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Opposing Actions of Notch1 and VEGF in Post-natal Cardiac Valve Endothelial Cells

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Abstract

The endothelium of the cardiac valves is unique compared the rest of the vasculature in its ability to undergo an endothelial-to-mesenchymal transformation (EMT) in vitro in response to transforming growth factor- β (TGF- β). EMT is a critical event during embryonic valve development, and both VEGF-A and Notch1 have been shown to function in this process. Here we investigate the effects of VEGF-A and Notch1 on EMT in clonal endothelial cell (EC) populations isolated from adult aortic valve leaflets. VEGF-A inhibited TGF- β -induced EMT. Endothelial growth, however, was not affected by VEGF-A or TGF- β . A positive role for Notch1 was revealed in three experiments: 1) TGF- β induced Notch1 mRNA in valve ECs, 2) a γ -secretase inhibitor of Notch1 signaling blocked EMT and 3) overexpression of a ligand-independent form of Notch1 induced EMT. These results demonstrate, for the first time, that VEGF-A and Notch1 play opposing roles in regulating EMT in post-natal valve endothelium.

Keywords

endothelial-to-mesenchymal transformation; valve endothelial cells; Notch1; VEGF-A; TGF- β

Introduction

Many congenital heart defects affect formation of the cardiac valves – structures needed to direct blood flow through the heart and prevent regurgitation. Heart valve defects such as bicuspid valves are relatively common but typically do not cause symptoms until adult life, at which time degeneration and calcification lead to deterioration of valve function. Understanding the cellular mechanisms needed to form, maintain and repair the valve leaflets throughout adult life will facilitate the development of new strategies to treat heart valve defects and disease.

During heart development, at embryonic day 9.5 in the mouse, the endothelium overlying the endocardial cushions is stimulated to undergo EMT to provide interstitial cells to the developing valve leaflets. The cellular hallmarks of EMT are loss of endothelial cell-cell contacts, delamination of ECs from the endothelium, cellular migration into the underlying extracellular matrix, and induction of α -smooth muscle actin (α -SMA). Studies from chick and mouse embryos have shown that paracrine factors from the myocardium, which include TGF- β family members, are responsible for initiating EMT [1]. Notch1 has also been shown to be critical for EMT in the developing valves [2]. Human mutations in Notch1 have been linked

to bicuspid aortic valves and to onset of calcification in adult life [3]. A complex role for VEGF in valve development has been revealed by numerous studies[4;5;6;7].

We reported evidence for EMT in post-natal aortic and pulmonary valves and suggested EMT in adult valves represents a mechanism for replenishing the valve interstitial cells (VICs) of the leaflets as needed for repair and/or regeneration through out adult life [8;9]. In the present study, we used clonally-derived cell populations from ovine aortic valve leaflets to study the interplay between TGF- β , VEGF and Notch1 in regulating EMT in post-natal valves. We show that Notch1 mediates TGF- β -induced EMT while VEGF inhibits this process. These results suggest that in post-natal life, the pathways that operate in the endocardial cushion ECs during valve development can be re-activated, which suggests a plausible mechanism for replenishing the VICs of the valve leaflets on an as-needed basis.

Materials and Methods

Materials

Endothelial basal medium (EBM), Cambrex Bio Science; fetal bovine serum (FBS), Hyclone; glutamine-penicillin-streptomycin sulfate (GPS), trypsin-EDTA and other cell culture materials, Gibco-BRL. Human TGF- β s1-3, soluble TGF-receptor type II-B- Fc, human VEGF-A₁₆₅, R&D Systems; basic fibroblast growth factor (bFGF), Roche Applied Biosciences; Dulbecco's phosphate saline solution (PBS) and mouse anti-human α -SMA monoclonal antibody, Sigma-Aldrich; goat anti-human CD31 polyclonal antibody (M-20), Santa Cruz Biotechnology. NuPAGE Novex Bis-Tris gels (4-12 %) and MOPS SDS running buffers, Invitrogen; N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester, abbreviated DAPT, CalBiochem.

Clonal Valve ECs

Primary cultures from ovine aortic valve leaflets from juvenile sheep were prepared as described [8;9]. To obtain homogenous cell populations, primary cultures were plated as single cells in 96-well plates [8]. Out of several clones, an ovine aortic valve EC clone designated wav-1 was expanded on 1% gelatin-coated dishes in "EBM-B" medium (EBM, 10 % heat-inactivated FBS, 1 % GPS and 2 ng/ml bFGF). Experiments were performed using wav-1 cells up to passage 13-14. Two clonal populations from human pulmonary valve leaflets, E6 and E2, were isolated and found to be similar to wav-1 (Supplemental Fig 1).

Western analysis for EMT

Cells were lysed directly on the plate with 4 M urea, 0.5% SDS, 0.5% Nonidet P-40, 100 mM Tris (pH 7.4) and 5mM EDTA on ice, homogenized by pipetting up and down, and centrifuged at 12,000 rpm for 30 min at 4 °C. 40-50 μ g protein was loaded on a 4-12% gradient gel and run at 100 V for 2 h in MOPS SDS running buffer. Western blot transfer to PVDF membranes (Millipore) in 25 mM Tris base (pH 8.5), 0.2 M glycine, and 20% methanol was carried out at 300 mA for 1.5 h. After washing with deionized water and TBST buffer (10 mM Tris, pH 7.6, 233 mM NaCl and 0.1% Tween-20), the membrane was incubated with blocking buffer, 5% nonfat dried milk in TBST buffer, for 1 h at RT. Mouse anti-human α -SMA (1:2000) and goat anti-human CD31 (1:1000) were used to detect α -SMA and CD31, respectively. (Both antibodies were shown to cross-react with their ovine homologs.) The membrane was incubated overnight at 4°C and washed with TBST buffer for 25 min (2 $\times$ 10 min, 5 min). HRP coupled anti-mouse IgG (1:3000) and anti -goat IgG (1:3000) were applied for 1 h at 37 °C, washed as above, and developed by ECL method (LumiGLO, KPL).

Cell proliferation assay

Wav-1 cells were plated at 2000 cells/cm² in EBM-B. The next day, fresh EBM-B without or with 10 ng/ml VEGF, 1 ng/ml TGF- β 1 or 10 ng/ml VEGF plus 1 ng/ml TGF- β 1 was added. Cells were trypsinized at each time point and counted. Each condition was performed in triplicate; data are presented as the mean $\pm$ S.D.

RNA extraction and quantitative real-time PCR analysis

Wav-1 cells were plated at a density of 2000 cells/cm². After 24 hr, cells were treated with 1 ng/ml TGF- β 1 in fresh media. Total RNA was isolated using an RNeasy Mini Kit (Qiagen). Two μ g of RNA was treated with DNase I (Invitrogen) for 15 min at RT. The digestion was stopped with 1 μ l of 2 mM EDTA and incubated for 10 min at 65 °C. Reverse transcription reaction was performed with oligo-dT primer and Superscript II RNase H reverse transcriptase (Invitrogen) in 20 μ l. PCR was performed for Notch1, Slug, and GAPDH with iQ Sybr Green Supermix (Bio-Rad) in 20 μ l reaction volume in triplicate. The primers for Notch1 were: forward 5'- GTGCAGGGCAAGAAGGTC - 3' and reverse 5'- CACGTCTGCAGGTAGCCAT - 3'. The primers for Slug were: forward 5'- GAGGCCATTGGGTAGCTG-3' and reverse 5'-GCCTGTCATACCACAACCAG-3'. The primers for GAPDH were: forward 5'- TGCACCACCAACTGCTTAG- 3' and reverse 5'- GATGCAGGGATGATGTTC - 3'. PCR products were sequenced using ABI DNA sequencer (Children's Hospital Boston core facility).

NOTCH1- Δ EC-GFP transfection and sorting of GFP-positive cells

A cDNA construct encoding a ligand-independent form of Notch1 lacking the extracellular domain, NOTCH1- Δ EC, and green fluorescent protein (GFP) was inserted into the EcoRI and XhoI site of EGFP-N3 vector[10;11] (kindly provided by Dr. Gerry Weinmaster, UCLA). Wav-1 cells were cultured for 3-5 days until 90-100 % confluent, washed twice with 10 ml of serum free medium and then fed with EBM containing 20 % FBS and 2 ng/ml bFGF. 20 μ l TransfectinTM lipid reagent (Bio-Rad) mixed with 900 μ l EBM was incubated for 10 min at RT. 8 μ g NOTCH1- Δ EC-GFP construct was added to the mixture for 25 min. The NOTCH1- Δ EC-GFP- TransfectinTM complex was added drop-wise, gently, to the cell monolayer. After 24 h, cells were trypsinized, washed once with PBS, and filtered with 100 μ m cell strainer. GFP-positive cells were collected by fluorescence-activated cells sorting (FACS). NOTCH1- Δ EC-GFP expressing cells were plated on a 1% gelatin-coated dish in EBM, 20% FBS, 1% GPS and 2 ng/ml bFGF for further analysis.

Results

VEGF inhibits TGF- β 1- induced EMT in aortic valve ECs

The first experiment was to test the role of VEGF in TGF- β -mediated EMT (Fig. 1A) using induction of α -SMA to measure EMT. In ovine wav-1 cells, TGF- β 1 induced expression of α -SMA at 5 and 8 days, but the induction was blocked by 5 ng/ml VEGF (Fig. 1A). A VEGF dose response curve showed that 1 ng/ml VEGF had a partial inhibitory effect and that the effect was saturated at 10 ng/ml VEGF (data not shown). VEGF inhibition was seen in human pulmonary valve clones E2 and E6 (Supplemental Fig 1C), as well as in a previously described human pulmonary valve EC clones [9]. The ability of VEGF to reverse the mesenchymal cell morphology induced by TGF- β is shown in Supplemental Fig 1D.

VEGF inhibitory effect on EMT is mediated by VEGF-R2/KDR

To gain insight into which VEGF-receptor is required for the VEGF-mediated inhibition of EMT, we tested VEGF-receptor-selective variants[12]. KDR-sel was shown to have wild-type affinity for VEGF-R2/KDR but 2000-fold reduced affinity for VEGF-R1/Flt-1 whereas Flt-1-

sel was shown to have expected affinity for VEGF-R1 but a 470-fold reduced affinity for VEGF-R2/KDR. Western analysis shows that TGF- β 1-induced EMT was inhibited by KDR-sel but not by Flt-1-sel (Fig 1B). To substantiate this finding, we tested whether a small molecule receptor tyrosine kinase inhibitor with high selectivity for KDR/VEGF-R2 would reverse the inhibitory effect of VEGF on EMT. Results in Fig 1C show that the inhibitor, PTK787[13], reversed the effect of VEGF (compare lane 6 to lane 3). These results strongly demonstrate an ability of VEGF-A to inhibit TGF- β -mediated EMT by signaling through KDR/VEGF-R2.

TGF- β and VEGF-induced changes in cellular differentiation do not affect proliferation

To determine whether the effects on differentiation coincide with effects on proliferation, we measured cell growth over the 5 day time course in EBM-B, which contains 10% serum and 2ng/ml bFGF. Cells for each condition were initially plated in EBM-B alone for 24 h so that cell adhesion would take place under uniform conditions. Soluble factors were added at 24 h and cell numbers were determined at 24 h intervals. Fresh medium with factor was added to cells every two days. Cellular proliferation was equivalent in control medium and control medium plus 10 ng/ml VEGF, plus 1 ng/ml TGF- β 1 or plus 10ng/ml VEGF and 1 ng/ml TGF- β 1 (Fig 2). A population doubling occurred approximately every 24 h under these culture conditions indicating the healthy proliferative capacity of the valve endothelium. We also analyzed cell proliferation in control medium without bFGF (Fig 2) and found a modest reduction cellular proliferation, which suggests, as expected, that bFGF contributes to in vitro growth of valve ECs.

Positive role for Notch1 in post-natal valve EMT

Notch1 is known to play a critical role in EMT during embryonic valve development, but there have been no studies on Notch1 in adult valve ECs. Therefore, we investigated the role of Notch1 in EMT in adult cardiac valve endothelium using our clonal aortic valve ECs as a model system. First, we examined expression levels of Notch1 mRNA in wav-1 cells treated for various times with TGF- β 1. The results in Fig 3A show induction of Notch1 after 1-2 h. The increase was found to be 3-fold by real-time quantitative PCR, with a modest drop at 4-8 h and a return to levels ~3-fold above untreated cells at 1-2 days of treatment (data not shown). Levels of Slug mRNA followed a similar pattern (Fig 3A). Slug is a known target of TGF- β during EMT in the chick endocardial cushions [14] and has also recently been shown to be a target of Notch1 [15].

We next tested the effect of the γ -secretase inhibitor DAPT, a potent inhibitor of Notch1 activation, on TGF- β 1-mediated EMT. In the experiment shown, the human pulmonary valve endothelial clone E6 was tested; similar results were obtained with wav-1 cells in three independent experiments. E6 cells were treated with 2 ng/ml of TGF- β 2 for 8 days in the presence or absence DAPT. Fresh media with indicated factors were added every 2-3 days. Cells treated with VEGF alone (10ng/ml) and TGF- β plus VEGF served as controls (Fig 3B, lanes 1-4). DAPT alone had no effect on CD31 expression and did not induce α -SMA (lane 5) but DAPT did effectively block TGF- β 1-induced EMT, as seen by the negligible α -SMA (lane 6), providing an indication that Notch1 activation is downstream of TGF- β 1 in the EMT cascade in adult valve ECs.

To proceed further, we tested the effect of expressing a ligand-independent form of Notch1 in wav-1 cells to determine if Notch1 was sufficient to induce EMT. The gene construct (NOTCH1- Δ EC) produces a membrane-associated form of Notch1 that lacks the extracellular domain, and is thus ligand-independent. Activation of this Notch construct requires γ -secretase cleavage to release the intracellular domain of Notch1, which can be inhibited by DAPT [11]. In addition, the gene construct is a GFP fusion so that expression in cells can be followed by

GFP fluorescence. Wav-1 cells were transfected with NOTCH1- Δ EC and FACS sorted for GFP-positive cells after 24 h. Approximately 20% of the cells were significantly positive for GFP (data not shown). Sorted cells were collected, cultured in EBM-B for 6 days, and analyzed by indirect immunofluorescence (Fig 3C) and by Western blot (Fig 3D) for α -SMA, as an indicator of EMT. NOTCH1- Δ EC-positive cells underwent EMT, which was blocked by DAPT but not by cyclosporin A (CsA), a calcineurin inhibitor, or by a soluble TGF- β -receptor II that acts as a TGF- β antagonist. The lack of effect seen with these latter reagents rules out 1) the calcineurin pathway mediating effects downstream of Notch1 and 2) the possibility of Notch1 affecting autocrine secretion or bioavailability of TGF- β . In summary, the potent inhibition of TGF- β -mediated EMT by DAPT and the induction of EMT by NOTCH1- Δ EC expression provide evidence that Notch1 is necessary and sufficient for EMT in post-natal valve ECs.

Discussion

TGF- β family members, Notch1 and VEGF have all been shown to have critical roles in EMT in valve development [1]. In this study, we show that Notch1 and VEGF have opposing roles in regulating EMT in post-natal valve endothelial cells (Fig 4). Notch1 promotes EMT downstream of TGF- β based on the following data: 1) Notch1 mRNA was induced by TGF- β , 2) a ligand-independent form of Notch1 was sufficient to induce EMT, and 3) blocking Notch1 activation with the γ -secretase inhibitor DAPT prevented TGF- β -mediated EMT. In contrast, VEGF inhibited TGF- β -mediated EMT. Neither VEGF nor TGF β significantly affected cellular proliferation. Down syndrome critical region-1 (DSCR1), although induced by VEGF in valve ECs, did not appear to play a role in the VEGF-mediated inhibition of EMT (Supplemental Fig 2A-E). Overall, results were consistent between ovine and human valve ECs, as in previous studies [8;9]. A difference is that ovine valve EC will undergo EMT in response to all three TGF- β isoforms while human valve ECs undergo EMT in response to TGF- β 2 (Supplemental Fig 1A) [8;9]. Here, as in our previous studies, we show that valve EC treated with TGF- β 2 (human clones) or the TGF- β isoforms 1,2 or 3 (ovine clones) take on a mesenchymal phenotype that resembles valve interstitial cells. Consistent with the TGF- β effects, we showed previously that valve ECs express TGF- β -receptors I, II and III[9].

VEGF-A is an important regulator of EMT and valve elongation. Initially, VEGF expression in the myocardium is suppressed, producing conditions that favor EMT [6]. At later time points, VEGF-A was shown promote morphological and cellular changes consistent with EMT [16]. Hence, it appears that a finely-tuned balance of VEGF-A signaling is required to achieve appropriate levels of EMT and maintain a continuous endothelium as valve remodeling and leaflet elongation occur[7]. The importance of VEGF in valve development is supported by recent studies identifying single nucleotide polymorphisms in the VEGF gene associated with congenital valve defects [17].

Notch family members are transmembrane proteins that, upon ligand binding, undergo two proteolytic cleavage events to generate an intracellular Notch domain that can travel into the nucleus to regulate transcription and cell fate decisions. In arterial endothelial cells, Notch and its ligands were shown to be upregulated by VEGF [18]. In zebrafish, VEGF induction of Notch was shown to be required for arterial specification [19]. Hence, in these cellular contexts, VEGF and Notch appear to function in series to promote endothelial differentiation. In the developing cardiac valves, Notch1 was shown to promote EMT in both murine and zebrafish models: over expression of Notch 1 resulted in hypercellular valves (too much EMT) while prevention of Notch1 signaling blocked valve development [2]. Interestingly, Schroeder et al. [20] showed that Notch1 signaling inhibits embryonic stem cell differentiation into Flk1+ mesodermal progenitor cells and CD31-positive endothelial cell differentiation and instead favors mural cell differentiation [20]. This result is consistent with Notch1 playing a positive role in

promoting a mesenchymal phenotype in valve ECs. In summary, in some cellular contexts VEGF and Notch appear to function in a coordinated fashion to promote endothelial differentiation and vascular development [21], whereas in valve development, Notch1 promotes EMT; this activity in turn is opposed by VEGF to maintain an endothelial phenotype.

Our results with post-natal valve ECs indicate the developmental program used to form cardiac valves can be re-activated in post-natal valve endothelium. Human semilunar valve leaflets are composed of three distinct layers - the ventricularis at the inflow surface, spongiosa in the center, and fibrosa at the outflow surface – each layer contains VICs which are thought to be responsible for synthesizing and assembling the ECM. Deterioration of the ECM underlies many types of heart valve disease, suggesting that proper synthesis, assembly and maintenance of the ECM is essential for heart valve function. Therefore, further studies to elucidate the specific pathways that regulate cellular differentiation and proliferation in post-natal valves will be important for understanding causes of valve disease and deterioration and may perhaps lead to the development of new therapeutic approaches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations

EMT	endothelial-to-mesenchymal transformation
ECs	endothelial cells
VICs	valve interstitial cells
EBM	endothelial cell basal medium

TGF-β	transforming growth factor- β
VEGF	vascular endothelial growth factor
bFGF	basic fibroblast growth factor
FBS	fetal bovine serum
α-SMA	smooth muscle actin
DSCR1	Down Syndrome critical region-1
GFP	green fluorescence protein
FACS	fluorescence-activated cell sorting
PCR	polymerase chain reaction
DAPT	N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester

Figure 1. VEGF inhibits TGF- β -mediated EMT

(A) Wav-1 cells were treated with 10 ng/ml VEGF, 1 ng/ml TGF- β 1, or 10 ng/ml VEGF and 1 ng/ml TGF- β 1 in EBM-B for 5 days or 8 days and analyzed by Western blot using mouse anti- α -SMA and goat anti-CD31.

(B) Wav-1 were treated with TGF- β 1 as above and with 10ng/ml VEGF-receptor specific variants Flt-1-sel and KDR-sel [12] for 6 days. Cell lysates were analyzed as in A.

(C) Wav-1 cells were treated as above without or with 2 μ M PTK 787 and analyzed as in A.

Figure 2. VEGF and TGF- β do not alter valve EC proliferation in vitro

Wav-1 proliferation was measured in EBM-B without bFGF, EBM-B (control medium), EBM-B with VEGF, EBM-B with TGF- β 1, and EBM-B with VEGF and TGF- β 1 over five days. All points were performed in triplicate. Cell numbers are presented as the mean $\pm$ S.D.

Fig.3. Notch1 in EMT

(A) TGF- β 1 induces expression of Notch1 and Slug. Wav-1 cells were treated with 1ng/ml TGF- β 1 for indicated times and total RNA prepared for RT-PCR.

(B) Pharmacologic inhibition of Notch1 activation prevents TGF- β 2-induced EMT. E6 cells were treated without or with 10 μ M DAPT in the presence of TGF- β 2 or VEGF and TGF- β 2 for 6 days. Cell lysates were analyzed by Western blot with goat anti-CD31 and mouse anti- α -SMA.

(C and D) Ligand independent Notch1 (Notch1- Δ EC) expressing cells undergo EMT in aortic valve ECs. Notch1- Δ EC-GFP-positive cells were treated without or with 10 μ M DAPT, 5 μ M CsA or 30 ng/ml soluble TGF- β receptor type II antagonist (Sol T β RII) for 8 days. (C) Immunofluorescence was performed with anti- α -SMA. Nuclei were stained by DAPI (a-e).

(D) Western analysis was performed with goat anti-CD31 and mouse anti- α -SMA.

Fig. 4. Schematic of opposing actions of Notch1 and VEGF in aortic valve ECs
Over-expression of Notch1 induces EMT, blocking Notch activation inhibits EMT, and TGF- β 1, a potent stimulator of EMT, increases Notch1 expression. VEGF inhibits EMT.