CD45 Expression in Mitral Valve Endothelial Cells After Myocardial Infarction


Rationale: Ischemic mitral regurgitation, a complication after myocardial infarction (MI), induces adaptive mitral valve (MV) responses that may be initially beneficial but eventually lead to leaflet fibrosis and MV dysfunction. We sought to examine the MV endothelial response and its potential contribution to ischemic mitral regurgitation.

Objective: Endothelial, interstitial, and hematopoietic cells in MVs from post-MI sheep were quantified. MV endothelial CD45, found post MI, was analyzed in vitro.

Methods and Results: Ovine MVs, harvested 6 months after inferior MI, showed CD45, a protein tyrosine phosphatase, colocalized with von Willebrand factor, an endothelial marker. Flow cytometry of MVs revealed significant increases in CD45+ endothelial cells (VE-cadherin+/CD45+/α-smooth muscle actin [SMA]+ and VE-cadherin+/CD45+/αSMA− cells) and possible fibrocytes (VE-cadherin+/CD45+/αSMA+) in inferior MI compared with sham-operated and normal sheep. CD45+ cells correlated with MV fibrosis and mitral regurgitation severity. VE-cadherin+/CD45+/αSMA− cells suggested that CD45 may be linked to endothelial-to-mesenchymal transition (EndMT). MV endothelial cells treated with transforming growth factor-β1 to induce EndMT expressed CD45 and fibrosis markers collagen 1 and 3 and transforming growth factor-β1 to 3, not observed in transforming growth factor-β1–treated arterial endothelial cells. A CD45 protein tyrosine phosphatase inhibitor blocked induction of EndMT and fibrosis markers and inhibited EndMT-associated migration of MV endothelial cells.

Conclusions: MV endothelial cells express CD45, both in vivo post MI and in vitro in response to transforming growth factor-β1. A CD45 phosphatase inhibitor blocked hallmarks of EndMT in MV endothelial cells. These results point to a novel, functional requirement for CD45 phosphatase activity in EndMT. The contribution of CD45+ endothelial cells to MV adaptation and fibrosis post MI warrants investigation. (Circ Res. 2016;119: 1215-1225. DOI: 10.1161/CIRCRESAHA.116.309598.)

Key Words: CD45 antigen ■ endothelial cell ■ endothelial cell differentiation ■ flow cytometry ■ mitral valve ■ myocardial infarction

Ischemic mitral regurgitation (IMR) is a common complication after myocardial infarction (MI) that doubles mortality.1 IMR is caused by left ventricular remodeling and dysfunction, which leads to papillary muscle displacement and tethering of the mitral valve (MV), restricting leaflet closure.2 Tethered MVs adapt by increasing their surface area, but this adaptation is often insufficient and seems to result in stiff, fibrotic valves, which may ultimately contribute to increasing IMR.3,5

Several cellular events occur in the IMR MV: MV endothelial cells (mitral VECs) undergo endothelial-to-mesenchymal transition (EndMT), MV interstitial cells (mitral VICs) are activated to become myofibroblasts that secrete and compact extracellular matrix, and there is evidence for valve neovascularization and leukocyte infiltration.2,5–8 Infiltrating macrophages and leukocytes release growth factors and cytokines, such as transforming growth factor-β (TGFβ) family members, which can promote angiogenesis, collagen

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production, attraction of additional inflammatory cells, and transform interstitial cells to myofibroblasts that also secrete growth factors and cytokines. Thus, TGFβ isoforms may also be produced endogenously by the MV cells.7,11

In this work, we used an ovine inferior MI (IMI) model to study the MV endothelium in the context of valve growth and fibrosis in response to IMR. Immunohistochemistry and flow cytometry of MV leaflets from infarcted sheep revealed endothelial cells positive for the leukocyte marker/protein tyrosine phosphatase (PTPase) CD45. Flow cytometry further revealed CD45+/α-SMA+ cells, which may be fibrocytes,12,13 and CD45+ hematopoietic cells in the MV, all of which might contribute to development of fibrosis in IMR. Fibrocytes are circulating CD45+ myeloid cells that migrate into sites of inflammation or tissue injury and, under the influence of cytokines and TGFβ, transform into α-SMA+ myofibroblast-like cells.14 In vitro, CD45 was induced in several ovine mitral VEC clones by the fibrogenic factor TGFβ1, which potently induces EndMT in these cells.14,15 Blocking CD45 PTPase activity pharmacologically inhibited expression of EndMT and fibrosis markers and EndMT-associated enhanced migration in mitral VEC. TGFβ did not induce CD45, EndMT, or fibrosis markers or migration in ovine carotid artery endothelial cells (CAEC), indicating specificity. Our findings uncover an unanticipated role for CD45 in TGFβ-stimulated EndMT and suggest that mitral VECs may be a source of fibroctic cells in IMR.

Methods

Animal Model

A total of 13 Dorsett hybrid sheep were analyzed. The IMI sheep (n=5) had ligation of their second and third obtuse marginal branches of the left circumflex coronary artery. Two- and 3-dimensional echocardiography was performed before MI and 30 to 60 minutes after MI. Six months post MI, echocardiograms were repeated to assess mitral regurgitation (MR), and the animals euthanized and their MVs excised. The excised MV leaflet tissue was immediately submerged in a solution of 5% heat-inactivated fetal bovine serum, 4% Penicillin/Streptomycin (R&D Systems; #FC004) and labeled in Flow Cytometry Permeabilization/Wash Buffer 1 (R&D Systems; #FC005) for 45 minutes (100000 cells/100 µL buffer I). Murine anti-sheep CD45-fluorescein isothiocyanate or -APC (1:50; AbD Serotec; # MCA220F, MCA220GA, and MCA896GA and LYNX Rapid APC Antibody Conjugation Kit; #LNK031APC), VE-cadherin-PE or -fluorescein isothiocyanate (1:100; R&D Systems; # FAB9381P and AbD Serotec; # AHP628F), and α-smooth muscle actin (α-SMA)-APC or -PE (1:100; R&D Systems; # IC1420A) were used. All antibodies were shown to cross-react with their ovine homologs.

Cell Culture

Ovine mitral VEC clones and CAEC were isolated14 and grown on 1% gelatin-coated dishes in EB-M-2 medium (Lonza Inc; #CC-3156) supplemented with 10% heat-inactivated fetal bovine serum, 1% glutamine–penicillin–streptomycin sulfate (Life Technologies; cat# 10378-016), and 2 ng/mL basic fibroblast growth factor (Roche Life Science, Inc; cat # 11123149001), henceforth referred to as EB-M-B.

EndMT Assay

Ovine mitral VEC and CAEC were plated on gelatin-coated plates at 10000 cells/cm². After 24 hours, EB-M-B was replaced with fresh EB-M-B-containing 1 ng/mL human TGFβ1 (R&D Systems; #100-B-001). Cells were harvested with Liberase (100 µL/cm²) 96 hours later and used for flow cytometry, quantitative polymerase chain reaction, or migration assays. For flow cytometry, cells were analyzed simultaneously for VE-cadherin, α-SMA, and CD45 as described above.

Quantitative Polymerase Chain Reaction

Ovine mitral VEC and CAEC were subjected to the EndMT assay in the presence or absence of 0.5 µmol/L N-(9,10-dioxo-9,10-dihydrophenanthren-2-yl)-2,2-dimethyl propionamide, a CD45-selective PTPase inhibitor (EMD Millipore Sigma, MA; # 540215).21 Total cellular RNA was extracted from mitral VEC clones (C5, D1, E5, E10, and E10-2) and CAEC (n=2) with an RNaseasy Micro extraction kit (Qiagen, Valencia, CA; #74004). Reverse transcriptase reactions were performed using an iScript cDNA Synthesis Kit (Bio-Rad, CA; #170-8890). Quantitative polymerase chain reaction was performed using Kapa Sybr Fast ABI Prism 2x quantitative polymerase chain reaction Master Mix (KAPA BioSystems, MA; # KK4604). Amplification was performed in an ABI 7500 (Applied Biosystems, Foster City, CA). A standard curve for each gene was generated to determine amplification efficiency. RPS9 was used as housekeeping gene expression reference. Fold increases in gene expression were
calculated according to ΔΔCt method,22,23 with each amplification reaction performed in triplicate.

Cellular Migration Assay
Mitral VEC clones (D1, E5, E10-2) or CAEC were treated±TGFβ for 4 days to induce EndMT. The cells were treated for 30 minutes in the CD45-selective PTPase inhibitor (1 μmol/L)21 before trypsinization. Twenty thousand cells (±PTPase inhibitor 1 μmol/L) in 0.1% BSA/EBM-2 (Lonza) were placed in the upper chamber of 6.5 mm Transwells containing fibronectin-coated (0.2 μg/cm2) polycarbonate membranes with 8.0 μm pores. The lower chambers contained 0.1% BSA/EBM-2 media alone or EBM with serum and basic fibroblast growth factor as chemoattractants. Cells were allowed to migrate for 6 hours at 37 °C. Cells that migrated through the pores were fixed with methanol and stained with Eosin-Y, Azure A, and Methylene Blue for visualization and quantification using Three Step Stain Set (VWR, PA; #48218–567). In parallel, an aliquot of cells used for the migration assay were also analyzed for CD45 by flow cytometry to verify response to TGFβ1.

Statistics
Sample variances were analyzed by Fisher tests to determine equal or unequal variances. A Fisher P value >0.05 was considered equal variance. In Table, equal variances were found for all comparisons. Two-tailed, 2-sample t tests were performed. In Figures 4 and 5, fold changes are reported as means±SD. In Figure 4, quantitative polymerase chain reaction data from 5 different MV VEC clones were standardized as described in the study by Willems et al.24 In Figures 4 and 5, data were analyzed by 1-way ANOVA, Fisher tests, and 2-tailed 2 independent sample t tests. Statistical programs were from Excel and XLStat Pro. In Figure 6, linear regression analysis was performed, and the R2 value was calculated to see how well the regression line fit the data. P<0.05 was considered significant.

Results
CD45 Expression in MV Endothelium Post MI
A total of 5 sheep underwent IMI and were euthanized 6 months after the procedure. All infarcted sheep developed MR, as determined by color-Doppler echocardiography at the time of euthanasia. Sham (n=5) and normal (n=3) sheep were analyzed as well (Online Table I). Because of the known inflammatory state associated with MI, we assessed the presence of leukocytes in MV by staining for CD45, a receptor-like protein tyrosine phosphatase (PTPase) expressed in all leukocytes. Strong immunostaining for CD45 was seen on the endothelial and subendothelial layers on the atrial side of the IMI MVs and the interstitial regions (Figure 1A and 1D). CD45 expression was less evident in control MVs (Figure 1B) and was not detected in IMI MV stained with an isotype-matched control IgG (Figure 1C). For comparison, CD14, expressed on macrophages and, to a lesser extent, neutrophils, was detected in the interstitial region of IMI MVs but not along the endothelium (Figure 1E). CD45 was not detected in MVs from a MR-only model, in which there was no MI, only a posterior leaflet retraction to produce MR (Figure 1F). Double immunofluorescent staining of IMI MVs showed CD45 colocalized with von Willebrand factor, a glycoprotein expressed in endothelial cells25 (Figure 1G-J). Results from these 2 immunostaining experiments indicated that mitral VECs might contribute to the CD45+ cell population in IMI MVs. CD45 was also partially colocalized with αSMA+ cells in IMI MV leaflets (Online Figure 1A). On the basis of this, we hypothesized that some of the CD45+ cells could be endothelial cells undergoing EndMT or fibrocytes, which are myeloid fibroblasts that express αSMA and produce collagen.12,26 αSMA and increased collagen are both hallmarks of MV adaptation to IMR.26

Quantification of CD45-Positive Cells in MVs by Triple-Label Flow Cytometry
To pursue the endothelial-localized CD45 expression, we devised a method to gently release cells from MV leaflet tissue immediately after removal from sheep and quantify the relative proportions of endothelial, interstitial, and hematopoietic cell populations. MV leaflet tissue was minced into small pieces and digested with a mix of type I and II collagenases for 30 minutes at 37 °C to generate a single-cell suspension. Cells were permeabilized and simultaneously stained. Cells were permeabilized and simultaneously stained.
significantly increased in IMI MVs compared with sham MVs (Table). VE-cadherin+/αSMA+ cells, which we designated fibrocytes,27,28 were also significantly increased in IMI MVs compared with sham MVs (Table).

The flow cytometric analysis provided additional information on the cellular composition of the ovine MV and how it changed after IMI. Cells with a quiescent VIC phenotype, VE-cadherin+/αSMA−, were the most abundant in the MV

Figure 1. CD45 detected in the mitral valve endothelium post myocardial infarction (MI). A–F. Immunohistochemical staining of mitral valve (MV) leaflets: anti-CD45 staining (A, B, D, F), control IgG staining (C), and anti-CD14 staining (E). Ovine MV leaflets from animals 6 mo after IMI (A, C, D, E), control animals (B), and mitral regurgitation (MR)–only animals (F). G–J, Immunofluorescence staining of 6-mo IMI MV leaflets. G, Double staining with anti-CD45 and anti–von Willebrand factor (vWF), an endothelial marker. Arrows mark endothelial cells coexpressing CD45 and vWF. H, Single staining with anti-vWF. I, Single staining with anti-CD45 and (J) DAPI staining only.

Figure 2. CD45/VE-cadherin/αSMA analysis of mitral valve (MV) cells 6 mo after inferior myocardial infarction (IMI). A representative flow cytometric analysis is shown. Top. The labeling for VE-cadherin, αSMA, and CD45. VE-cadherin+/CD45+ cells (pink-shaded box) were analyzed in the APC-channel (green-shaded box) for αSMA-positive cells. This shows the distribution of VE-cadherin+/CD45+ cells into αSMA and αSMA−subpopulations. Bottom row shows cells labeled with isotype-matched IgGs to establish background staining. A summary of the complete flow cytometric analysis is shown in Table.
but were not significantly different between IMI and sham MVs (Table). Activated VICs (VE-cadherin−/CD45−/αSMA+) were detected in both sham and IMI valves but also did not differ significantly by this method of analysis. Hematopoietic cells, defined as VE-cadherin−/CD45+/αSMA−, were present and not significantly changed. From Table, one can calculate that MV endothelial cells constituted 64% of the total CD45+ cells, hematopoietic cells 21%, and fibrocytes 14% in this 6-month IMI model. This demonstrates that at 6 months post IMI, the majority of the CD45+ cells in the MV are endothelial cells.

Figure 3. Flow cytometry of mitral valve endothelial cell (VEC) clones and nonvalvular carotid artery endothelial cell (CAEC). Mitral VEC clone E10 (A and B) and CAECs (C and D) treated with EBM-B (A and C) or EBM-B+1 ng/mL transforming growth factor-β1 (TGFβ1; B and D) for 4 d to induce EndMT. E, Four additional mitral VEC clones treated with EBM-B+1 ng/mL TGFβ1 for 4 d. Each mitral VEC clone, E5, D1, I4, and C5, was analyzed for VE-cadherin and CD45 to show the range of CD45 expression among mitral VEC clones.
For comparison, the flow cytometry analysis was also performed on MVs from normal sheep (Online Table II). The normal MVs comprised primarily VE-cadherin+/CD45−/αSMA− endothelial cells (38%) and VE-cadherin−/CD45−/αSMA− cells (57%), which we designate quiescent VICs. There were few CD45+ or α-SMA+ cells indicating a quiescent, noninflammatory, nonfibrotic state.

**TGFβ1 Induces CD45 in Mitral VEC, Coincident With EndMT**

The presence of CD45 and αSMA in VE-cadherin–positive cells in IMI MVs suggested that CD45 induction may coincide with EndMT processes. Our previous work showed increased EndMT, also called EMT, in tethered MVs in an ovine model and suggested EndMT as a possible contributor to growth of MV leaflets to minimize MR. To assess the link between EndMT and CD45, mitral VEC clones (prepared by expansion from a single mitral VEC) and CAECs (incapable of undergoing EndMT) were tested for CD45 expression in response to TGFβ1. In vitro treatment of mitral VEC clone E10 with TGFβ1 led to strong induction of CD45 and αSMA, detected by flow cytometry (Figure 3A). Interestingly, 23% to 25% of the mitral VEC clone E10 cells showed a low level of CD45 expression before TGFβ1 treatment (Figure 3A).

**Figure 4. Increased endothelial-to-mesenchymal transition (EndMT) and fibrosis markers blocked by CD45 protein tyrosine phosphatase (PTPase) inhibitor.** A and B, CD45, VE-cadherin, and αSMA mRNA levels in mitral valve endothelial cell (VEC) clones and carotid artery endothelial cell (CAEC) measured by quantitative polymerase chain reaction (qPCR). Cells were treated for 4 d without (gray bars) or with (black bars) transforming growth factor-β1 (TGFβ1). C, Mitral VEC clones were treated±TGFβ1 (1 ng/mL) and ±CD45 PTPase (0.5 µmol/L) for 4 d. mRNA levels of αSMA (black), Slug (blue), MMP-2 (orange), NFATc1 (teal), collagen 1 (yellow), collagen 3 (gray), TGFβ1 (red), TGFβ2 (green), and TGFβ3 (light blue) were measured by qPCR. Closed symbols indicate P values between control and TGFβ1 treatment groups; open symbols indicate P values between TGFβ1 and TGFβ1+CD45 PTPase inhibitor treatment groups. D, CAECs were analyzed as in (C).
In TGFβ1 nontreated VEC clones, CD45+ cells ranged from 3% to 40% (not shown). In total, 7 mitral VEC clones were studied: CD45 was significantly increased after 4-day exposure to TGFβ1 \( (P=0.029 \text{ by paired } t \text{ test}). \) αSMA was also significantly increased in TGFβ1-treated mitral VEC clones, as expected \( (P=0.007 \text{ by paired } t \text{ test}; \text{ data not shown}). \)

To determine whether other hematopoietic markers were increased in TGFβ1-treated mitral VEC, we analyzed expression of CD11b (expressed on monocytes, neutrophils, natural killer cells, granulocytes, and macrophages) and CD14 (expressed on macrophages, neutrophils, and dendritic cells) by flow cytometry (Online Figure II). No expression was detected, whereas CD45 was increased as expected. These results demonstrate that purified mitral VECs specifically express CD45, and the levels are significantly increased by TGFβ1.

**CD45 PTPase Inhibitor Blocks Expression of EndMT and Fibrosis Markers**

To verify the increased CD45 detected by flow cytometry, we analyzed CD45 mRNA in TGFβ1-treated mitral VEC and CAEC by quantitative polymerase chain reaction (Figure 4A, B). In parallel, VE-cadherin and αSMA were measured to assess EndMT. Data compiled from 5 different mitral VEC clones showed significant increases in CD45 \( (P=0.0001) \) and αSMA mRNA \( (P=0.0131; \text{ Figure } 4A). \) No changes in CD45, VE-cadherin, or αSMA mRNA levels were seen in TGFβ1-treated CAEC (Figure 4B). Well-established EndMT markers, Slug and MMP-2, and NFATc1, which is negatively correlated with EndMT, were modulated as expected in TGFβ1-treated mitral VEC, consistent with our previous study. Collagen 1 and collagen 3 mRNA transcripts and TGFβ1, TGFβ2, and TGFβ3 were also increased in TGFβ1-treated mitral VEC clones (Figure 4C). Inclusion of a CD45-selective PTPase (0.5 μmol/L) during the 4-day TGFβ1 treatment significantly reduced αSMA, Slug, MMP-2, collagen 1, collagen 3, TGFβ1, TGFβ2, and TGFβ3 mRNA levels and restored NFATc1 mRNA (Figure 4C). P values for Figure 4C are provided in Online Table III. In contrast, no changes in these markers were seen in TGFβ1-treated CAEC, in the presence or absence of the CD45 PTPase inhibitor (Figure 4D). These
results suggest that CD45 plays a functional role in EndMT and transition of mitral VEC to a fibrotic phenotype.

**CD45 PTPase Inhibition Reduced EndMT-Associated Migration**

Increased migration is a hallmark of endothelial cells undergoing EndMT. Therefore, we examined the requirement for ongoing CD45 phosphatase activity in migration of endothelial cells induced to undergo EndMT (Figure 5). Mitral VEC and CAEC were treated without (gray bars) or with TGFβ1 for 4 days (black bars). Cells were then treated with or without CD45 PTPase inhibitor (1.0 μmol/L) for 30 minutes, removed from culture dishes with trypsin, and resuspended in EBM without serum and growth factors. Cells were assayed for migration toward serum and basic fibroblast growth factor in the 6-hour migration assay (n=3) showed significantly increased migration toward serum and basic fibroblast growth factor (P=0.0149), which was significantly inhibited by the CD45 PTPase inhibitor (P=0.0100; Figure 5A). Both control and TGFβ1-treated CAEC showed modest but significantly increased migration toward serum and basic fibroblast growth factor in the 6-hour migration assay (P=0.0249; P=0.0141, respectively) but showed no increase in migration after TGFβ treatment and no response to the CD45 PTPase inhibitor (Figure 5B). An aliquot of mitral VEC and CAEC used for the migration assay was assayed for CD45 by flow cytometry to verify that CD45 was expressed in the mitral VEC and not in the CAEC (data not shown). These results further suggest that CD45 plays a functional role in EndMT.

**Increase in CD45+ Cells at 6 Months Post IMI**

To determine whether CD45+ MV cells were associated with detrimental impacts, MVs were analyzed histologically and functionally. To assess collagen accumulation, which would stiffen the MV leaflets and impair their ability to form an effective seal to prevent MR, MV sections from sham (n=5) and 6-month IMI (n=5) sheep were analyzed by Masson trichrome stain (Figure 6A). Quantification of the percent positive area of positively stained areas of collagen showed significantly increased collagen in the 6-month IMI MV (Figure 6B), which correlated with increased CD45+ cells as in (E), G. Ejection fraction (%) plotted against the same CD45+ cells as in (E) and (F). D–G, Linear regression analysis was performed, and the R² value was calculated to see how well the regression line fit the data. P<0.05 was considered significant.
measuring the total LV endocardial surface area ratio between immediately post MI (T1) and after 6 months (T2) by 3-dimensional echocardiography. 16–19 This remodeling ratio showed a strong positive correlation with the MV CD45+ cells (R²=0.79; P<0.001). This suggests that not only the infarct size but left ventricular remodeling exerts an effect on the MV and in turn the number of CD45-expressing cells in the leaflets. The cell-modifying stimulus could be attributed to prolonged cytokine release occurring after LV damage and failure, as has been described. 26 CD45+ cells showed a negative correlation with ejection fraction (R²=0.71; P=0.002; Figure 6G).

Discussion

In this study, we identify an unanticipated expression of CD45 in mitral VEC both in vivo post MI and in vitro in response to TGFβ1. At 6 months post MI, CD45+ endothelial cells were the most abundant CD45+ cell population in the MV. This was determined by flow cytometry, an objective and quantitative method performed on single-cell preparations from collagenase-digested anterior and posterior MV leaflets. A large fraction of the CD45+ endothelial cells coexpressed αSMA, which suggested that the cells were undergoing EndMT. CD45+/αSMA+ cells were also detected and significantly increased in 6-month IMI MVs. The increases in CD45+ cells correlated with MV fibrosis, MR severity, and infarct size. In vitro, ovine mitral VECs expressed a low level of endogenous CD45, which was increased significantly by TGFβ1, with concomitant, significant increases in αSMA, additional EndMT markers, and collagen 1, collagen 3, TGFβ1, TGFβ1, and TGFβ3; such cytokine self-amplification has been described previously. 26 A CD45-selective PTPase inhibitor modulated expression of all of these markers. The CD45 PTPase inhibitor also blocked EndMT-associated increased migration of mitral VEC. These assays were conducted on several different mitral VEC clones, indicating robust findings. Combined, these results suggest a functional role for CD45 in EndMT and induction of a collagen-producing, TGFβ-producing cellular phenotype that could portend fibrosis.

Consistent with this idea, increased collagen accumulation, indicative of fibrosis, was coincident with increased CD45+ cells in the 6-month IMI MVs. CD45+ cells showed increased Src and phosphorylated Erk. 37 An analysis of Src family kinases as potential targets of VEC CD45 would be a topic of future studies.

CD45 is not normally expressed on endothelium or endothelial cells. An exception is during embryonic development when specific sites within the yolk sac, the placenta, and the dorsal aorta become hemogenic for a narrow window of time. CD45+ VE-cadherin+ cells bud from the hemogenic endothelium to give rise to hematopoietic stem cells. 20,21 Purified cultures of human endothelial cells are typically devoid of CD45+ cells, 22 although we detected a limited window of hemogenic activity in human umbilical cord blood CD133-selected endothelial colony-forming cells. 43 No CD45+ adult endothelium has been described to date. Therefore, our discovery of CD45+ endothelial cells in MVs at 6 months post IMI and in cultured mitral VECs is novel. Furthermore, we show that CD45 is not expressed in arterial endothelial cells treated with or without TGFβ1, indicating selectivity. We also showed that hematopoietic markers, such as CD14 and CD11b, were not increased in mitral VEC by TGFβ1, nor was CD14 detected along the endothelium in MVs from IMI sheep at 6 months.

We postulate that endothelial CD45 is important in the MV adaptive response post MI, and further, the response encompasses both pro- and maladaptive processes. We recently reported a constellation of cellular changes in MV leaflets in an ovine model of tethering plus MI, which included significantly increased CD45+ cells at 2 months, as well as significantly increased EndMT. 8 In that study, we had not yet developed the triple-label flow cytometric method to quantify the 8 distinct cell populations in MV and therefore do not know the distribution of CD45+ cells among endothelial cells, hematopoietic cells, and fibrocytes. In the current study, we show that in the 6-month IMI model, MV endothelial cells constituted 64% of the total CD45+ cells, hematopoietic cells 21%, and fibrocytes 14%. We postulate that increased endothelial CD45, with its intrinsic phosphatase activity, may decrease adhesion and increase migration of mitral VECs, as CD45 has been implicated in these cellular activities. 20,38,39 Indeed, increased migration is a hallmark of cells undergoing EndMT, 29 which we speculate is an important process used to replenish mitral VCs and increase MV leaflet area. 35 The EndMT could be proadaptive if it proceeded in a regulated manner to produce VICs; on the contrary, if EndMT is uncontrolled, it may result in maladaptive valve growth. EndMT has recently been implicated other maladaptive settings, such as atherosclerotic lesions and plaque instability. 44

Approximately 15% of the CD45+ cells in the 6-month IMI MVs may be fibrocytes, also known as myeloid fibroblasts, based on the coexpression of CD45 and αSMA and lack of VE-cadherin. CD45 suggests a hematopoietic origin of fibrocytes/myeloid fibroblasts, and αSMA indicates myofibroblastic functionality. 12 Despite their relatively low number in IMI MVs, the fibrocytes may be an important factor in the maladaptive MV response to MI, as these cells can release inflammatory and fibrogenic growth factors, including TNFα, IL6, IL8, TGFβ1 to 3, collagen 1 and 3, and fibronectin 44 and importantly have been shown to contribute to cardiac fibrosis, 26 including fibrosis post MI. 36,38 The possibility that MV CD45+ cells after MI contribute to a self-reinforcing fibrotic cycle merits exploration for potential therapeutic directions; targets could include both fibrocytes...
and CD45+ VEC undergoing EndMT. In myxomatous MVs, fibrocytes are increased under proinflammatory conditions, supporting their role in collagen deposition in the valves. Hajdu et al identified a fibrocyte-like population in normal murine MV leaflets that are spindle shaped, CD45+, and bone marrow derived. They further characterized the cells as vimentin positive but endothelial and leukocyte marker negative. They concluded that bone marrow-derived cells contribute to the VIC population under normal homeostatic conditions. The increased CD45+/αSMA+ cell population in the ovine IMI MVs may be related to this phenomenon but would likely reflect an enhancement or exaggeration of the steady-state influx of bone marrow cells into the MV. An alternative hypothesis, given the capability of mitral VEC to express collagen I, collagen 3, TGFβ, and CD45, may be an indicator of an endothelial population suggests that upregulated endothelial cell plasticity of mitral VECs, CD45 may be an indicator of an endothelial population. Multiple cell types in MVs at 6 months post MI using an objective and unbiased method, similar to a recent report on murine cardiac cellular composition. The identification of a CD45+ endothelial population suggests that upregulated endothelial CD45 can play a role in adaptation of MV cells post MI. Given the plasticity of mitral VECs, CD45 may be an indicator of an adaptive phenotype. Finally, these results identify a functional role for CD45 PTPase in EndMT, an entirely novel finding that warrants further investigation. The fact that CD45+ cells were found associated with adverse outcomes including leaflet fibrosis, MR severity, LV remodeling, and reduced ejection fraction provides a strong rationale for pursuit of such studies.

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Disclosures
None.

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CD45 in Mitral Valve Endothelial Cells After MI
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What is Known?
• After inferior myocardial infarction, progressive mitral valve (MV)
regurgitation develops that increases heart failure and mortality and is
difficult to repair.
• Although MV leaflet tethering causes compensatory valve enlargement,
associated with endothelial-to-mesenchymal transition (EndMT), myocar-
dial infarction (MI) combined with leaflet tethering induces counterproduc-
tive MV fibrosis that impairs leaflet closure and increases regurgitation.
• This post-MI fibrotic process is associated with excessive EndMT and
CD45+ cells in the MV and transforming growth factor-β (TGFβ) protein
in MV cells.
What New Information Does This Article Contribute?
• Unexpectedly, the CD45+ cells in the ovine 6-month post-MI MV co-
express endothelial markers, many localize within the endothelium or
subendothelium and express markers consistent with EndMT.
• The proportion of CD45+ cells in these valves correlates with valve
fibrosis (collagen content) and mitral regurgitation severity and infarct
size and left ventricular remodeling (dilatation).
• In vitro, TGFβ specifically induces ovine MV endothelial cells to un-
dergo EndMT, migrate, and express CD45, collagen, and intrinsic TGFβ1,
all blocked by a CD45-selective protein tyrosine phosphatase inhibitor.

Novelty and Significance

MI is accompanied by adaptive enlargement of the tethered MV
combined with maladaptive fibrosis that limits leaflet expan-
sion and promotes mitral regurgitation through as yet unknown
cellular processes. MV EndMT seems to play a role in the ini-
tial enlargement, and new findings here indicate that it may be
a substrate for fibrosis. There are substantially increased cells
expressing CD45, a protein tyrosine phosphatase, in the MV at
6 months post inferior MI. The majority of the MV CD45+ cells
have an intrinsic endothelial phenotype with indicators of ongo-
ing EndMT. The increase in CD45+ cells is correlated with MV
collagen deposition, infarct size, and mitral regurgitation severity
in the ovine inferior MI model. In vitro, TGFβ1, which is released in
the post-MI inflammatory state, induced CD45+ expression in
MV endothelial cells, coincident with increased cell migration, a
hallmark of EndMT, and expression of EndMT markers and the
fibrosis markers collagens 1 and 2 and endogenous TGFβ1 to
3. A CD45-selective phosphatase inhibitor blocked the increased
migration and expression of EndMT and fibrosis markers, which
suggests a functional role for CD45 in EndMT and the production
of fibrotic cells. These findings suggest new avenues for under-
standing mechanisms driving MV regurgitation and point to new
therapeutic opportunities.
CD45 Expression in Mitral Valve Endothelial Cells After Myocardial Infarction

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SUPPLEMENTARY METHODS

- Animal model and data acquisition

Thirteen Dorsett hybrid sheep were used in this study. In the inferior myocardial infarction (IMI) model, 5 sheep were induced with Propofol (0.5 to 1.5 mg/kg intravenously), and the trachea intubated and ventilated at 15 ml/kg with a mixture of 3% isoflurane and oxygen. Animals were loaded with antiarrhythmic drug Amiodarone 200mg PO daily for three days prior to surgery. Intravenous Glycopyrrolate (0.4 mg), Buprenorphine (0.3 mg), and Cefazolin (1 g) were administered 30 minutes before chest wall incision.

Bupivacaine 0.75% was administered locally to block the intercostal nerves prior to chest retractor placement. A left thoracotomy was performed between the 4th and 5th intercostal space to access the heart. Following chest retractor placement, the heart was exposed and the pericardium opened. Epicardial 2- and 3-dimensional echocardiography imaging and left ventricular hemodynamic parameters were acquired at baseline and 30-60 minutes post myocardial infarction (MI). Left ventricular hemodynamic data were recorded with a 5F Scisense catheter inserted through the apex. Basic echo views were obtained using a Philips iE33 scanner and a 5-MHz transducer. Images were analyzed offline using QLab 10.5 (Philips, Andover, Mass). LV end-diastolic volume, end-systolic volume, and ejection fraction were measured using 3D full volume data set. MR was quantified by the width of the proximal jet (vena contracta) in the apical long-axis view. The infarct surface area and total LV endocardial surface area were measured at end-diastole using customized 3D analysis software (Omni4D). The second and third obtuse marginal branches of the left circumflex coronary artery were ligated at their origin to create myocardial infarction (MI) that results mitral regurgitation. Lidocaine 0.8mg/kg intravenous bolus(es) were administered prior to coronary artery ligation and after coronary artery ligation. Sham animals were treated identically except that the coronary arteries were not ligated and thus no myocardial infarction created. Three-dimensional (3D) echocardiographic analysis included LV end-systolic and end-diastolic volumes integrated from multiple rotated views derived from the full 3D dataset using Omni4D software (M.D.H., Boston, MA); infarct size as endocardial surface area (ESA) measured at end diastole based on visualized wall motion hinge points; and total LV remodeling reflected by the increase in total LV ESA from immediately to 6 months post-MI1-4.

Following 6 months after MI creation, prior to euthanasia, the animals were anesthetized as described above and echocardiography and hemodynamic data repeated to assess severity of mitral regurgitation and hemodynamics. Upon completion of data collection, animals were euthanized via intravenous injection of 100 mg/kg IV pentobarbital (while on 3% isoflurane). Under sterile conditions, the hearts were excised and the mitral valve leaflets immediately submerged in a solution of 5% heat inactivated FBS, 4% Penicillin/Streptomycin/Amphotericin B, 1% L-Glutamine and 0.2% gentamycin sulfate in EBM-2 medium (Lonza Inc., GA, USA, #CC-3156), and kept on ice at 4°C until processed. Animals were monitored by qualified AAALAC-certified veterinary staff. These studies conform to National Institutions of Health guidelines for animal care and received Institutional Animal Care Committee approval.

- Immunohistochemistry

A portion of each excised mitral valves was frozen in OCT compound (Sakura Finetek, Tokyo, Japan) and sectioned in 7 µm slices. Sections were then incubated with 0.3% hydrogen
peroxide to inhibit endogenous peroxidase activity, and then incubated with primary mouse anti-sheep CD45 (1:10 dilution; AbD Serotec, NC, USA, cat# MCA2220GA), anti-sheep CD14 (1:10 dilution; AbD Serotec, NC USA, cat# MCA920GA) or anti-human α-SMA (1:150 dilution; DAKO, CA, USA, cat#, M0851) antibodies. After washing with PBS, secondary antibody (biotinylated anti-mouse IgG, 1:100 dilution; Vector Laboratories, CA, USA) was applied, followed by avidin-peroxidase complex (Vectastain ABC kit; Vector Laboratories, CA, USA). The reaction was visualized with 3-amino-9-ethyl carbazole substrate (AEC; Sigma-Aldrich, MO, USA). Sections were counterstained with Mayer's hematoxylin solution (Sigma-Aldrich, MO, USA) and mounted. Isotype-matched IgGs applied to the tissue sections served as control. Images were captured with a digital camera (Eclipse 50i, Nikon Instruments, Melville, New York) with a cooled CCD camera (DS-Fi1c, Nikon Instruments, Melville, New York) using imaging software NIS-Elements (version 3.1). Fluorescence microscopy visualized simultaneously fluorescein-labeled endothelial cells (von Willebrand Factor, DAKO, CA, USA, cat# A0082) detected by fluorescein-conjugated streptavidin (Alexa Fluor 488 dye, Life Technologies, CA, USA) and CD45 (Alexa Fluor 594 dye, Life Technologies, CA, USA). Nuclei were counterstained with 4′,6-Diamidino-2-phenylindole (DAPI, Vector Laboratories, CA, USA). Images were captured and processed with the epifluorescence microscope (Eclipse Ti-E, Nikon Instruments, NY, USA).

Fibrosis: Sections from each sham and IMI-6 MV) were stained with Masson Trichrome; five high powered fields (HPF) were analyzed from each section to determine % Positive Area for collagen. In parallel adjacent sections were stained for CD45+ cells. Sections were analyzed at 400X magnification (Eclipse 50i, Nikon Instruments, Melville, NY, USA) with a cooled CCD camera (DS-Fi1c, Nikon Instruments, Melville, NY, USA) using imaging software NIS-Elements AR (Advanced Research) 3.1 (Nikon Instruments, Melville, NY, USA).

- Flow cytometry

Ovine MVs were minced and digested for 30 minutes with Liberase (Roche Diagnostics, IN, USA), a blend of highly purified collagenases I and II, in a 37°C water bath. Tissue pieces were rinsed with wash buffer (5% FBS, 1% Penicillin/Streptomycin/Amphotericin B, 1X Hank’s Basic Salt Solution, 126 µmol/L CaCl₂ and 80 µmol/L MgSO₄ in H₂O) 4 times, to extract the cells, and the isolated cells were fixed using Flow Cytometry Fixation Buffer (R&D Systems, MN, USA) and labeled in Flow Cytometry Permeabilization/Wash Buffer I (R&D Systems, MN, USA #FC005) for 45 minutes (100,000 cells/100 µl buffer), following the manufacturer’s protocol. Murine anti-sheep CD45-FITC and -APC (1:50 dilution; AbD Serotec, NC, USA, cat# MCA2220F, #MCA896GA, LYNX Rapid APC Antibody Conjugation Kit, #LNK031APC), murine anti-human VE-Cadherin-PE and -FITC (1:100; R&D Systems, MN, USA FAB9381P and AbD Serotec, NC, USA, # AHP628F, respectively), and murine anti-human α-smooth muscle actin-APC and -PE (1:100; R&D Systems, MN, USA, # IC1420P and # IC1420A) were used to detect CD45, VE-Cadherin, and αSMA respectively. All antibodies were shown to cross-react with their ovine homologs. Flow cytometry samples were analyzed on a BD FACS Calibur system. Data were analyzed using FlowJo version 10 software (Tree Star, Inc. Ashland, OR).

- RNA isolation and quantitative real-time PCR (qPCR)

Total cellular RNA was extracted from mitral VEC clones (D1, E5 and E10-2) and CAEC (n=2) with an RNeasy Micro extraction kit (Qiagen, Valencia, CA). Reverse transcriptase reactions were performed using an iScript cDNA Synthesis Kit (Bio-Rad, CA, USA #170-8890). qPCR was performed using Kapa Sybr Fast ABI Prism 2x qPCR Master Mix (KAPA BioSystems, MA, USA # KK4604). Amplification was carried out in an ABI 7500 (Applied Biosystems, Foster City, CA). A standard curve for each gene was generated to determine amplification efficiency. RPS9 was used as housekeeping gene expression reference. Fold increases in gene
expression were calculated according to 2 delta C_{T} method \(^5,6\), with each amplification reaction performed in triplicate.

- Migration assay

Mitral VEC clones (D1, E5, E10-2) or CAEC were treated ± TGFβ for 4 days to induce EndMT. The cells were treated for 30 minutes ± the CD45-selective PTPase inhibitor (1µM)\(^7\) prior to trypsinization. 20,000 cells (± PTPase inhibitor 1µM) in 0.1% BSA/EBM-2 (Lonza) were placed in the upper chamber of 6.5mm Transwells containing fibronectin-coated (0.2 ug/cm\(^2\)) polycarbonate membranes with 8.0µm pores. The lower chambers contained 0.1% BSA/EBM-2 media alone or EBM with serum and basic FGF as a chemoattractants. Cells were allowed to migrate for 6 hours at 37°C. Cells that migrated through the pores were fixed with methanol and stained with Eosin-Y, Azure A and Methylene Blue for visualization and quantification using Three Step Stain Set (VWR, PA, USA #48218-567). In parallel, an aliquot of cells used for the migration assay were also analyzed for CD45 by flow cytometry to verify response to TGFβ1.
Online Table I

<table>
<thead>
<tr>
<th>Sheep ID</th>
<th>EDV(mL)</th>
<th>ESV(mL)</th>
<th>SV(mL)</th>
<th>EF(%)</th>
<th>VC(mm)</th>
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<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>94</td>
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<td>70</td>
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<td>105</td>
<td>51</td>
<td>54</td>
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</table>

- Sheep 5149 was larger (67kg) than other sheep in the study (~45kg), which could account for the larger LV

**Online Table I** - Echocardiographic measurements ischemic, sham and normal sheep. Volumes were calculated using three-dimensional data (full volume acquisition) on an Echomachine Philips IE33, using Philips Qlab software. EDV = End-diastolic volume; ESV = End-systolic volume; SV = Stroke volume; EF = Ejection fraction; VC = Vena contracta of the MR jet (proximal dimension reflecting orifice size). Sheep 2095 and 2137 were analyzed at 6 months and 4048, 4064, 4033 at 2 months.
Online Table II

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>Normal n = 4</th>
<th>6 months IMI n = 5</th>
<th>Statistical Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEC VE-Cadherin+/CD45-/αSMA-</td>
<td>38.7 ± 9.7</td>
<td>4.6 ± 6.6</td>
<td>P = 0.0004</td>
</tr>
<tr>
<td>VEC^{CD45+} VE-Cadherin+/CD45+/αSMA-</td>
<td>1.5 ± 1.9</td>
<td>9.0 ± 5.1</td>
<td>P = 0.028</td>
</tr>
<tr>
<td>VEC-EndMT VE-Cadherin+/CD45+/αSMA-</td>
<td>1.5 ± 1.5</td>
<td>3.8 ± 3.6</td>
<td>P = 0.272</td>
</tr>
<tr>
<td>VEC^{CD45+}-EndMT VE-Cadherin+/CD45+/αSMA-</td>
<td>0.4 ± 0.3</td>
<td>17.4 ± 4.6</td>
<td>P = 0.001</td>
</tr>
<tr>
<td>Quiescent VIC VE-Cadherin+/CD45+/αSMA-</td>
<td>57.5 ± 9.8</td>
<td>47.1 ± 12.8</td>
<td>P = 0.222</td>
</tr>
<tr>
<td>Activated VIC VE-Cadherin+/CD45+/αSMA-</td>
<td>0.1 ± 0.2</td>
<td>5.3 ± 5.1</td>
<td>P = 0.082</td>
</tr>
<tr>
<td>Hematopoietic cells VE-Cadherin+/CD45+/αSMA-</td>
<td>0.5 ± 0.8</td>
<td>8.8 ± 6.0</td>
<td>P = 0.036</td>
</tr>
<tr>
<td>Fibrocytes VE-Cadherin+/CD45+/αSMA-</td>
<td>0.0 ± 0.0</td>
<td>5.8 ± 2.9</td>
<td>P = 0.011</td>
</tr>
</tbody>
</table>

Online Table II. Endothelial, interstitial and hematopoietic cell populations in MVs from IMI infarcted sheep versus normal sheep as percentage of total cells within each cell population. Statistical significance measured using student t-test analysis.

Online Table III

Student t-tests performed on fold increases (mean ± SD) in Figure 4C.

<table>
<thead>
<tr>
<th>Control vs TGFβ1 (p values)</th>
<th>VE-cadherin</th>
<th>αSMA</th>
<th>Slug</th>
<th>MMP2</th>
<th>NFATc1</th>
<th>Collagen1</th>
<th>Collagen3</th>
<th>TGFβ1</th>
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<td>0.0079</td>
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</table>

<table>
<thead>
<tr>
<th>TGFβ1 vs TGFβ1 + PTPase (p values)</th>
<th>VE-cadherin</th>
<th>αSMA</th>
<th>Slug</th>
<th>MMP2</th>
<th>NFATc1</th>
<th>Collagen1</th>
<th>Collagen3</th>
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<th>TGFβ2</th>
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<tr>
<td></td>
<td>0.1666</td>
<td>0.0143</td>
<td>0.0060</td>
<td>0.0196</td>
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<td>0.0179</td>
<td>0.0344</td>
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<td>0.0051</td>
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</table>
Supplementary Online Figures

Online Figure I - A) αSMA and B) CD45 in the endothelial and subendothelial layers of IMI mitral valves Adjacent sections of valve leaflets were analyzed by immunohistochemistry. Arrows mark sites of potential co-localization. Scale bar, 100 µm.
Online Figure II – Mitral VEC clone E10 treated without (control) or with TGFβ1 for 4 days to induce EndMT. Cells were analyzed by flow cytometry using anti-CD45-FITC (top row), anti-CD11b-FITC (middle row) or anti-CD14-FITC (bottom row). IgG isotype-matched controls-conjugated to FITC were used to establish background. As expected, mitral VEC clone E10 expressed CD45 at a low level without TGFβ1 and at a higher level (31%) after TGFβ1 treatment, but neither CD11b nor CD14 were detected. The anti-CD11b and anti-CD14 were validated using ovine peripheral blood leukocytes as a positive control (not shown).

References