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Losartan Inhibits Endothelial-to-Mesenchymal Transformation in Mitral Valve Endothelial Cells by Blocking Transforming Growth Factor- β -induced Phosphorylation of ERK

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Abstract

Adult cardiac valve endothelial cells (VEC) undergo endothelial to mesenchymal transformation (EndMT) in response to transforming growth factor- β (TGF β). EndMT has been proposed as a mechanism to replenish interstitial cells that reside within the leaflets and further, as an adaptive response that increases the size of mitral valve leaflets after myocardial infarction. To better understand valvular EndMT, we investigated TGF β -induced signaling in mitral VEC, and carotid artery endothelial cells (CAEC) as a control. Expression of EndMT target genes α -smooth muscle actin (α -SMA), Snai1, Slug, and MMP-2 were used to monitor EndMT. We show that TGF β -induced EndMT increases phosphorylation of ERK (p-ERK), and this is blocked by Losartan, an FDA-approved antagonist of the angiotensin II type 1 receptor (AT1), that is known to indirectly inhibit phosphorylation of ERK (p-ERK). Blocking TGF- β -induced p-ERK directly with the MEK1/2 inhibitor RDEA119 was sufficient to prevent EndMT. In mitral VECs, TGF β had only modest effects on phosphorylation of the canonical TGF- β signaling mediator mothers against decapentaplegic homolog 3 (SMAD3). These results indicate a predominance of the non-canonical p-ERK pathway in TGF β -mediated EndMT in mitral VECs. AT1 and angiotensin II type 2 (AT2) were detected in mitral VEC, and high concentrations of angiotensin II (AngII) stimulated EndMT, which was blocked by Losartan. The ability of Losartan or MEK1/2 inhibitors to block EndMT suggests these drugs may be useful in manipulating EndMT to prevent excessive growth and fibrosis that occurs in the leaflets after myocardial infarction.

Keywords

mitral valve; endothelial cells; endothelial to mesenchymal transformation; TGF β ; ERK; Losartan

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Introduction

EndMT is an essential step in cardiac valve formation during embryogenesis. A subset of endothelial cells lining the endocardial cushions in arterioventricular canal and outflow tract disengage from their neighbors, increase expression of α -SMA, migrate into the interstitial region between the endocardium and myocardium, and synthesize valve-specific extracellular matrix. TGF β is one important mediator of this orchestrated process[1]. Of note, EndMT is a relatively new term in the literature that refers specifically to endothelial-to-mesenchymal transformation[2], as opposed to EMT, which encompasses all types of epithelial to mesenchymal transformation.

In post-natal adult valves, endothelial cells that appear to be undergoing EndMT are seen in focal regions [3,4]. In vitro studies of aortic, pulmonary and mitral valve endothelial cells (VEC) demonstrated that ovine and human adult VEC undergo hallmarks of EndMT when treated with TGF β 1, 2 or 3 (ovine VECs) or TGF β 2 (human VECs) [3,4,5]. The potential relevance of post-natal EndMT was revealed in an experimental ovine model designed mimic mechanical forces imposed on the leaflets after myocardial infarction. A mechanical stretch imposed over 2 months on mitral valve leaflets significantly increased EndMT, coincident with increased size of the leaflets in vivo[5]. This lead us to propose that EndMT is part of an adaptive mechanism to increase leaflet size and thereby prevent or minimize mitral regurgitation after myocardial infarction.

TGF- β -stimulated EndMT has been examined in many types of cultured endothelial cells. Ghosh and colleagues showed signaling through the canonical SMAD pathway in murine cardiac endothelial cells with little involvement of the non-canonical ERK pathway [6]. SMAD signaling was also shown to be operative in a murine MS-1 endothelial cell line[7]. Several pathways - SMAD, MEK, PI3K, and p38 – were stimulated by TGF β 2 and shown to be required for EndMT in human skin endothelial cells[8]. In human umbilical vein endothelial cells, TGF β increased microRNA-21 and EndMT through an AKT-dependent mechanism[9]. This array of signaling pathways suggests that perhaps the endothelial cell type and the environmental context influence the signaling pathways used to initiate EndMT.

We focus on EndMT in mitral VEC as part of an on-going effort to understand how the mitral valve endothelium responds over time to the myriad of changes that occur in the heart after myocardial infarction. We were intrigued by the elegant studies by Dietz and colleagues[10,11,12] which showed a critical role for non-canonical TGF β signaling in aortic aneurysm formation in a murine model of Marfan syndrome. Excessive TGF β signaling in this model could be blocked by Losartan, a selective inhibitor of angiotensin II receptor-1 (AT1). By inhibiting AT1, Losartan shunts AngII signaling to AT2, which in turn provides a robust block on the phosphorylation of ERK. Indeed, Habashi and coauthors showed that AT2-mediated antagonism of ERK activity is required for Losartan to prevent aortic aneurysms in Marfan mice[11]. Thus, Losartan inhibits non-canonical TGF β signaling by an indirect inhibition of ERK activation.

Materials and methods

Mitral valve endothelial cells (VEC)

Clonal VEC populations from mitral valve leaflets from sheep were prepared as described [13] and expanded on 1% gelatin-coated dishes in endothelial basal medium-2 (EBM-2 (Lonza, cat # 3156), 10 % heat-inactivated FBS (Hyclone), 1X glutamine/penicillin/streptomycin (Life Technologies, Inc) and 2 ng/ml basic FGF (Roche Applied Science). This medium is referred to as EBM-B. Experiments were performed with mitral VEC clone C4 at passages 10 and 11, mitral VEC clone C5 at passages 9–12 and mitral VEC clone E10 at passages 11 and 12.

Non-valvular EC

Ovine carotid artery EC (CAEC), isolated as described [13], and endothelial colony forming cells (ECFC), isolated from ovine peripheral blood as described [14] served as a non-valvular endothelial controls. CAEC and ECFC were cultured under identical conditions as the mitral VECs for all experiments.

Inhibitors

Losartan (Cayman Chemical), dissolved in DMSO at 11.8mM, was tested at 1–50μM. RDEA119, an inhibitor of MEK [15] provided by Dr. Craig J. Thomas, NIH Chemical Genomics Center, NHGRI, National Institutes of Health, was dissolved in DMSO at 10mM and tested at 5–100 μM. SB-431542 hydrate (Sigma Aldrich) was dissolved in DMSO at 26mM. Inhibitors were stored in aliquots at –80C.

EndMT assay

Ovine mitral VEC and CAEC were plated at 10,000 cells/cm² on 1% gelatin-coated dishes in EBM-B. 24 hours after plating, fresh EBM-B containing 1ng/ml human recombinant TGFβ1 (R&D Systems, Inc) was added. Inhibitors were added 30 minutes before adding TGFβ1. Four days later cells were harvested for Western blots or for isolation of mRNA for reverse transcriptase-quantitative PCR (qPCR).

Western Blots

Cells were lysed, fractionated by SDS-PAGE and transferred to 0.45 PVDF membranes as described [13]. Blots were probed with goat anti-human CD31 (1:300) (M-20, Santa Cruz Biotechnology), goat anti-human VE-cadherin (1:300) (C-19, Santa Cruz Biotechnology, murine anti-human α-SMA (1:2000) (Sigma, mAb clone 1A4) and anti-tubulin (Sigma). Two different anti-p-ERK antibodies were used. The first was a murine mAb anti-phospho-44/42 MAPK (Erk1/2) (Thr202/Tyr204) and the second was a rabbit polyclonal anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204). Total ERK was detected using anti-p44/42 MAPK (ERK) mAb. Anti-phosphorylated-SMAD3 (p-SMAD3) (Ser423/425(clone C25A9) and anti-SMAD2/3 (3102), and all ERK pathway antibodies, were from Cell Signaling Technology. All antibodies were shown to cross-react with their ovine homologs. Band intensities on the western blots were quantified using Image Studio Lite software (LI-COR).

RNA extraction and Qpcr

Total RNA was isolated using an RNeasy Mini Kit (Qiagen), 1µg was treated with DNase I (Life Technologies). Reverse transcription was performed with Superscript III Reverse Transcriptase Kit (Life Technologies). qPCR was performed with Kapa Sybr Fast ABI Prism 2X qPCR Master Mix (Kapa Biosystems) in triplicate using StepOne Plus 96 well Real Time PCR system (Applied Biosystems). Results were normalized to ribosomal protein S9 amplified in the same experimental run. All PCR products were sequenced using ABI DNA sequencer (Dana Farber/Harvard Cancer Center DNA Resource Core) to verify the sequence corresponded to the gene of interest. Oligonucleotide primer sequences are shown in Supplemental Table 1.

Results

TGFβ1 induces rapid phosphorylation of ERK in mitral VEC

Ovine mitral VEC clones exhibit cobblestone morphology (Figure 1A), and express an endothelial phenotype [13]. Mitral VEC treated with TGFβ1 for 5 minutes showed robust p-ERK, which was inhibited when the cells were pre-incubated with the AT1 receptor antagonist Losartan (Figure 1B). The phosphorylation of ERK by TGFβ1 treatment was, as expected, blocked by the MEK1/2 inhibitor RDEA119 (Figure 1B). TGFβ1-induced p-ERK occurred within 5 minutes and was diminished by 15 minutes, while the onset of TGFβ-induced p-ERK in CAEC required 10 minutes, with increased p-ERK seen at 15 minutes (Figure 1D). Thus, TGFβ-induced activation of the non-canonical ERK pathway occurred rapidly in mitral VEC. Controls in which cells were moved out of the incubator, PBS added instead of TGFβ1, and the cells returned to the incubator for 5, 10 and 15 minutes verified that the rapid phosphorylation of ERK was not due to movement of the cells between the incubator and cell culture hood (data not shown).

Blocking TGFβ1-induced phosphorylation of ERK inhibits EndMT in mitral VEC

Mitral VECs were shown previously to undergo TGFβ1-mediated EndMT [5,13]. To determine whether EndMT relies on the non-canonical ERK signaling pathway, we tested the effect of Losartan and the MEK1/2 inhibitor RDEA-119 on TGFβ1-induced EndMT. Because a small number of contaminating fibroblasts in a primary culture of endothelial cells could contribute to the apparent increase in α-SMA in TGFβ-treated cultures, we used clonal populations of mitral VECs to insure that EndMT, and not fibroblast to myofibroblast activation, was assessed. Each experiment was conducted, and results confirmed, with three different ovine mitral VEC clones – C4, C5 and E10. A time course experiment showed that α-SMA expression, a marker of EndMT, was first detected in mitral VECs after 3 days of culture in TGFβ1, with maximal expression after 4 days (data not shown). This is the basis for analyzing EndMT after 4 days.

Mitral VECs were treated without or with TGFβ1 for 4 days in the presence or absence of Losartan or RDEA119. TGFβ1 strongly induced expression of α-SMA protein and caused a small decrease in VE-cadherin, both of which were blocked by inclusion of either Losartan or RDEA-119 over the 4 days (Figure 2A). The rapid phosphorylation of ERK in mitral VEC seen in Figure 1 prompted us to ask whether a short burst of TGFβ signaling would be

sufficient to induce EndMT. Mitral VEC were pulsed with 1ng/ml TGF β 1 for 30 minutes, TGF β 1 was removed, and cells were cultured for 4 days in EBM-B. α SMA was not induced (Figure 2B), which indicates that a 30 minute pulse of TGF β 1 signaling is not sufficient to induce EndMT.

We assessed additional markers of EndMT – the transcription factors Slug, Snai1 and NFATc1 and the matrix metalloproteinase MMP-2 - by qPCR in mitral VEC clones and CAEC treated for TGF β 1 for 4 days in the presence or absence of inhibitors. qPCR was performed instead of western blot because of a lack of commercially available anti-ovine antibodies for these proteins. Slug, Snai1 and MMP-2 were increased when mitral VEC C4 were treated with TGF β 1 for 4 days but not when cells were also treated with Losartan or RDEA-119 (Figure 2C). NFATc1 is a transcription factor expressed in the endocardial cushions that has been shown to mark endothelial cells that do not undergo EndMT and instead proliferate [16,17]; this indicates that NFATc1 is inversely correlated with EndMT. Indeed, NFATc1 levels were reduced in mitral VEC C4 treated with TGF β 1, but not when the cells were treated with TGF β 1 plus Losartan or plus RDEA-119. The same trends in induction of EndMT markers and suppression of NFATc1 can be appreciated when three experiments on three different mitral VEC clones (E10, C5 and C4) were compiled into one graph (Figure 2D). In contrast, mRNA levels of Slug, Snai1, MMP-2 and NFATc1 were not modulated in non-valvular CAEC treated for 4 days under identical conditions (Figure 2E). The inset verifies that α SMA was not induced in CAEC treated with TGF β 1 for 4 days. These results demonstrate that Losartan, which effectively blocks TGF β 1-mediated p-ERK, blocked EndMT. RDEA-119, a specific inhibitor of MEK1/2, which directly phosphorylates ERK, blocked EndMT as well. Thus, the non-canonical TGF β signaling pathway plays a critical role in initiating EndMT in mitral VEC.

TGF β 1-induced phosphorylation of ERK and SMAD3 in mitral VEC over four days

Additional experiments were performed to analyze TGF β -induced phosphorylation SMAD3 – the canonical pathway – in mitral VEC treated with or without TGF β 1 for 15 minutes, with or without Losartan. Consistent with Figure 1, Losartan blocked the TGF β 1-mediated increase in p-ERK but levels of p-SMAD3 were not modulated by TGF β 1 or by the inhibitors (Figure 3A). CAEC were analyzed in parallel to provide a positive control for the phosphorylation of SMAD3 and the effectiveness of the anti-p-SMAD3 mAb (Figure 3B). The TGF β R signaling inhibitor SB431542 decreased the level of p-SMAD3 in CAEC.

We assessed the phosphorylation status of ERK and SMAD3 in mitral VEC treated with TGF β 1 over a 4 day time course, in the presence or absence of Losartan or RDEA119, to determine the extent to which one or both pathways was active during EndMT. Cell lysates were prepared at 24, 48, 72 and 96 hours. The western blots and quantifications were similar for 24 and 48 hour time points, and for the 72 and 96 hour time points. Therefore we show the 48 and 96 hour time points only in Figure 3C and D. p-ERK was strongly increased by TGF β at 48 hours and modestly increased at 96 hours. Losartan and RDEA119 reduced TGF β -induced p-ERK at 48 and 96 hours and reduced basal levels of p-ERK at 96 hours. Levels of p-SMAD3 were increased less than two-fold by TGF β 1 at 48 hours and not at all at 96 hours. The fold increase was less than 2-fold at 24 hours and there was no increase at

72 hours (data not shown). In summary, analysis of p-ERK and p-SMAD3 levels over 4 days in response to TGF β revealed a marked increase in p-ERK at 24 and 48 hours, which was inhibited by Losartan or RDEA119. The drugs continued to reduce levels of p-ERK at 96 hours. Mitral VEC treated with TGF β 1 showed induction of α -SMA at 96 hours (Figure 3D), as expected. These results indicate that TGF β induced signaling through the non-canonical ERK pathway is ongoing and required for EndMT in this in vitro model.

Angiotensin II receptors in mitral VEC

Our finding that Losartan inhibited TGF β 1-mediated EndMT indicated that the ovine mitral VEC should express AT1 and AT2 receptors. Habashi and colleagues showed Losartan inhibition of AT1 in Marfan syndrome mice enhances signaling through AT2. Further, they showed that AT2 receptor-mediated inhibition of p-ERK is required for the beneficial effect of Losartan in preventing aortic aneurysm in these mice[11]. The schematic in Figure 4A depicts the shift from AT1 to AT2 signaling in the presence of Losartan. AT1 and AT2 receptors were detected by qPCR in three mitral VEC clones, CAEC and ovine peripheral blood endothelial colony forming cells (ECFC) (Figure 4B). Since AngII signaling through AT1 is known to stimulate expression of TGF β ligands and receptors, we tested the ability of angiotensin II to induce EndMT in mitral VECs over 4 days. Angiotensin II induced strong expression of α -SMA at 50uM (Figure 4C), but had no effect at lower concentrations of 0.01–10 uM (not shown). Angiotensin II induced expression of α -SMA was effectively blocked by Losartan (Figure 4C). These results indicate the AT1 and AT2 receptors are functional in mitral VECs.

Discussion

We show that EndMT in mitral VEC requires non-canonical TGF β 1 signaling via p-ERK. The canonical pathway, TGF β -induced phosphorylation of SMAD3, was detected, but at modest levels in comparison to TGF β -induced p-ERK. Losartan, an FDA-approved “ARB” (angiotensin receptor blocker) that dampens TGF β signaling by indirect mechanisms (Figure 4A), blocked TGF β 1-induced EndMT in mitral VEC. Direct inhibition of p-ERK with the MEK1/2 inhibitor RDEA119 also blocked EndMT. These results demonstrate a critical role for the non-canonical TGF β -induced EndMT in mitral VEC.

While SMAD-dependent TGF β signaling has been studied extensively, roles for p-ERK in TGF β signaling, specifically in the context of epithelial to mesenchymal transition (EMT) and EndMT, have emerged slowly. A report in 2004 first revealed a role for p-ERK in TGF β 1-induced EMT in normal murine mammary gland epithelial cells[18]. In 2006, phosphorylation of ERK was shown to be required for EndMT in the atrioventricular cushions of developing murine embryos [19]. Several studies on TGF β -induced EndMT in cultured cells were published in 2012 [6,7,8,9]. SMAD-dependent signaling was implicated in three of these studies, while one study indicated that multiple pathways including SMAD, ERK, PI3K and p38 MAPK were activated in EndMT in human endothelial microvascular endothelial cells[8].

We focus on EndMT in a specific cellular context –the mitral valve endothelium – and when and how VEC re-activate EndMT to adapt to changing hemodynamics and/or cytokines after

myocardial infarction. In humans and in sheep, the mitral valve leaflets both lengthen and thicken over time after myocardial infarction due to displacement of the papillary muscles caused by remodeling in the left ventricular myocardium. The increase in leaflet size is initially beneficial as it can minimize mitral regurgitation, but over time fibrosis sets in, disrupting the mitral valve seal. We asked whether EndMT might occur as part of this adaptive response and found that EndMT was significantly increased in mitral valve endothelium in an ovine model in which mechanical stretch was imposed on the mitral valve leaflets for 2 months [5]. This study was the first to show evidence for EndMT in the mitral valve endothelium, associated with increased leaflet size, and suggested that active manipulation of EndMT may provide a therapeutic approach to boost or modulate compensatory mechanisms.

This in vivo finding prompted us to examine the effect of Losartan on EndMT in mitral VEC in vitro, to probe intracellular signaling pathways that might be involved in this process. Losartan is an FDA-approved “ARB” or angiotensin receptor blocker. It is used widely to treat hypertension and is also being tested in clinical trials for ability to prevent aortic aneurysms in patients with heritable disorders that result in too much TGF β signaling in the aortic wall[20]. Losartan proved to be a potent inhibitor of EndMT in mitral VEC. Whether Losartan could be used strategically in vivo to modulate EndMT in the mitral valve will require in vivo studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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The abbreviations used are

VEC	valve endothelial cells
EndMT	endothelial to mesenchymal transformation
TGFβ	transforming growth factor- β
CAEC	carotid artery endothelial cells
α-SMA	α -smooth muscle actin
AT1	angiotensin II type 1 receptor
AT2	angiotensin II type 2 receptor
SMAD	signaling mediator mothers against decapentaplegic homolog
Ang II	angiotensin II
EBM	endothelial basal medium

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Highlights

- TGF β 1 induces rapid phosphorylation of ERK1/2 but not SMAD3 in mitral valve endothelial cells.
- Losartan, a drug known to dampen TGF β signaling, blocks phosphorylation of ERK1/2.
- Losartan blocks EndMT in mitral valve endothelial cells.
- TGF β 1-induced phosphorylation of ERK1/2 is required for mitral valve EndMT.
- Losartan may be useful for modulating excessive EndMT in mitral valve diseases.

FIGURE 1. TGFβ₁ induced phosphorylation of ERK in mitral VEC

A, Phase contrast image of ovine mitral VEC grown in EBM-B. B, Western blot of P-ERK and ERK in mitral VEC pre-treated for 30 minutes with 0, 1 or 10 μM Losartan, as indicated, before addition of 1ng/ml TGFβ₁ for 5 minutes. C, Western blot of P-ERK and ERK in mitral VEC pretreated for 30 minutes with 60μM RDEA-119 before addition of TGFβ₁ for 5 minutes. D, Time course of phosphorylation of ERK in mitral VEC and carotid artery EC (CAEC) treated with 1 or 5 ng/ml TGFβ₁. Tubulin, loading control.

FIGURE 2. Losartan and the MEK1/2 inhibitor RDEA-119 inhibit TGFβ1-induced EndMT in mitral VEC

A, Western blot of VE-cadherin, α-SMA and tubulin in mitral VEC treated ± TGFβ1 for 4 days without or with Losartan, without or with RDEA-119 as indicated. B, Mitral VEC as in A, but treated with TGFβ1 for 30 minutes. After 30 minutes, VEC were washed with PBS, and cultured for 4 days in EBM-B. C, Mitral VEC clone 4 (C4) treated as described in A. Untreated cells (Control), cells treated with TGFβ1 for 4 days (TGFβ1), cells treated with TGFβ1 + Losartan (+Losartan) and cells treated with TGFβ1 + RDEA-119 (+RDEA-119) were analyzed by qPCR for EndMT markers Slug (black bars), Snail1 (light grey bars), MMP-2 (white bars) and NFATc1 (dark grey bars). Ribosomal protein S9 was analyzed in parallel as a house keeping gene and used for normalization. D, qPCR data compiled from experiment in A for mitral valve clones C4, C5 and E10. E,

CAEC treated as in A, analyzed by qPCR. Inset shows western blot for CD31 (endothelial marker) and α SMA (EndMT marker) in CAEC treated $\pm$ TGF β 1 for 4 days.

FIGURE 3. P-ERK and P-SMAD3 in mitral VEC

A, Western blot of P-ERK, ERK, P-SMAD3 and SMAD2/3 in mitral VEC C5 pretreated $\pm$ 50 μ M Losartan for 30 minutes and treated $\pm$ 1ng/ml TGFβ₁ for 15 minutes, as indicated. B, Western blot of p-SMAD3 and SMAD2/3 in CAEC pretreated for 30 minutes $\pm$ 10 μ M SB-431542 and treated $\pm$ 1ng/ml TGFβ₁ for 15 minutes, as indicated. C and D, Western blots of mitral VEC clone E10 treated for 48 hours (C) or 96 hours (D) $\pm$ TGFβ₁, $\pm$ Losartan, $\pm$ RDEA-119 as indicated. Tubulin, loading control. Band intensities were quantified using Image Studio Lite software (LI-COR). The P-ERK/ERK and P-SMAD3/SMAD2/3 levels and ratios in untreated (control) mitral VEC were set to 1.0.

FIGURE 4. AT1 and AT2 receptors in mitral VEC

A, Schematic showing Losartan effects on AT1 and AT2 receptor signaling and pERK. From “Angiotensin II Type 2 Receptor Signaling Attenuates Aortic Aneurysm in Mice Through ERK Antagonism” by Jennifer P. Habashi, Jefferson J. Doyle, Tammy M. Holm, Hamza Aziz, Florian Schoenhoff, Djahida Bedja, YiChun Chen, Alexandra N. Modiri, Daniel P. Judge, Harry C. Dietz, published in *Science*, 2011, vol. 332, issue 6027. Printed with permission from AAAS. B, Quantitative RT-PCR of AT1 (black bars) and AT2 (gray bars) mRNA in ovine CAEC, ECFC and three clones of mitral VEC, normalized to β-actin. C,

Western blot for VE-cadherin, α -SMA and tubulin in mitral VEC clone E10 $\pm$ 50uM Losartan and $\pm$ angiotensin II (50uM) for 4 days. Tubulin, loading control.