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The Role of the Cysteine-Rich Domain and Netrin-Like Domain of Secreted Frizzled-Related Protein 4 in Angiogenesis Inhibition In Vitro

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Secreted frizzled-related protein 4 (sFRP4) is a Wnt signaling antagonist. Classically, sFRP4 antagonizes the canonical Wnt signaling pathway, resulting in decreased cellular proliferation and increased apoptosis. Recent research from our laboratory has established that sFRP4 inhibits angiogenesis by decreasing proliferation, migration, and tube formation of endothelial cells. The objective of this study was to examine the role of sFRP4's cysteine-rich domain (CRD) and netrin-like domain (NLD) in angiogenesis inhibition. Experiments were carried out to examine cell death and tube formation of endothelial cells after treatment with the CRD and the NLD. The CRD was seen to inhibit tube formation of endothelial cells, which suggests that this domain is important to sFRP4's antiangiogenesis property. In addition, the NLD promoted endothelial cell death and may also inhibit angiogenesis. Furthermore, treatment with the CRD and the NLD increased endothelial intracellular calcium levels. Our findings implicate a role for both the CRD and NLD in angiogenesis inhibition by sFRP4. It is suggestive of alternative antiangiogenic downstream targets of canonical Wnt signaling and a possible importance of the noncanonical Ca^{2+} Wnt signaling pathway in sFRP4-mediated angiogenesis inhibition.

Key words: Secreted firzzled-related protein 4 (sFRP4); Angiogenesis; Apoptosis; Calcium signaling

INTRODUCTION

The Wnt signaling pathways are regulators of cellular proliferation, migration, differentiation, and survival, and recent literature suggests the Wnt signaling pathways have an increasingly important role in angiogenesis (1,2). Secreted frizzled-related proteins (sFRPs) are secreted glycoproteins that antagonize the Wnt signaling pathway (3,4). A recent study from our laboratory has discovered that secreted frizzled-related protein 4 (sFRP4) inhibits angiogenesis by decreasing the proliferation, migration, and tube formation of endothelial cells (5). However, the structural motifs and the cellular signaling involved in sFRP4's angiogenesis inhibition have not yet been established.

sFRP4 is comprised largely of two domains: a cysteine-rich domain (CRD) and a netrin-like domain (NLD), both of which have been implicated in Wnt inhibition and sFRP function (6,7). This study extends previous research on sFRP4's angiogenesis inhibition by exploring the effects of the CRD and the NLD on angiogenesis and determining what, if any, these effects are.

MATERIALS AND METHODS

Cell Lines and Reagents

ECV304 cells were used as an endothelial cell line in the apoptosis study because they function similarly to primary endothelial cells (8–10). Human umbilical vein endothelial cells (HUVECs) were used for the Fura-2 assay and tube formation assay. All reagents used for maintenance of ECV304 cells were obtained from Invitrogen-Gibco® Laboratories (North Andover, MA). Cells were grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM penicillin, and 2 mM streptomycin. The cells were incubated at 37°C/5% CO_2. HUVEC were grown in endothelial basal medium-2 (EBM-2) (Clonetics® Lonza) supplemented with an EGM™-2 Single Quot bullet kit (Clonetics® Lonza) and 10% FBS (Invitrogen-Gibco®). All other reagents used to maintain HUVECs were obtained from Gibco® Laboratories. Both the HUVEC and ECV304 cells were obtained from Dr. Chooi-May Lai, Lions Eye Institute, The University of Western Australia.

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Transient Transfections

The CRD- and the NLD-containing conditioned media were produced by transiently transfecting ECV304 cells with a pEGFP-N1 plasmid vector containing DNA for the CRD or the NLD. sFRP4's signaling sequence was included in the CRD and NLD DNA sequence. The proteins produced were secreted proteins and, after translation, they were secreted into the conditioned media, which was collected. The conditioned media were used as treatments since there is no purified CRD and NLD protein available. Transient transfections were performed on ECV304 cells using Lipofectamine™ 2000 (Invitrogen) and a pEGFP-N1 plasmid vector (Clontech Inc.), which has the cDNA of the CRD inserted into the *Kpn*I site, or the NLD inserted into the *Acc*I site (amino acid sequence for the CRD was gvr gapceavrip mcrhmpwnit rmpnhlhhst qenailaieq yeelvdvncs avlrfflcam yapictlefl hdpikpcksv cqrarddcep lmkmynhswp slacdelpv ydrgvci aivt; and the amino acid sequence for the NLD was aty lsknysyvih akikavqrsg cnevttvvdv keifkssspi prtqvplitnsscqcphilp hqdvlimcye wrsrmmllen clvekwrdql skrsiqweer lqeqrr). ECV304 cells were also transfected with an empty pEFGP-N_1 plasmid vector (empty vector) to provide an experimental control. The conditioned media, containing the CRD and the NLD, were collected 48 h after transfection and used as treatments to assess the antiangiogenic properties of sFRP4 and each domain. Transfection efficiency was calculated by comparing total cell counts and fluorescence cell counts for the same field of view. Transfection efficiency was assessed by cellular GFP expression and was found to be high (~40%) for all treatments (data not shown).

Matrigel Tube Formation Assay

The antiangiogenic effects of the CRD and the NLD were tested on HUVEC blood vessel network formation. A Matrigel tube formation assay (5) was used to assess blood vessel network formation after 6 h of treatment. Forty-five microliters of a 2.87 µg/ml Matrigel solution was pipetted into a 96-well plate, which was incubated at 37°C/5% CO_2 for 36 h. A 100,000 cell/ml HUVEC suspension was made and 600 µl of this was pipetted into six 1.5-ml tubes. The tubes were spun at 2,500 × *g* for 5 min and the media were removed, leaving the cell pellet. Five hundred and fifty microliters of each treatment (empty vector, CRD, or NLD) was used to suspend one cell pellet; 100 µl of each cell suspension treatment was added to five Matrigel-coated wells. The plate was then incubated for 6 h at 37°C/5% CO_2 and the wells were imaged using a Nikon inverted microscope under 4× objective lens. Angiogenesis inhibition was assessed using Image J image analysis software (Release Alpha 4.0 3.2)

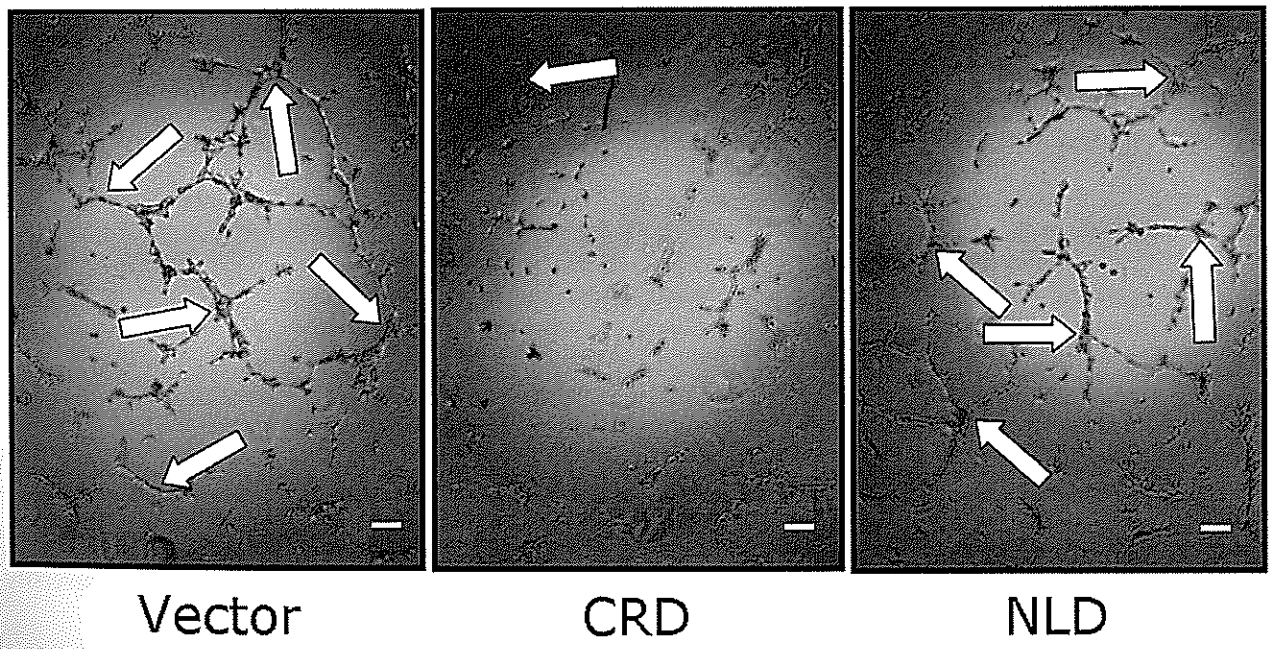

Figure 1. HUVEC tube network inhibition by sFRP4 and the CRD (bright field images). HUVECs treated with empty vector containing media showing a strong blood vessel network. Tube network formation following treatment with medium containing the CRD is inhibited relative to empty vector. No inhibition of network formation was seen following treatment with media containing the NLD. Arrows indicate areas of branching of endothelial tubes. Scale bars: 400 µm.

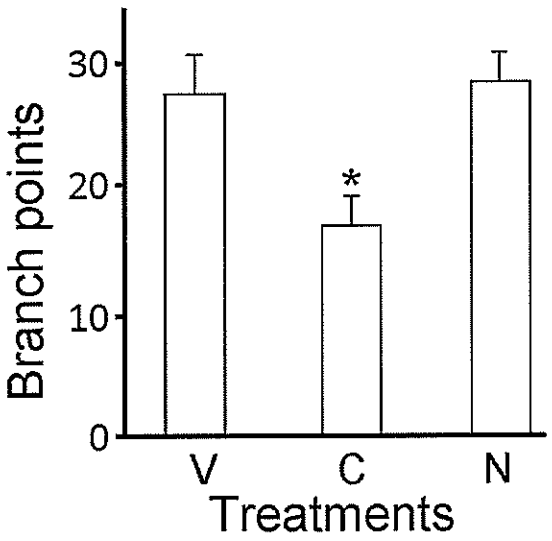

Figure 2. Mean ± SEM of branch points. HUVECs were treated for 6 h and branch points were counted as a measure of tube network complexity. Conditioned media containing the CRD (C) significantly reduced the number of branch points compared to the empty vector (V) and NLD (N). *$p < 0.01$.

and by counting branch points formed by the HUVECs as a measure of blood vessel network complexity.

JC-1 Apoptosis Assay

ECV304 cell death was examined using the JC-1 assay after 24 and 48 h of treatment. The fluorescent cationic dye 5,5′6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1) is capable of measuring mitochondrial membrane potential collapse and has been used to detect cell death (11). In healthy cells, the JC-1 cationic dye accumulates in the mitochondria and forms polymers that fluoresce red. In a dying cell, the mitochondrial membrane potential is destroyed and the JC-1 dye remains in the cytoplasm as a monomer that fluoresces green. Consequently, a decrease in the red/green fluorescence intensity ratio is indicative of increased cell death. This study used the MitoProbe™ JC-1 assay kit (Invitrogen) to detect ECV304 cell death after treatment with conditioned media containing the empty vector, CRD, and the NLD for 24 and 48 h.

Intracellular Calcium Measurements

Intracellular calcium was monitored using the fluorescent indicator Fura-2 AM (1 μM, ex 340/380 nm, em 510 nm; Molecular Probes) in PBS at 37°C as previously described (12). Fluorescent signal was measured on a Hamamatsu Orca ER digital camera attached to an inverted Nikon TE2000-U microscope. Data were analyzed using Metamorph 6.3. Ratiometric 340/380 nm fluorescence was recorded before and after exposure of HUVECs to 1 μl, then 50 μl, then 100 μl of media containing EV, CRD, or NLD. The duration of each exposure was 10 min. Ratiometric 340/380 nm fluorescence recorded over the final 3 min of each 10-min exposure was averaged and alterations in fluorescent ratio were

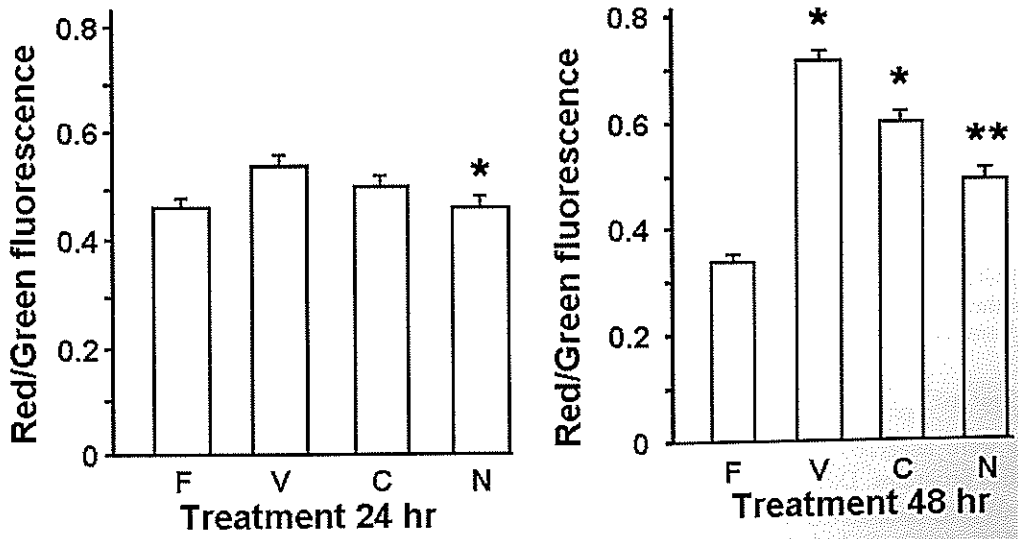

Figure 3. Endothelial cell apoptosis is increased by the CRD and the NLD. Mean and SEM of red/green fluorescence ratio. The ratio of red (590 nm) and green (527 nm) light emitted by the JC-1 dye is indicative of the number of dying cells. After 24 h the NLD (N) increased apoptosis compared to the empty vector (V) and CRD (C). After 48 h of treatment both the CRD and the NLD increased apoptosis. Carbonylcyanide-4-trifluoromethoxyphenylhydrazone (F) was used as a positive control. *$p < 0.05$, **$p < 0.01$.

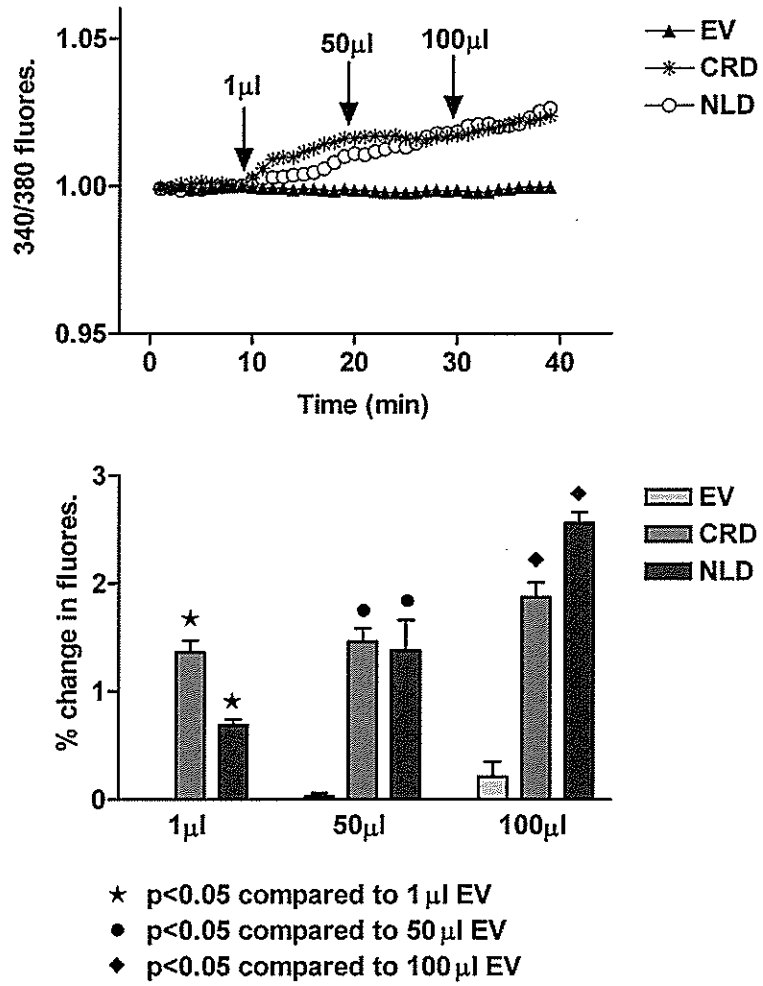

Figure 4. Fura-2 340/380 nm ratiometric fluorescence (340/380 fluores) is increased by the CRD and the NLD. Top: 340/380 fluorescence recorded from individual HUVECs before and after exposure to 1 μl, then 50 μl, then 100 μl of media containing empty vector (EV), CRD, or NLD. Arrows indicate when treatment was added. Bottom: Mean ± SE of changes in 340/380 nm fluorescence (% change in fluores.) for HUVECs exposed to EV, CRD, or NLD as indicated is shown. Mean ± SE for HUVECs for each treatment ($n = 5$).

reported as a percentage increase from the baseline average. Ratiometric 340/380 nm fluorescence was plotted relative to the pretreatment fluorescence assigned a value of 1.0.

Statistical Analysis

Statistics were analyzed using Microsoft Excel and SPSS statistics programs. Data were expressed as means and SEMs. When there were multiple treatments, one-way ANOVA was used to establish significance between treatments groups. If statistical significance was found, a subsequent post hoc test was completed to compare treatments to controls. In this study post hoc test included a Dunnetts T3 test, carried out in SPSS, and Student two-tailed t-test assuming unequal variance, carried out in Excel. For singular treatments two-tailed unpaired t-tests assuming unequal variance were used. Differences were considered significant when $p < 0.05$ and in some assays in which a strong trend was observed but significance was not reached then $p < 0.1$ was considered significant.

RESULTS

Matrigel Tube Formation Assay

There was the development of a complex network that was not affected by the addition of media containing the empty vector (Fig. 1). However, after 6 h of treatment with conditioned media containing the CRD ($n =$

9, $p < 0.01$) there was significant inhibition of blood vessel network formation when compared to the empty vector control (Figs. 1 and 2). Interestingly, in all repeats ($n = 10$), ring formation was not inhibited by the NLD (Figs. 1 and 2).

JC-1 Apoptosis Assay

ECV304 cells were treated with conditioned media and examined at 24 and 48 h. At this time point, cells treated with media containing the NLD showed a significant degree of apoptosis compared to both the empty vector control and CRD media-treated cells ($p < 0.05$) (Fig. 3). After 48 h the CRD significantly increased apoptosis compared to the empty vector control ($p < 0.05$) but not to the same degree as the NLD ($p < 0.01$).

Intracellular Calcium Measurements

HUVECs were treated with empty vector, the CRD, and the NLD using increasing volumes of media. At each time point (10 min) both the CRD and NLD significantly increased Fura-2 340/380 nm ratiometric fluorescence compared to empty vector control at each titration ($p < 0.05$). Furthermore, each treatment produced a positive relationship between dose and response (Fig. 4).

DISCUSSION

The primary objective of this study was to analyze the effect of the CRD and NLD of sFRP4 on endothelial cells. The process of angiogenesis involves several well-identified steps (13); one of the final and important stages involves tube/network formation. Having previously demonstrated that sFRP4 was capable of significantly reducing the number of endothelial connections made in vitro (5), we investigated the effect of the CRD and NLD on this critical phase of angiogenesis.The CRD was found to be responsible for inhibition of endothelial cell spreading, development of pseudopodia, and endothelial network formation, which are important for the successful initiation and development of angiogenesis.

The NLD is highly conserved in sFRPs (14). Conservation of a domain is often indicative of an important role in a protein's function. However, the role of the NLD remains poorly addressed in the available literature (15). The JC-1 assays carried out during this study provide evidence that the NLD promotes endothelial cell death. Upregulation of cell death, specifically through increased apoptosis, is a characteristic function of sFPR4 and other sFRPs (16). To our knowledge, this is the first study that implicates the NLD as an important structural component in endothelial cell death promotion by sFRP4, and suggests that the NLD may function similarly in other sFRPs.

The noncanonical Wnt Ca^{2+} signaling pathway has been shown to antagonize canonical Wnt signaling (17,18) and could be providing a block on angiogenic factor transcription by β-catenin. One study by Ishitani et al. (19) found that Wnt-5a activated noncanonical Wnt Ca^{2+} signaling and upregulated NLK, which inhibited TCF transcription. It is also possible that sFRP4 is inhibiting angiogenesis independently of Wnt signaling as other studies have shown that sFRP-NLD can bring about actions without Wnt signaling participation: for example, by interacting with fibronectin and integrin (20) and altering JNK, RANKL, and BMP signaling (21).

In conclusion, we have reported the novel findings that the CRD of sFRP4 is responsible for its ability to interfere with vessel network formation while the NLD induces apoptosis of endothelial cells. Furthermore, both the CRD and NLD are involved in activating the Wnt Ca^{2+} signaling pathway by increasing intracellular calcium levels. These findings may facilitate the use of sFRP4's domains as a targeted therapeutic intervention.

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