A Recombinant Mutant Vascular Endothelial Growth Factor-C that Has Lost Vascular Endothelial Growth Factor Receptor-2 Binding, Activation, and Vascular Permeability Activities*

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The vascular endothelial growth factor (VEGF) and the VEGF-C promote growth of blood vessels and lymphatic vessels, respectively. VEGF activates the endothelial VEGF receptors (VEGFR) 1 and 2, and VEGF-C activates VEGFR-3 and VEGFR-2. Both VEGF and VEGF-C are also potent vascular permeability factors. Here we have analyzed the receptor binding and activating properties of several cysteine mutants of VEGF-C including those (Cys156 and Cys165), which in other platelet-derived growth factor/VEGF family members mediate interchain disulfide bonding. Surprisingly, we found that the recombinant mature VEGF-C in which Cys156 was replaced by a Ser residue is a selective agonist of VEGFR-3. This mutant, designated ΔNAC156S, binds and activates VEGFR-3 but neither binds VEGFR-2 nor activates its autophosphorylation or downstream signaling to the ERK/MAPK pathway. Unlike VEGF-C, ΔNAC156S neither induces vascular permeability in vivo nor stimulates migration of bovine capillary endothelial cells in culture. These data point out the critical role of VEGFR-2-mediated signal transduction for the vascular permeability activity of VEGF-C and strongly suggest that the redundant biological effects of VEGF and VEGF-C depend on binding and activation of VEGFR-2. The ΔNAC156S mutant may provide a valuable tool for the analysis of VEGF-C effects mediated selectively via VEGFR-3. The ability of ΔNAC156S to form homodimers also emphasizes differences in the structural requirements for VEGF and VEGF-C dimerization.

The PDGF/VEGF family of growth factors currently includes seven members: PDGF-A, PDGF-B (1, 2), VEGF (3, 4), placenta growth factor (PlGF) (5), VEGF-B/VEGF-related factor (6, 7), VEGF-C/VEGF-related protein (8, 9), and c-fos-induced growth factor/VEGF-D (10). All members of the family share a common structure in that they contain eight characteristically spaced cysteine residues in the core domain. PDGF-A and PDGF-B promote the growth of several cell types, whereas VEGF, PlGF, and VEGF-C regulate almost exclusively endothelial cells, which express the corresponding receptors. VEGF binds VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), whereas PlGF and VEGF-B bind only VEGFR-1 (3, 12, 13).

VEGF-C and VEGF-D are ligands and activators of VEGFR-3 (8, 9, 11). Mature VEGF-C and VEGF-D, which are generated by proteolytic processing of precursor polypeptides, also activate VEGFR-2. VEGF and VEGF-C are distinct in their specificity toward endothelial cells. VEGF specifically stimulates proliferation of the endothelial cells of blood vessels (14), whereas VEGF-C preferentially promotes growth of lymphatic endothelium (15, 16). On the other hand, there are certain similarities in the biological activities of VEGF and the mature form of VEGF-C in that both factors are potent inducers of vascular permeability (8, 17, 18). In addition, at higher concentrations VEGF-C, similarly to VEGF, also stimulates proliferation and migration of vascular endothelial cells in culture (8, 18). These data addressed a question of whether certain redundancy in VEGF and VEGF-C activities might be mediated via VEGFR-2, which is used by both of these two growth factors.

The previously known members of the PDGF/VEGF family form homodimers via disulfide bonds between the second and fourth of the eight conserved cysteine residues. These bonds are crucial for the dimerization and biological activity of VEGF but not for the activity of PDGF-BB (19–21). Unlike these factors, the recombinant mature VEGF-C forms mostly noncovalent homodimers. It also contains an unpaired extra cysteine residue (Cys137) located between the first and second conserved cysteine residues (18). Another unpaired Cys residue (Cys87) is located in the N-terminal VEGF-C propeptide, which is cleaved off during the proteolytic processing of VEGF-C precursor. To evaluate the significance of disulfide bonds for VEGF-C homodimerization and activity, we converted selected cysteine residues to serine residues either in the VEGF-C precursor or in the recombinant “processed” VEGF-C (ΔNAC, described in Joukov et al. (18)) and analyzed these mutants for their abilities to bind and to activate VEGFR-3 and VEGFR-2. This led to the identification of a ligand for VEGFR-3 that is devoid of VEGF-C stimulating properties and does not possess VEGF-like activities in vitro or in vivo.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfections, and Antibodies—293 EBNA cells were cultured in DMEM/10% FCS, porcine aortic endothelial (PAE/VEGFR-2 (22), and PAE/VEGFR-3 (23) cells in Ham’s F-12 medium/

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¶ The abbreviations used are: PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; DMEM, Dulbecco’s modified Eagle’s medium; wt, wild type.

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A VEGFR-3-specific VEGF-C Mutant

RESULTS

Binding of VEGF-C Mutants to VEGFR-3 and VEGFR-2—Taking into account the importance of the second and the fourth characteristic cysteine residues for VEGF activity, we replaced these residues either separately or in combination with Ser residues in the VEGF-C precursor and in the recombinant mature VEGF-C (ΔNAC). The mutants were expressed in 293 EBNA cells, and the conditioned media were studied for their ability to compete with \[^{125}\text{I}]\text{ΔNAC} for VEGF-C 3 and VEGFR-2 binding. Replacement of Cys165 alone or together with Cys156 in ΔNAC or in wt VEGF-C abolished the ability of the proteins to bind VEGF-3 or VEGFR-2 (Fig. 1 and data not shown). On the other hand, the C83S,C137S mutant displaced \[^{125}\text{I}]\text{ΔNAC} from VEGFR-3 and ΔNAC156S from VEGFR-2 by ΔNAC and ΔNAC156S. A, stimulation of tyrosine phosphorylation of VEGF-3 and VEGFR-2 by ΔNAC and ΔNAC156S. PAE/VEGFR-3 (upper panel) and PAE/VEGFR-2 (middle panel) cells were treated with 100 ng/ml of the indicated purified factor or 10% FCS added to the DMEM. Cells were lysed after 5 min, and ERK1 and ERK2 phosphorylation was analyzed by Western blotting using phospho-specific p44/42 MAPK antibodies. Blots were then stripped and reprobed with p44/42 MAPK antibodies to detect total protein. p44/42 and phospho-specific p44/42 antibodies were purchased from New England BioLabs and used according to the manufacturer’s instructions.

Analysis of VEGF-C Biological Activity—To eliminate the effect of trace amounts of co-purified VEGF, the conditioned media, purified ΔNAC, ΔNAC156S, and the material similarly purified from the mock transfected cells were pretreated for 1 h at room temperature with anti-human VEGF neutralizing antibody (R & D Systems) and in nonreducing conditions with subsequent Western blotting and detection using the antisera 882. Chemical cross-linking of the \[^{35}\text{S}]\text{metabolically labeled VEGF-C mutants with disuccinimidyl suberate (Pierce) was carried out as described (18).
VEGFR-3 or VEGFR-2, whereas C83S,C137S was nearly as active as wt VEGF-C in this assay (data not shown). Surprisingly, the VEGFR-3 phosphorylation of VEGFR-3 and VEGFR-2 were compared. In conditioned media, and their abilities to stimulate the tyrosine phosphorylation (Fig. 2A). The migration toward the material purified from the 293 EBNA cells was left untreated or pretreated with anti-human VEGF neutralizing antibody and injected intradermally to the back skin of guinea pigs. B, migration of BCE cells in collagen gel. The diagram shows the number of cells migrating to four different distances (in µm with 0.6-µm steps) starting from the left (marked by vertical ticks). The migration toward the material purified from the 29NAC, 29NAC156S, and the mock-transfected cells is shown by the open, filled, and hatched bars, respectively.

29NAC156S,C165S also failed to induce tyrosine phosphorylation of VEGFR-3 or VEGFR-2, whereas C83S,C137S was nearly as active as wt VEGF-C in this assay (data not shown). Surprisingly, the 29NAC156S growth factor mutant efficiently bound VEGFR-3 but that it lacks activity toward VEGFR-3 (Fig. 2A). As can be seen from Fig. 3B, 29NAC dose-dependently stimulated the migration of BCE cells in collagen gel, whereas 29NAC156S had no significant activity in this assay. Taken together, these data indicate that the inability of 29NAC156S to activate VEGFR-2 correlates with the lack of vascular permeability and endothelial cell migration inducing activities.

29NAC156S Forms Partially Disulfide-bonded Homodimers—Replacement of the second and/or the fourth cysteine residues of VEGF abolishes its dimer formation and biological activity (21). We investigated the dimeric nature of the VEGF-C mutants. No homodimers were obtained when 29NAC156S,C165S or 29NAC156S
were chemically cross-linked (Fig. 4A, lanes 1–4). On the other hand, about half of both cross-linked ΔNΔC (18) and ΔNΔC156S (lane 6) migrated as dimers. This indicates that ΔNΔC156S forms homodimers. Moreover, unlike ΔNΔC, which forms preferentially noncovalently bound dimers, a fraction of ΔNΔC156S was disulfide bonded, as detected by SDS-PAGE in nonreducing conditions (Fig. 4B). These data suggest that homodimerization is required for VEGF-3 activation by VEGF-C and indicate that the inability of ΔNΔC156S to activate VEGFR-2 and to induce VEGF-like effects is not due to an inability of this mutant to form homodimers.

**DISCUSSION**

Here we describe a VEGF-C point mutant that is active toward VEGFR-3 but, unlike wt VEGF-C, is unable to bind to and to activate signaling through VEGFR-2. This mutant (ΔNΔC156S) was generated by replacement of the second conserved Cys residue of the recombinant processed VEGF-C by a Ser residue. ΔNΔC156S was inactive in the vascular permeability assay and did not increase migration of the capillary endothelial cells, indicating that these VEGF-like effects of VEGF-C require VEGF-2 binding. Interaction with VEGFR-2 has been shown to be a critical requirement for the full spectrum of biological responses induced by VEGF (22, 27, 28). Taking into account that VEGF-2 is the only known receptor shared by VEGF and VEGF-C, one can speculate that the ability of VEGF-C to increase vascular permeability and the ability to stimulate the migration of capillary endothelial cells are mediated via VEGFR-2 and that the activation of VEGF-2 is sufficient to induce these biological effects. Moreover, downstream signaling from VEGFR-2 requires activation of the MAPK pathway through at least ERK1 and ERK2 (29). However, the possibility remains that there are additional, as yet unknown receptors for VEGF and VEGF-C, which could mediate the vascular permeability effect of VEGF-C instead of or in addition to VEGFR-2.

Interestingly, the structural requirements for binding and activation of VEGF-2 by VEGF and VEGF-C are different. Despite the prominent similarity between the mature VEGF-C and VEGF 

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