FrzA, a Secreted Frizzled Related Protein, Induced Angiogenic Response

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Background—The secreted frizzled related proteins (sFRP) are soluble proteins thought to interfere with the Wnt signaling. Our group previously demonstrated that one of these members, sFRP-1/FrzA, is strongly expressed during early phases of the vascularization process in embryonic vasculature and in the endothelium of arteries and capillaries in adults and modulated vascular cell proliferation.

Methods and Results—Analysis of the expression of sFRP-1 during cyclic ovarian angiogenesis revealed that sFRP-1 is expressed during the formation of neovessels and becomes undetectable when the vasculature is fully matured. We then studied the role of FrzA in several distinct angiogenic models. FrzA induced angiogenesis in a chick chorioallantoic membrane model. Moreover, gene transfer of AdFrzA in grafted mesenchymal and glioma cells increased vessel density and tumor growth. FrzA induced formation of vessels, which were enlarged, longer, and appeared to be more mature compared with vessels formed under control treatments. In vitro, FrzA increased migration and tube formation of endothelial cells and seemed to protect them from apoptosis. FrzA-angiogenic effect in vitro was independent of vascular endothelial growth factor, fibroblast growth factor-2, or angiopiotin-1 induction and Akt activation. In contrast, FrzA decreased glycogen synthase kinase-3 phosphorylation.

Conclusions—These results showed that FrzA has proangiogenic effects and suggest that Wnt signaling may be involved in normal differentiation as well as in the pathological development of vasculature. (Circulation. 2002;106:3097-3103.)

Key Words: angiogenesis • endothelium • muscle, smooth

Angiogenesis and vascular remodeling occur throughout growth and development and can be induced by a variety of cytokines. Wnt constitute a family of secreted proteins involved in the remodeling processes, including cell polarity, proliferation, and differentiation. Recent evidence implicated members of the frizzled family as Wnt receptors and suggests a role of the Wnt/frizzled cascade on vascular cell proliferation and differentiation, mechanisms evolved to control the formation and differentiation of the vasculature. Wnt receptor gene, Fzd5, is essential for yolk sac and placental angiogenesis. After myocardial infarction, dishevelled and β-catenin, upstream elements of the Wnt pathway, were translocated into the cytoplasm of vascular endothelium, reflecting an activation of the Wnt/frizzled pathway during neovessel formation. Moreover, overexpression of Wnt-1 endothelial cell (EC) culture resulted in proliferation of these cells with an increase in cytoplasmic β-catenin.

A family set of secreted frizzled related protein (sFRP) has been recently described. These proteins contain a cysteine-rich domain similar to the Wnt-binding site of frizzled receptors. sFRP are able to bind to Wnt and are thought to function as antagonists for Wnt signaling. We have isolated one sFRP member, FrzA, from bovine aortic ECs. FrzA has been reported to form a biochemical complex with Wg protein in vitro. FrzA and its human orthologue were shown to interfere with Wnt signaling and to antagonize the effects of Wnt-1, Wg, and Xwnt-8. Our laboratory previously reported that sFRP-1 is expressed during embryonic vascular development in the inner lining of the ECs of the visceral yolk sac at 9.5 dpc and disappears by 12.5 dpc. In the adult, FrzA is expressed in the endothelium and the media of arteries and capillaries. Experimental data demonstrated that FrzA overexpression controlled in vitro vascular cell proliferation. All these data prompted us to additionally investigate the role of FrzA in the process of angiogenesis.

Methods

Development of Ovarian Follicles and Corpus Luteum

Hyperstimulation of the ovaries of 8-week-old C57BL6 mice was induced by injecting 10 UI of pregnant mare’s serum gonadotropin (PMSF, Sigma), followed 44 hours later by 10 UI human chorionic...
gonadotropin (hCG, Sigma). Ovaries were collected for reverse transcriptase–polymerase chain reaction (RT-PCR) or immunohistochemistry analysis.

**Analysis of RNA by RT-PCR**

Total RNA and RT-PCR reactions were carried out as previously described. In pilot experiments, cycle numbers were checked to determine that the fragments being amplified were in the exponential phase of amplification.

**Immunohistochemistry Analysis**

Animals were killed with an overdose of sodium pentobarbital. For immunohistochemistry, tissues were fixed in methanol and embedded in paraffin as previously described. Endogenous sFRP1 was stained with polyclonal antibody, as previously described. CD31 (Pharmingen) antibodies were used to detect ECs, α-actin and PAL-conjugated α-actin (Sigma) to detect smooth muscle cells, and β-gal (Chemicon) to detect β-galactosidase.

**Recombinant Adenoviral Infection**

Cells in cultures were infected with Adβgal, AdFrzA, or AdVEGF (gift from Dr B. Annex, University of Michigan Health System, Ann Arbor, Mich) at 500 πg/cell for 2 hours. Infected cells were then cultured in fresh culture medium for 48 hours and then either lysed with RIPA buffer for Western blot analysis or Trizol reagent (Invitrogen) for RNA extraction or harvested for in vivo angiogenesis assays.

**Mesenchymal and Tumor Cell Grafts in Immunodeficient Mice**

One million mesenchymal fibroblasts 10T1/2 or glioma cells (U87 MG) were mixed in 100 μL of cold collagen (BD Biosciences) and injected intradermally into abdominal subcutaneous tissue of 8-week-old RAG2/γc male mice. The plugs containing 10T1/2 and U87 MG infected with the different adenovirus were excised after 5 and 20 days, respectively.

**Chick Chorioallantoic Membrane Assay**

CHO and CHO-FrzA stably transfected cell lines 10⁶ cells were resuspended in 50 μL of serum-free medium and seeded onto the membranes of 10-day-old chick embryos. After 2 days, chorioallantoic membranes (CAMs) were harvested by fixation with 3% of PFA and embedded into paraffin. Sections were stained with Alizarin to label red blood cells. Vessel number per surface around the graft were counted at ×25 magnification.

**Cell Culture**

Human umbilical vein ECs (HUVECs), bovine aortic ECs, and bovine smooth muscle cells (SMCs) were cultured in M199 medium (Invitrogen) supplemented with 10% FCS, 2 mmol/L L-glutamine, and 200 U/mL hCG (Sigma). Ovaries were collected for reverse transcriptase–polymerase chain reaction (RT-PCR) or immunohistochemistry analysis.

**Cell Migration Assays**

Cell chemotactic migration was carried out with 8-μm pore size in a 12-well Transwell migration chamber (Costar) with membranes coated with 0.1% gelatin. Radial migration was performed using cell sedimentation manifold for migration assay (Creative Scientific Methods, Inc) coated with 0.1% gelatin. EAHY926 at 50 000 cells/well was plated for 1 hour in serum-free medium (SFM) or plated for 20 ng/mL (R&D system) in SFM during 6 or 24 hours. Fixed cells were counterstained to count migrating cells and measure migration diameter by microscopy with a CCD camera. Each experiment was done in triplicate.

**In Vitro Morphogenesis**

ECs were plated on Matrigel (Becton Dickinson) at 2×10⁵ cells/24 well in the absence or presence of recombinant FrzA protein or VEGF 20 ng/mL in 10% of serum. After 24 hours, tube formation and branching were quantified in 5 random fields (10-fold magnification). Each experiment was done in triplicate.

**Apoptosis Assay**

Confluent cells on labteck (Polylabo) were incubated during 18 hours, with or without purified recombinant FrzA protein in SFM with 0.1% of BSA, fixed in 2% PFA, and stained with Hoechst 33342. The percentage of pyknotic nuclei was determined by counting 5 different fields per well (×40 objective). Each experiment was done in quadruplicate.

**Statistical Analysis**

All results are expressed as mean±SEM. Differences were analyzed by ANOVA test and considered statistically significant at P<0.05.

**Results**

**sFRP-1 Expression During Angiogenesis in Hormone-Stimulated Ovary**

RT-PCR analysis showed that sFRP-1 mRNA expression increased slowly during follicle development and reached peak expression during the lutein phase, 20 hours after hCG injection (Figure 1A). Previous characterizations of this model have shown that during the cycle of follicle development and CL formation, vascular changes are tightly regulated. During the first phase, follicles acquire individual vascular networks originating at vessels residing in the medulla, which then extend toward each growing follicle (Figure 1B, parts b and d). sFRP-1 was expressed in the interstitial tissue around prenatal follicles and in the theca layers of growing follicles (Figure 1B, a). During a second angiogenic phase, capillaries invade the CL (Figure 1B, f) and then become muscularized and form an elaborate network whereby each lutein cell resides in proximity to blood capillaries (Figure 1B, parts j, l, n, and p). Interestingly, sFRP-1 was strongly expressed by luteal cells in early CL in areas of invading vessels arising from the thecal vascular plexus (Figure 1B, parts e and i). With additional CL maturation, sFRP-1 expression remained strong in lutein cells and pericytes, whereas low sFRP-1 expression was observed in ECs (Figure 1B, parts m, n, and p). In parallel, immunohistochemical experiments were conducted to compare its expression to VEGF (Figure 1B, parts c, g, k, and o), revealing a matching spatial and temporal expression pattern during vessel development. These results revealed that the pattern of sFRP-1 expression correlated with physiological angiogenesis.
FrzA-Angiogenic Response in Chick CAM Assay

Untransfected or FrzA stably transfected CHO cells were grafted onto chick embryo CAMs (Figure 2A). Two days later, control cells did not provoke angiogenesis in the CAM, whereas the FrzA transfectants induced an angiogenic response. Neovascular response quantified by counting functional vessel number showed that FrzA induced a 3.25-fold increase of vessel number compared with control (Figure 2B and 2C, \( P < 0.05, n = 4 \)).

Plug and Tumoral Assays in Immunodeficient Mice

The activity of FrzA was examined in mouse plug and tumoral assays using gene transfer of AdFrzA of multipotent 10T1/2 mesenchymal cells and human U87 MG (U87) glioma cells. Infection with AdVEGF was used as positive control for angiogenesis.

Before implantation assays, we checked that FrzA infection did not modify the expression of angiogenic factors angiopoietin-1, FGF-2, and VEGF (Figure 3A) and that neither purified recombinant FrzA protein treatment nor AdFrzA infection had an effect on proliferation of 10T1/2 and U87 cells (\( n = 4 \)).

We investigated the effect of FrzA during tumor angiogenesis using U87 cells implanted in RAG2/γ mice (\( n = 12 \)). Tumor size and weight after AdFrzA infection were significantly increased compared with those with Adβgal infection (Figure 3B and 3C). AdFrzA treatment induced strong tumor vascularity compared with Adβgal treatment (\( P < 0.0001 \), Figure 3D), with a significant induction in vessel length (\( P < 0.001 \), Figure 3E). In this model, combined treatment with AdFrzA and AdVEGF did not result in a synergistic effect compared with AdFrzA alone (Figure 3).

Collagen containing adenovirus-infected 10T1/2 cells forms a plug in subcutaneous space. Vessel density evaluated with CD31 staining (Figure 4A, b) was significantly increased in AdFrzA- or AdVEGF-treated 10T1/2 cells compared with Adβgal-treated cells (Figure 4B, \( P < 0.005 \), \( n = 6 \) for each group). AdFrzA angiogenic response resulted in larger vessels. In the AdFrzA-treated group, 41% of blood vessels had a diameter >500 μm², whereas no vessels of that...
caliber were detected in the control group (Figure 4C). Double immunohistostaining for β-actin/CD31 demonstrated that FrzA increased vessel maturation, as did VEGF (Figure 4D). To investigate whether 10T1/2 recruitment and differentiation in neovessels could participate in the FrzA-induced angiogenesis process, we implanted AdFrzA-infected 10T1/2 cells stably transfected for expression of nuclear β-galactosidase.\textsuperscript{18} Double immunostaining for β-gal/α-actin did not revealed double-labeled 10T1/2 cells in neovessels (Figure 4A, d), suggesting that FrzA promoted the recruitment of host vascular cells for neovessel formation. Taken together, these results support the notion that FrzA secreted from AdFrzA-infected cells induced a strong angiogenic response in vivo.

**Functional Analysis of FrzA In Vitro**

To provide additional support for the role of FrzA in angiogenesis, in vitro EC migration, branching, and apoptosis assays were performed.

Recombinant FrzA protein increased EAhy926 motility and migration in a radial migration assay and a Transwell chemotaxis assay \( (P<0.05, n=4, \text{Figure 5A and 5B}) \). No additive effect of FrzA combined with VEGF protein was observed on EC migration. Untreated EAhy926 cells demonstrated a typical anastomosed cellular network 24 hours after plating on Matrigel. Addition of recombinant FrzA increased significantly the amount of branching compared with control \( (P<0.005, n=3, \text{Figure 5C}) \). Moreover, FrzA protected HUVECs from apoptosis. When HUVECs were cultured in SFM with 0.5% BSA, FrzA treatment decreased the frequency of pyknotic nuclei events after 6 and 18 hours \( (P<0.05, n=3, \text{Figure 5D}) \). Together these findings support the in vivo observations that FrzA has angiogenic potentiality.

![Figure 2](image) FrzA induces angiogenesis in CAM assay. Western blot for FrzA on FrzA stably transfected CHO and native CHO lysates (A). Microphotograph represents paraffin sections of CAM engrafted with CHO or CHO-FrzA cells (E) after Alizarin-blood cells staining and hematoxylin counterstaining (B). Magnification \( \times 40 \). Vessels numbers per surface were quantified and results were expressed as percent of control \( (n=4, ^* P<0.05) \) (C).

![Figure 3](image) Effects of FrzA on growth of U87 MG cells in vivo. U87 MG cells were infected with Adβgal, AdFrzA, AdVEGF, or AdFrzA + AdVEGF and injected subcutaneously. Before being injected, adenoviruses-infected U87 was lysed to perform Western blot for FrzA, VEGF, angiopoietin-1, and FGF-2 expressions (A). Representative microphotograph of tumor sizes after 20 days (line=2 mm) (B). Graphs represent tumor weight (mg) \( (n=12, ^* P<0.05) \) (C). CD31-stained blood vessels were evaluated in 3 different \( \times 20 \) fields in sections obtained from 5 different tumors for each group, \( ^* P<0.001 \) (D). Effect of FrzA and VEGF on the length of vessels, \( ^* P<0.001 \) (E).
Effect of FrzA on Angiogenic Factor Expression in Vascular Cells and on Akt and GSK-3 Pathways

In vitro experiments were conducted to discern the mechanisms underlying FrzA-induced angiogenesis. First, we studied the modification of angiogenic factor expression induced by FrzA in vascular cells. Activation by FrzA did not modify VEGF, FGF-2, nor angiopoietin-1 expression in ECs (Figure 6C). In contrast, an increase in VEGF mRNA (Figure 6B) and protein (not shown) was detected in the SMCs after their exposure to AdFrzA. Second, we investigated modification of the intracellular pathway, Akt, and GSK3β phosphorylation, which are respectively activated by angiogenic factors and Wnt pathway in EC. FrzA did not modify phospho-Akt levels after 15 minutes to 2 hours (Figure 6D), whereas FrzA stimulation decreased phospho-GSK3β levels, supporting an antagonist role of FrzA on the Wnt/GSK3β pathway in ECs.

Discussion

In the present study, we sought to assess whether FrzA/sFRP-1, a secreted inhibitor of the Wnt/frizzled pathway, could participate in the formation and the maturation of neovessels. We provided evidence that sFRP-1 was expressed during the physiological angiogenic process in the ovary. Furthermore, using several in vivo models, we demonstrated that FrzA induced a strong angiogenic response. In vitro, FrzA promoted activation of ECs, which is required for angiogenesis. FrzA increased migration and organization into capillary-like structures and protected ECs from apoptosis. Activation of ECs by FrzA involves a signaling pathway independent of VEGF/FGF-2 and Akt activation. These results are the first demonstration of a role of FrzA on the Wnt/GSK3β pathway in ECs.
and induced during follicular development. sFRP-1 expression was linked to new vessel growth in the interstitial tissue and in the theca layers and followed the front of invading ECs during CL maturation. When the microvasculature was fully covered with pericytes, sFRP-1 expression decreased in capillary ECs that will lately undergo regression processes. This model demonstrates a striking relationship between sFRP-1 expression and vessel formation, thus suggesting that vessel maturation depends in part on FrzA-mediated signal expression.

We tested the impact of FrzA on angiogenesis in vivo using different angiogenic models. FrzA induced a strong increase in functional vessels in CAM assays, in mesenchymal cell graft model, and in a malignant glioblastoma tumor model. FrzA increased tumor volume and weight, which was attributable to a dramatic increase in vessel density. In contrast, a previous study did not reveal a significant effect of sFRP-1 on tumors implanted in the brain.19 These differences may be explained by differences in host vascular cells recruited or in cytokine secretion attributable to differences in implantation microenvironments.20 The angiogenesis process could be divided into an early step where ECs migrate, proliferate, and form an endothelial plexus. In a later stage, pericytes are recruited, stabilize the vasculature, and mark the end of the immature plasticity window.21 In an attempt to evaluate the impact of FrzA on the distinct angiogenic events, we analyzed qualitative and quantitative features of the FrzA-induced neovascularure. FrzA overexpression allowed the formation of blood vessels that were enlarged, longer, and more associated with pericytes. Moreover, we demonstrated that in vitro FrzA could induce VEGF expression in SMCs and could facilitate the recruitment of pericytes to ECs. In a previous study, we demonstrated that FrzA transiently delayed the SMC and EC entrance into the cell cycle through an effect on G1 phase (T. Couffinhal, unpublished data, 2002). These experiments suggest that FrzA could be considered a potent angiogenic factor that might be required for vascular remodeling and maturation.

To clarify the functional significance of FrzA effect on angiogenesis, we examined the capability of FrzA to modulate EC functions. FrzA is able to exert angiogenic effects on ECs, inducing their migration and organization into capillary-like structures and protecting them from apoptosis. These angiogenic effects on EC were independent of VEGF, FGF-2, or angiopoietin-1 induction by ECs. The existing literature regarding the effect of sFRP on migration and apoptosis has been puzzling. It has been reported that overexpression of sFRP-1 and -2 in glioma cells increased their clonogenicity, enhanced their survival under serum withdrawal conditions, and decreased cellular motility.19 In contrast, sFRP-1 expression was associated with apoptosis in MCF7 lines,22,23 and its downregulation was correlated with breast malignancy, thereby suggesting that sFRP-1 may act as a tumor-suppressor gene.23,24 The discrepant data may result from the different cell types used and from differences in sFRP levels locally obtained. Specific frizzled receptors are able to lead to apoptosis,25 and sFRP-1 had an opposite effect at low concentration and at high concentrations on Wnt-dependent stabilization of β-catenin, known to be a key regulator of cell proliferation.26

Recently, a link between the serine-threonine kinase Akt/PKB involved in regulation of angiogenesis27 and the Wnt-frizzled pathway was demonstrated.28 Also we explored the possibility that FrzA could exert a direct effect on EC angiogenic properties via an Akt activation–dependent pathway. Contrary to our expectation, FrzA stimulation did not activate Akt pathway. In contrast, FrzA was able to inhibit the canonical Wnt/frizzled/GSK3β pathway on ECs.13

In conclusion, our data indicate that FrzA has a direct role during angiogenesis. FrzA may be required for normal vascular morphogenesis and may control the formation of functional blood vessels promoting pericyte recruitment. These results suggest that Wnt signaling may be involved in

Figure 6. FrzA effects on EC are independent of VEGF/FGF-2 modulation and Akt activation. FrzA mRNA expression was quantified by RT-PCR after VEGF or DFO (100 μmol/L) activation during 12, 24, and 48 hours in bovine EC (A) and SMC (B). VEGF, FGF-2, and angiopoietin-1 expressions were studied by Western blot after EAhy926 cell infection with Adβgal, AdFrzA, AdVEGF, or AdFrzA+AdVEGF (C). Deprived confluent EAhy926 cells were treated without (-) or with (+) FrzA during 15 minutes, 30 minutes, and 2 hours. Cell lysates were analyzed by Western blot with antibodies against P-GSK3β, P-Akt, and total Akt (D).
normal differentiation and in the pathological development of the vasculature, where endothelial properties are modified.

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