

Dissection of the effects induced by VEGF₁₆₅, VEGF₁₂₁ and Semaphorin 3A in endothelial cells

ABSTRACT

The development of new blood vessels is a complex and highly regulated process that requires the coordinated action of different growth factors. Vascular Endothelial Growth Factor (VEGF) is a powerful inducer of angiogenesis, acting through the metabolic activation, proliferation and migration of endothelial cells.

The major angiogenic member of the VEGF family is VEGF-A, a gene which is transcribed to give rise to at least 4 major splicing variants, of 206, 189, 165 and 121 amino acids in humans. The angiogenic effect of the most abundant isoform (VEGF₁₆₅) is basically mediated by its interaction with VEGFR2 (KDR) and VEGFR1 (Flt-1) as well as with the co-receptor Neuropilin-1 (NP-1). The shortest isoform (VEGF₁₂₁) binds VEGFR1 and VEGFR2 only, but not NP-1. NP-1, together with PlexinA1, also acts as a receptor of Semaphorin 3A (Sema3A), a factor also involved in vascular patterning, besides its very well known role in axonal guidance.

The aim of this project has been the detailed analysis of the peculiarity/specificity of the effect of VEGF₁₆₅, VEGF₁₂₁ and Sema3A in endothelial cells.

We first exploited an Adeno Associated Virus (AAV)-based gene delivery to unravel the *in vivo* effect of these cytokines. In particular, we observed that the prolonged expression of the main isoform of VEGF, VEGF₁₆₅, acted as a powerful inducer of angiogenesis, stimulating the development of both a larger capillary network and the formation of an impressive new set of arterioles. Most surprisingly, an unexpected effect of VEGF₁₆₅ was also the recruitment to the sites of its expression, of a vast set of mononuclear cells. These cells derived from the bone marrow and expressed a broad set of monocytic markers (CD45+, CD11b+); their presence correlated with the formation of arterioles and the maturation of the VEGF-induced vasculature. Strikingly, VEGF₁₂₁ (the shorter form unable to bind NP-1) was, on the contrary, unable to induce maturation of the newly formed capillaries to mature arteries and to recruit cells, an observation that suggested that these monocytes might be essential in blood vessel maturation. We found that cell recruitment both *in vitro* and *in vivo* strictly depends on NP-1 and that Sema3A, a NP-1 activator, acts as a powerful recruiter of these cells. The expression of VEGF and Sema3A receptors by CD11b+ cells together with the VEGFR2 interaction with NP-1, indicate that these cells could be target of a peculiar VEGF and/or Sema3A induced signalling pathway.

Taken together, these results prompted us to develop an *in vitro* model to unravel the signalling features elicited by the VEGF₁₆₅, VEGF₁₂₁ and Sema3A on endothelial cells. In particular, we wanted to dissect these pathways through a proteomic approach involving the detection of the phosphoproteome of endothelial cells expressing specific receptor after treatment with the various ligands of interest.

Due to the large amount of growth factors required for this kind of experimentation, we first established a Baculovirus-based system to generate the recombinant factors. For this purpose, we engineered suitable plasmids encoding VEGF₁₆₅, VEGF₁₂₁ and Sema3A endowed of a cleavable histidine tag at the N-terminus. Once the recombinant protein expression conditions were set up, we moved to optimize protein purification by affinity chromatography, together with the removal of the tag. The functionality of baculovirus-expressed VEGF₁₆₅ and VEGF₁₂₁ was ascertained by the analysis of VEGF receptor phosphorylation, as well as proliferation assays on endothelial cells; a cell contraction assay confirmed that the recombinant Sema3A produced was as active as protein expressed in mammalian cells.

To dissect the differential signalling induced by each of the recombinant factors, we explored their transduction pathways in endothelial cell lines expressing the main receptors (KDR and NP1). We set up the lysis conditions and bi-dimensional SDS-PAGE, together with the optimal phospho-proteome staining. The findings obtained, together with phospho-enrichment approaches, strongly supported that peculiar signalling pathways exist in endothelial cells, selectively triggered by VEGF₁₆₅, VEGF₁₂₁ and Sema3A, as demonstrated by the differential pattern of phosphorylated proteins induced by the recombinant proteins. Furthermore, we first demonstrated a close similarity in the phospho-tyrosine proteome induced by VEGF₁₆₅ and Sema3A, at least in the cellular system examined.