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Paracrine and autocrine mechanisms of apelin signaling govern embryonic and tumor angiogenesis

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Abstract

Apelin and its G protein-coupled receptor APJ play important roles in blood pressure regulation, body fluid homeostasis, and possibly the modulation of immune responses. Here, we report that apelin-APJ signaling is essential for embryonic angiogenesis and upregulated during tumor angiogenesis. A detailed expression analysis demonstrates that both paracrine and autocrine mechanisms mark areas of embryonic and tumor angiogenesis. Knockdown studies in *Xenopus* reveal that apelin-APJ signaling is required for intersomitic vessel angiogenesis. Moreover, ectopic expression of apelin but not vascular endothelial growth factor A (VEGFA) is sufficient to trigger premature angiogenesis. *In vitro*, apelin is non-mitogenic for primary human endothelial cells but promotes chemotaxis. Epistasis studies in *Xenopus* embryos suggest that apelin-APJ signaling functions downstream of VEGFA. Finally, we show that apelin and APJ expression is highly upregulated in microvascular proliferations of brain tumors such as malignant gliomas. Thus, our results define apelin and APJ as genes of potential diagnostic value and promising targets for the development of a new generation of anti-tumor angiogenic drugs.

Keywords: Angiogenesis; Apelin; APJ; Cancer; Glioblastoma; Mouse; Signal transduction; VEGF; Xenopus

Introduction

Glioblastoma multiforme (GBM) is the most malignant glioma with an invasive and destructive growth pattern. It presents with increased mitotic activity, tumor necrosis, and pronounced angiogenesis (Kleihues et al., 2002). Clinically, patients diagnosed with GBM show a median survival of less than a year despite aggressive surgery, radiation, and chemotherapy (Holland, 2001). Current anti-GBM treatment modalities including radiotherapy plus concomitant and adjuvant temozolomide (Stupp et al., 2005) provide a modest survival advantage at best and, hence, alternative therapeutic approaches are urgently needed.

Tumor angiogenesis is one of the pathological hallmarks of malignant gliomas (Plate and Risau, 1995), which, in turn, makes the control of tumor blood supply a promising therapeutic target. Vascular endothelial growth factors, particularly VEGFA, are considered to be the most important proangiogenic factors in pathologic angiogenesis (Ferrara et al., 2003). VEGFA is overexpressed in most GBMs, and its receptors are present at high levels on the tumor vessels (Hatva et al., 1995; Plate et al., 1992). Furthermore, studies using orthotopic animal models indicate that glioma growth and vascularization is strongly VEGFA dependent and, vice versa, inhibition of VEGFA signaling significantly halts glioma growth by blocking neovascularization and proliferation (Goldbrunner et al., 2000; Goldbrunner et al., 2004).

Given the importance of VEGFA in tumor angiogenesis, much attention has focused on developing anti-VEGF or anti-VEGF receptor (VEGFR) therapies to treat a variety of cancers. In the last years, various clinical trials have demonstrated the efficiency of anti-VEGF therapies (Kerbel, 2006). More recently, however, drug resistance by evasion of VEGF inhibition

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through upregulation of additional angiogenic factors has been reported to occur in preclinical settings (Casanovas et al., 2005; Mizukami et al., 2005). Hence, the identification and development of alternative drug targets to prolong the effectiveness of antiangiogenic drugs is pertinent.

APJ (putative receptor protein related to the angiotensin receptor AT1; angiotensin II related receptor-like 1—Agtrl1) and its amphibian orthologue Msr encode members of the G protein-coupled receptor (GPCR) gene family that have been implicated in the control of blood vessel development (Devic et al., 1996; Devic et al., 1999). Originally identified as orphan GPCRs, APJ and Msr are structurally related to the angiotensin II receptor type I (AT1R) (Devic et al., 1996; O'Dowd et al., 1993) but despite the significant structural homology, angiotensin II does not bind to APJ (Tatemoto et al., 1998). Apelin, the natural ligand of APJ was isolated from bovine stomach (Tatemoto et al., 1998). In mammals, the apelin gene encodes a secreted preprotein of 77 amino acids with a signal peptide, a prodomain, and a C-terminal peptide, which upon proteolytic maturation generates a number of apelin polypeptides (Tatemoto et al., 1998). Apelin-36, comprised of amino acids 42–77, and apelin-13 (amino acids 65–77) represent the predominant and most active isoforms (Hosoya et al., 2000; Tatemoto et al., 1998). In the adult, apelin and its receptor are expressed both in the brain and in the periphery, particularly in the gastrointestinal tract, adipose tissues, lung, kidney, liver, and the skeletal muscle. In addition, APJ and apelin are highly expressed in the cardiovascular system, where they play important physiological roles in the regulation of blood pressure and cardiac contractility (Kleinz and Davenport, 2005; Masri et al., 2005). During postnatal development, apelin signaling is also associated with retinal blood vessels, where it may function to regulate angiogenesis (Kasai et al., 2004; Saint-Geniez et al., 2002). The role of apelin-APJ signaling in embryonic angiogenesis and its significance for pathologic angiogenesis has, however, remained largely elusive. While this manuscript was in preparation, two studies reporting contradicting results about the role of apelin signaling during cardiovascular development in the *Xenopus* embryo were published (Cox et al., 2006; Inui et al., 2006). Cox et al. demonstrate that apelin and APJ play important roles in blood vessel morphogenesis, where they are required for the formation of intersomitic vessels. In contrast, the study of Inui et al. suggests that apelin and APJ play more general roles in cardiovascular development by regulating differentiation of endothelia, hematopoietic cells, and cardiomyocytes.

Here we provide novel mechanistic insights into the role of apelin-APJ signaling during blood vessel formation under normal and pathological conditions. Apelin expression is associated with angiogenic blood vessel growth, where it acts via paracrine and autocrine mechanisms. We demonstrate that apelin-APJ signaling is necessary and sufficient to promote angiogenic blood vessel growth *in vivo*. Interestingly, we failed to observe in our loss-of-function studies any evidence of general defects in cardiovascular development supporting the view of a primary role of apelin signaling in angiogenesis. Epistasis analysis in *Xenopus* embryos suggests that apelin signaling functions downstream of VEGFA. Finally, we provide evidence for an important role of apelin and APJ in tumor angiogenesis, namely to pathological vessel formation in GBM. Taken together, our studies identify apelin and its receptor APJ as novel targets for anti-angiogenic tumor therapies.

Materials and methods

Cloning of cDNAs, sequencing, and sequence analysis

Expressed sequence tag (EST) databases were screened for potential *Xenopus laevis* cDNAs related to human apelin, APJ and VEGFA. For each gene, multiple cDNAs were identified and retrieved. Sequence comparisons revealed that each human gene was represented by two classes of cDNAs, which are likely to be derived from two pseudoallelic gene variants present in the *X. laevis* genome. *Xenopus* cDNAs for apelin-a (GenBank Acc. No. BE680255), APJa (AW460831), APJb (BQ399449, BQ724849, BQ726502), VEGFAb (BF426570) were obtained from the RZPD German Resource Center for Genome Research. *Xenopus* cDNAs for apelin-b (BJ030743) and APJb (BJ033869) were kindly provided by the NIBB *X. laevis* EST Project, Japan. Double-stranded DNA sequencing was either performed in-house or by Primm srl, Italy. Assembly of nucleotide sequence traces, analysis of nucleotide and protein sequences was performed using the DNAStar Lasergene software package (version 6.0). Amino acid sequence alignments were performed with MegAlign (DNAStar) using the Clustal W algorithm and the PAM250 residue weight table. The alignments then were used to construct phylogenetic trees with the Neighbor-Joining algorithm (Saitou and Nei, 1987). The described *Xenopus* nucleotide sequences were deposited with GenBank: apelin-a (GenBank Acc. No. DQ471852), apelin-b (DQ471853), APJa (DQ473441), APJb (DQ473442), and VEGFAb168 (DQ481238).

Plasmid constructs

The following constructs were generated for *in vitro* coupled transcription–translation reactions and/or *in vitro* RNA synthesis. Plasmid Apelin-ORF contains the ORF of *Xenopus* apelin-a (Gene Bank Acc. No. DQ471852). Plasmid Apelin-(5′)-UTR encodes 18 nucleotides of the 5′UTR followed by the ORF of apelin-a. Plasmid Apelin (F13A) contains the complete ORF of apelin-a but the carboxy-terminal phenylalanine was substituted by alanine. Plasmid APJa-ORF contains the ORF plus 118 nucleotides of the 3′UTR of *Xenopus* APJa (DQ473441). Plasmid APJa-(5′)-UTR contains 36 nucleotides of the 5′UTR and the ORF of APJa. Plasmid APJb-ORF contains 43 nucleotides of the 5′UTR, the ORF, and 89 nucleotides of the 3′UTR of *Xenopus* APJb (DQ473442). Plasmid VEGFA-ORF contains the ORF of *Xenopus* VEGFAb (isoform 4, VEGFA168, DQ481238). Plasmid VEGFA-MIS contains essentially the ORF of *Xenopus* VEGFAb (DQ481238), but seven point mutations were introduced which encode the original amino acid sequence but no longer serve as a target for the VEGFA-MO (see below). All plasmids were generated by PCR using the Expand High Fidelity PCR System (Roche Diagnostics) and subcloned into the pCS2+ vector (Turner and Weintraub, 1994). The constructs were confirmed by DNA sequencing.

Xenopus embryo manipulations, in situ hybridization, and sectioning

In vitro fertilization, culture and staging of *Xenopus* embryo were performed as described (Brändli and Kirschner, 1995; Helbling et al., 1998). Probe synthesis, whole mount *in situ* hybridization, β-galactosidase staining, and bleaching of *Xenopus* embryos were carried out as described (Helbling et al., 1999; Helbling et al., 1998; Saulnier et al., 2002). In some cases, embryos were immersed into clearing solution (2:1 benzyl benzoate/benzyl alcohol) to visualize staining of internal structures. Digoxigenin-labeled probes were synthesized from linearized plasmids encoding *Xenopus* Msr (Devic et al., 1996), APJb (GenBank Acc. No. DQ473442), apelin-a (DQ471852), Erg (Baltzinger et al., 1999), Fli1 (Meyer et al., 1995), VEGFR2 (BJ075253), VEGFR1 (BQ736 324), Gata3 (Bertwistle et al., 1996), αT3-globin (Banville and Williams, 1985), Pecam1 (BF612503), and Tie2 (Iraha et al., 2002). Sense strand controls were prepared from all plasmids and tested negative by *in situ* hybridization.