for construction of the library. 'Touchdown' PCR was used (Don *et al.*, 1991). The annealing temperature of the two first cycles was 62°C and subsequently 1°C less in steps of two cycles until a final temperature of 53°C was reached, at which temperature 16 additional cycles were carried out. Annealing time was 1 min and extension at 72°C for 1 min. The products of the first amplification (1 μl of a 1:100 dilution in water) were used in the second amplification reaction employing the nested

primers 5. 5′-TCACTATAGGGAGACCCAAGC-3′ and 6. 5′-GTTGTAGTGTGCTGCAGCGAATTT-3′. The annealing temperature was decreased in Touchdown PCR from 72°C to 66°C and continued with 18 additional cycles at 66°C. The annealing time was 1 min and extension at 72°C for 2 min. A product of ~220 bp was cloned into the pCR II vector, sequenced and found to contain the 5′-end of the VEGFR-3 ligand cDNA. This fragment was digested with *Eco*RI, and the resulting 153 bp fragment was labelled with [^{32}P]dCTP and used as a probe for hybridization screening of the amplified PC-3 cell cDNA library.

Expression and detection of recombinant VEGF-C

The 2.0 kb insert of the VEGF-C clone in pcDNAI vector was cut out from the vector using *Hin*dIII and *Not*I restriction enzymes and ligated into the corresponding sites in the pREP7 expression vector (Invitrogen). The resulting plasmid was transfected into 293 EBNA cells using a calcium phosphate precipitation method. An equivalent amount of the pREP7 plasmid without insert was used in mock transfections. The culture medium was changed to DMEM–0.2% BSA 48–72 h after transfection and after an additional 24 h this medium was collected, clarified by centrifugation and used for studies of the effects of VEGF-C. In some cases CM was concentrated using Centriprep-10 devices (Amicon).

Metabolic labelling of 293 EBNA cells transfected with the VEGF-C construct was carried out by addition of 100 μCi/ml of Pro-mix^{TM} L-[^{35}S] *in vitro* cell labelling mix (Amersham) to the culture medium devoid of cysteine and methionine. After 2 h the cell layers were washed twice with PBS and the medium was then replaced with DMEM–0.2% BSA. After 1, 3, 6, 12 and 24 h of subsequent incubation the culture medium was collected, clarified by centrifugation, concentrated and VEGF-C was bound to 30 μl of a slur of Flt4EC–Sepharose overnight at 4°C, followed by three washes in PBS, two washes in 20 mM Tris–HCl pH 7.5, alkylation, SDS–PAGE and autoradiography.

Endothelial cell migration in three-dimensional collagen gel

The collagen gels were prepared by mixing type I collagen stock solution (5 mg/ml in 1 mM HCl) with an equal volume of 2× MEM and two volumes of MEM containing 10% newborn calf serum (NCS) to give a final collagen concentration of 1.25 mg/ml. The tissue culture plates (5 cm diameter) were coated with ~1 mm thick layer of the solution, which was allowed to polymerize at 37°C. BCE cells were seeded on top of this layer. For the migration assays, the cells were allowed to attach inside a plastic ring (1 cm diameter) placed on top of the first collagen layer. After 30 min the ring was removed and unattached cells were rinsed away. A second layer of collagen and a layer of growth medium (5% NCS) solidified by 0.75% low melting point agar (FMC BioProducts, Rockland, ME) were added. A well (3 mm diameter) was punched through all the layers on both sides of the cell spot at a distance of 4 mm and the media were daily pipetted into the wells. Photomicrographs of the cells migrating out from the spot edge were taken after 6 days through an Olympus CK 2 inverted microscope equipped with phase-contrast optics. The migrating cells were counted after nuclear staining with the fluorescent dye bisbenzimide (1 μg/ml, Hoechst 33258, Sigma).

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