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Mesenchymal Stem Cell Adhesion to Cardiac Microvascular Endothelium: Activators and Mechanisms

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Running Head: Adhesion of MSCs to cardiac endothelium.

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Abstract

Objective. Circulating stem cells home within the myocardium, probably as the first step of a tissue regeneration process. This step requires adhesion to cardiac microvascular endothelium (CMVE). In this study, we studied mechanisms of adhesion between CMVE and mesenchymal stem cells (MSCs). *Methods.* Adhesion was studied in vitro and in vivo. Isolated DiI-labeled rat MSCs were allowed to adhere to cultured CMVE in static and dynamic conditions. Either CMVE or MSCs were pre-treated with cytokines (IL-1β, IL-3, IL-6, SCF, SDF-1 or TNF-α, 10 ng/ml). Control or TNF-α-treated MSCs were injected intracavitary in rat hearts in vivo. *Results.* In baseline in vitro conditions, the number of MSCs that adhered to CMVE was highly dependent on the flow rate of the superfusing medium, but remained significant at venous and capillary shear stress amplitudes. Activation of both CMVE and MSCs with TNF-α or IL-1β prior to adhesion concentration-dependently increased adhesion of MSCs at each studied level of shear stress. Consistently, in vivo, activation of MSCs with TNF-α prior to injection significantly enhanced cardiac homing of MSCs. TNF-α-induced adhesion could be completely blocked by pre-treating either CMVE or MSCs with anti-VCAM-1 monoclonal antibodies but not by anti-ICAM-1 antibodies. *Conclusions.* Adhesion of circulating MSCs in the heart appears to be an endothelium-dependent process, and is sensitive to modulation by activators of both MSCs and endothelium. Inflammation and the expression of VCAM-1 but not ICAM-1 on both cell types have a regulatory effect on MSC homing in the heart.

Keywords: cardiac endothelium, stem cells, cardiac regeneration, heart failure

Introduction

Adult stem cells have been considered to be restricted in their differentiative and regenerative potential to the tissues in which they reside. Recently, this traditional view of adult stem cell function has been challenged.(4) For example, it has been shown that mesenchymal stem cells (MSCs), usually residing in the adult bone marrow together with haematopoietic stem cells, were highly plastic and may differentiate into cartilage, bone, fat, muscle(23) and cardiomyocytes.(18; 29) These findings suggest that circulation and homing of these cells in various organs is part of a previously unrecognized regeneration process. Hence, the physiological relevance of MSCs residing in cardiac tissue was initially ascribed to the replacement of damaged cardiomyocytes. Recent reports indicate, however, that stimulation of neovascularisation and paracrine effects of MSCs may be of equal importance.(24; 28)

MSCs have been detected in circulating blood of mammalian species(14) and found to migrate into different organs such as heart, liver, spleen or lungs.(3; 22) The process of homing of circulating stem cells in the bone marrow has been studied in detail. This process is clinically highly relevant as it is crucial for therapeutic transplantation of haematopoietic stem cells. In the bone marrow, homing is a multi-step process that shares components with the extravasation of leukocytes at inflammatory sites with a significant role for interactions between stem cells and bone marrow endothelium.(26) Only few studies, however, have examined interaction of stem cells with endothelial cells in the vasculature outside the bone marrow.(12; 21; 25)

Cardiac endothelial cells in the endocardium and myocardial capillaries play a modulatory role on cardiac development and pump performance. At these locations, cardiac endothelial cells directly interact with surrounding cardiomyocytes through paracrine and

non-paracrine mechanisms.(5; 8) From this point of view, the heart functions as a pluricellular, multifunctional organ, in which the endothelium is a crucial functional component in cardiac metabolism, growth, contractile performance and rhythmicity.(5) In the present study we hypothesize that besides these established functions, cardiac endothelium may also have a modulatory role in cardiac regeneration. More specifically, we postulate that cardiac microvascular endothelium (CMVE) may play a role in homing and migration of circulating stem cells to myocardium and, as such, may act as a modulator of cardiac cellular homeostasis. Hence, we examined adhesion of mesenchymal stem cells to cardiac endothelium and determined whether a selected set of cytokines affected this process.

The inflammatory cytokines TNF-α and IL-1β were selected because they increase adhesion molecule expression in endothelial cells(20) and are released after myocardial damage(7). IL-3, IL-6 and stem cell factor (SCF) were tested because these cytokines play a role in homing of hematopoietic stem cells(31). IL-6 was selected because it is markedly upregulated in cardiac failure and has a protective role in the progression of pump failure(1), whereas stromal cell derived factor-1 (SDF-1) was tested because it has been proposed to participate in homing of stem cells to the heart(10).

Materials and Methods

Cell cultures.

Endothelial cell cultures. The investigation conforms to the *Guide for the care and use of laboratory animals* published by the US National Institutes of Health (NIH Publication NO 85-23, revised 1996). CMVE and aortic endothelial cells (AE) were isolated and cultured from adult Sprague-Dawley rats as previously described.(11)

Mesenchymal stem cell cultures. Femur and tibia of Sprague-Dawley rats were excised and connective tissue was removed.(3) Bone marrow cavity was flushed with complete culture medium. Marrow plug suspension was dispersed by passing it through subsequent pipettes of decreasing sizes. Once a homogenous cell suspension was achieved, mononuclear cells were isolated using density gradient centrifugation (Ficoll-Paque, Amersham Biosciences), mononuclear cells were plated at $8*10^6$ cells/cm² and non-adherent cells were removed after 4h. The mesenchymal population was isolated based on plastic adherence and was cultured in RPMI-1640 with 10% FCS of a selected batch (Gibco, Invitrogen). At 90% confluence, the cells were trypsinized (0.25% trypsin-EDTA) and passaged at 1:9 ratios. For adhesion experiments MSCs at passage 4 and later were used.

Adhesion assays.

Adhesion assays were performed using 8-well glass chamber-slides (Falcon CultureSlide) on which $2*10^4$ CMVE (Passage 2 to 4) were plated reaching confluence after 3 days. Unless otherwise stated, all experiments were performed with $8*10^3$ MSCs labeled with DiI (Molecular Probes) and diluted in 0.15 ml RPMI. After incubation at 37°C (1 to 16h), wells were gently washed 3 times with PBS and adherent cells were counted in 10 fields (250 fold magnification) per well using fluorescence microscopy (Zeiss). The number of adhering

cells was normalized to the total number of added cells. In some experiments, MSCs or CMVE were pre-treated to adhesion studies with one of the following substances: TNF-α (0.1 to 100 ng/ml), IL-1β (0.1 to 100 ng/ml), stem cell factor (SCF, 10 ng/ml), stromal cell derived factor-1 (SDF-1, 10ng/ml), IL-3 (10 ng/ml), IL-6 (10 ng/ml) (Sigma), anti-VCAM-1 antibody (mouse monoclonal anti-rat, clone 5F10, 10 µg/ml, Eurogentec), anti-ICAM-1 antibody (mouse monoclonal anti-rat, clone 1A29, 10 µg/ml, Res. Diagnostics).

Flow chamber adhesion assays.

Flow adhesion experiments were performed in a parallel plate flow microchamber with a slit height of 0.25 mm and slit width of 10 mm. CMVE (passage 2 to 4) were cultured to confluence on poly-L-lysine and collagen (Sigma) coated glass microscope slides. These slides were attached to the bottom of the flow chamber 15 min before flow experiments. Perfusion of flow chambers at different levels of shear stress (0.5, 1, 2, 5 or 10 dyne/cm^2) was conducted for 2h with circulating RPMI medium (37°C) containing 200 MSCs per µl. MSCs were labeled with DiI as described earlier. Thereafter, chambers were perfused for 15 min with cell-free medium at 2 dyne/cm^2 to remove loose cells. Adherent MSCs were counted with fluorescence microscopy (5 fields at 100X magnification). In some experiments either MSCs or CMVE were pre-treated with TNF-α (10 ng/ml) for 24h prior to adhesion assays. Experiments were repeated at least 3 times.

Rat model of in vivo adhesion of MSCs.

Sixteen adult Sprague-Dawley rats (150g) were randomized into 2 groups. One group received intraventricular injection of control MSCs, the other group received MSCs pre-treated with TNF-α (10 ng/ml, 24 h). For MSCs injection, rats were anaesthetized with fentanyl (IM, 0.05 mg/kg, Janssen-Cilag), diazepam (5 mg/kg, Roche) and haloperidol (3

mg/kg, Janssen-Cilag) and subsequently intubated endotracheally. A left lateral thoracotomy and pericardiotomy was performed, exposing the heart and ascending aorta. In a separate group of 6 rats, a procedure of myocardial ischemia (30 min) – reperfusion injury was induced by temporary LAD ligation. 24 h later, MSCs were injected following the same procedure as described above.

MSCs labeled with DiI were prepared for infusion by detaching the cells from the culture plates by 10-minutes incubation with 0.25% trypsin-EDTA. Cell solution was passed through a 40µm Nylon cell strainer (BD Falcon) to remove cell aggregates and centrifuged (1000 rpm, 7 min). $1.5*10^6$ MSCs were diluted in 0.5 ml PBS and were injected into the left ventricular cavity while the ascending aorta was clamped (15s) to mimic intra-coronary injection. The rats were monitored for 2 hours post-operatively and received an additional subcutaneous dose of fentanyl (0.03 mg/kg) before returning to their cages. All rats survived the procedure.

24 h after injection hearts were removed, fixed with 4% paraformaldehyde solution through Langendorff perfusion and left overnight in a 20% sucrose solution. Three cryosections per heart, separated by at least 10 sections, were made in the mid-ventricular region. Three digital images were made with fluorescence microscopy (low magnification, 40X) and an observer unaware of the experimental protocol (control vs TNF-α-treated MSCs) counted cells in the sections.

Immunofluorescent staining.

Endothelial cells in cryosections (20µm) were stained with mouse monoclonal anti-RECA (Rat endothelial cell antigen, Serotec) antibody and secondary Goat anti-mouse coupled to Alexa Fluor 488 (Molecular Probes) antibody. Cryosections (10 to 20µm) of infarcted hearts were stained with Phalloidin-Alexa Fluor 488 for F-actin to discriminate

normal from damaged cardiomyocytes. Cellular nuclei were counterstained with Hoechst. Images were captured with fluorescence microscopy (Zeiss Axioplan 2, Apotome, Axiocam HRm and Axiovision software). ZVI-images were exported as TIFF or JPG files and further processed in Adobe Photoshop.

RT-PCR.

mRNA was isolated in Trizol®Reagent (Invitrogen, Life Technologies) following instructions of the manufacturer. RT-PCR was performed with One-Step RT-PCR System (Life Technologies) in 25 µl reaction volume containing: 0.5 µl total RNA (10-100ng), 12.5 µl reaction mix, 400 nM of both primers and 0.5 µl Superscript II/ Taq mix (Life Technologies). After initial incubation of 30 min at 50°C and 2 min at 94°C, 35 PCR-cycles were performed which consisted of 15 s denaturation at 94°C, 30 s annealing at 55°C and a 1 min extension at 72°C, with a final extension step of 7 min. Primers used are listed in table 1.

Flow cytometry.

Cells were harvested using trypsin/EDTA and incubated 10 min with 10 µg/ml FITC-labeled monoclonal antibody in RPMI per $5*10^4$ cells at room temperature in the dark. Anti-rat CD34 (mouse monoclonal anti-rat, clone ICO115, Santa Cruz Biotechnology), anti-rat CD45 (Leukocyte common antigen, mouse monoclonal anti-rat, clone OX-1, BD Biosciences Pharmingen), anti-rat CD29 (β1-integrin, Armenian hamster monoclonal anti-rat, clone Ha2/5, BD Biosciences Pharmingen) and anti-rat CD90 (Thy-1, mouse monoclonal anti-rat, clone OX-7, Abcam) were used. Cells were washed twice with PBS/0.1% BSA. Labeled samples were examined with a Coulter Epics XL-MCL flow cytometer (Coulter). At least 10000 events were analyzed and compared to isotype-controls.

Real-Time PCR.

Real-Time PCR was performed for vascular cell adhesion molecule-1 (VCAM-1) and very late antigen-4 (VLA-4, α_4-chain). CMVE and MSCs were cultured on 6 well plates (Falcon, VWR international) for 2 days and serum starved for the last 7h during which some of products used in adhesion assays were added. mRNA was isolated in Trizol®Reagent (Invitrogen). Real-Time PCR was performed with TaqMan® One-Step RT-PCR System (Applied Biosystems) in 25 µl reaction volume containing: 1 µl total RNA (10-100ng), 12.5 µl One-Step RT-PCR Master Mix, 100 to 800 nM of both primers, 200 nM TaqMan Probe and 0.5 µl RNAse inhibitor. TaqMan® probes were labeled with FAM reporter dye and TAMRA quencher dye. After initial incubation of 30 min at 48°C and 10 min at 95°C, 45 PCR-cycles were performed which consisted of 15 s denaturation at 95°C, 60 s annealing and extension at 60°C. Primers and Taqman® probes used are listed in table 1. Expression of VCAM-1 and VLA-4 mRNA was normalized to expression of GAPDH mRNA.

Statistical analysis.

Adhesion assays were performed in duplicates. Mean values were compared with one-way ANOVA with Bonferroni correction for multiple comparisons. Real-time PCR experiments were performed 4 times and differences in CT values between VCAM-1 or VLA-4 and GAPDH were compared with one-way ANOVA with Bonferroni correction for multiple comparisons. Statistical analysis was performed with SPSS software (SPSS).

Results

Validation of MSC culture.

MSCs presented as a homogeneous fibroblastoid cell population. Expression of stem cell markers assessed with RT-PCR showed that after passage 2 these cells were completely negative for haematopoietic cell markers (CD34 and CD45) and positive for CD 90, CD105, CD166 (ALCAM), which are markers of MSCs (RT-PCR, Figure 1, panel A).(22) Flow cytometric analysis of passage 4 cells confirmed that cells were negative for CD34 and CD45 and that cells were positive for CD29 (β1-integrin) and CD90 (Thy-1) (Figure 1, panel B). Only cells at passage 4 and further passages were used for adhesion experiments.

Adhesion of MSCs to cardiac microvascular endothelium.

In static conditions, adhesion of MSCs to CMVE was dependent on the period that cells were allowed to interact, reaching a maximum after 4h of incubation (n=4, 1h: 8 ± 3%, 2h: 17 ± 1%, 4h: 25 ± 3%). Percentage of MSCs that adhered to CMVE was fairly independent of the number of MSCs incubated, at least between 4000 and 16000 cells added per well (not shown). When adhesion experiments were performed in dynamic conditions in which medium containing MSCs was superfused during 2h (200 MSCs /µl), increasing flow velocity (shear stress from 0.5 dyne/cm^2 to 5 dyne/cm^2) exponentially decreased adhesion of MSCs to CMVE from 2063 ± 360 to 113 ± 24 cells/cm^2 (Figure 3). At higher levels of shear stress adhesion remained unchanged between 5 dyne/cm^2 and 10 dyne/cm^2 (75 ± 17 cells/cm^2).

Influence of cytokines on adhesion of MSCs to CMVE.

A first screening experiment in static conditions, in which CMVE were treated with 6 different cytokines (10 ng/ml, 24h), showed that only pre-treatment of CMVE with TNF-α or IL-1β increased adhesion of MSCs (Figure 2A). Pre-treating CMVE with IL-3, IL-6, SCF or SDF-1 did not affect adhesion efficiencies (n=6, Figure 2A). The effect of TNF-α or IL-1β was confirmed in dynamic conditions (Figure 3) and appeared to be concentration-dependent. At 100 ng/ml TNF-α for example, the percentage of MSCs that adhered to CMVE increased from 26 ± 3% to 52 ± 5% (n=6, $p<0.001$, Figure 2B). Similar results were observed when CMVE were pre-treated with IL-1β (at 100 ng/ml, the percentage of MSCs adhering to CMVE increased from 23 ± 1% to 63 ± 6%, n=6, $p=0.002$, Figure 2C). When MSCs, instead of CMVE, were pre-treated with TNF-α during 24h prior to incubation with CMVE, adhesion of MSCs to CMVE also increased (n=6, increase from 28 ± 2% to 72 ± 2%, Figure 2D). When both MSCs and CMVE were pre-treated adhesion was still a little greater but effects were not additive. In flow-chamber experiments, pre-treatment of CMVE or MSCs with TNF-α (10 ng/ml, 24h) enhanced adhesion of MSC at every studied level of shear stress (Figure 3, values of TNF-α treated groups are significantly different from control at all shear stress levels, $p<0.05$, n=3). MSCs also adhered to confluent cultures of aortic endothelial cells (AE), and degree of adhesion did not differ significantly from adhesion to CMVE, neither in basal conditions (24 ± 2% for CMVE, 18 ± 2% for AE, n=6, p=1), nor after stimulation with TNF-α (48 ± 4% for CMVE, 49 ± 6% for AE, p=1, Figure 2E).

In vivo adhesion of MSCs.

The in vitro experiments indicated that MSCs adhere to CMVE in flow conditions and that adhesion is sensitive to pro-inflammatory cytokines. As a next step, DiI-labeled MSCs were injected into the left ventricular cavity of anesthetized rats, while the ascending aorta was clamped. In 6 of the 12 rats, MSCs had been pre-treated with TNF-α (10 ng/ml, 24h). 24h

after injection, MSCs residing in the myocardium were visualized by fluorescence microscopy. Compared to hearts injected with control MSCs hearts injected with TNF-α-treated cells contained 3 times more MSCs (23 ± 7 vs. 70 ± 19 cells/field, p= 0.046, Figure 4C). Sections for confocal microscopy were stained with anti-RECA antibody to evaluate spatial relationships between MSCs and cardiac endothelial cells (Figure 4A,B,D,E). Most of the MSCs were observed inside capillaries (Figure 4D), whereas some of them already crossed the endothelial barrier (Figure 4E). No MSCs were observed in larger vessels. There were no obvious differences in localization between control MSCs and TNF-treated MSCs (data not shown). MSCs injected 24h after ischemia (30min) – reperfusion injury homed abundantly in infarcted tissue (Figure 4F).

Role of VCAM-1 and ICAM-1.

The hyperbolic relation between flow velocity and MSC adhesion to CMVE suggests a role for α4-integrins (including α4β1-integrin= VLA-4, most important ligand of VCAM-1) in mediating cell adhesion.(13) Furthermore, adhesion above 0.5 dyne/cm^2 favors against involvement of β2-integrins, which are ligands to ICAM-1.(13) Therefore, monoclonal blocking antibodies against vascular cell adhesion molecule-1 (VCAM-1), were added to CMVE 1h prior to MSC incubation. VCAM-1 antibody completely abolished the TNF-α-induced increase of MSC adhesion (n=6, after stimulation with TNF-α, from 52 ± 5% to 28 ± 4%, p=0.013). Monoclonal blocking antibodies against intercellular adhesion molecule-1 (ICAM-1), however, had a slight but non-significant effect (n=6, after stimulation with TNF-α, from 52 ± 5% to 38 ± 5%, p=1) (Figure 5A). Similarly, anti-VCAM-1 antibodies, added to MSCs 1h prior to adhesion assays, inhibited the TNF-α-induced increase of MSC adhesion (n=6, after stimulation with TNF-α, from 66 ± 9% to 5 ± 1%, p=0.001) and also reduced adhesion in basal conditions (n=6, from 24 ± 4% in control to 5 ± 1%, p=0.006). Again, anti-

ICAM-1 antibodies added to MSCs prior to adhesion assays had no effect on MSC adhesion (Figure 5B).

Consistent with these observations, treatment of CMVE with TNF-α (10 ng/ml) or IL-1β (10 ng/ml) robustly induced VCAM-1 mRNA expression levels by respectively 88 ± 31 (n=4, p=0.005) or 60 ± 15 (n=4, p=0.002) fold (Figure 6). Similarly, treatment of MSCs with TNF-α or IL-1β resulted in a 41 ± 8 (n=4, $p<0.001$) or 31 ± 8 (n=4, $p<0.001$) fold induction of VCAM-1 mRNA expression respectively. Compared with VCAM-1 expression, mRNA levels of VLA-4, the principal VCAM-1-ligand, were not affected by TNF-α, neither in CMVE nor in MSCs (data not shown). Of note, basal expression levels of VLA-4 mRNA were similar in CMVE and MSCs. However, basal expression levels of VCAM-1 mRNA was 114 times (95% confidence interval from 38 to 341 times) higher in CMVE than in MSCs.

Discussion

Until recently it was generally accepted that the damaged heart did not regenerate. Meanwhile, several investigators have demonstrated that bone marrow stem cells including MSCs are capable of transdifferentiating into cardiomyocytes in vivo(29) and in vitro.(18) From these observations, it has been hypothesized that circulating mesenchymal stem cells(14) could serve as a source for regeneration of damaged myocardial cells. Moreover, MSCs have been shown to increase neovascularisation(24) and to mediate paracrine effects.(9; 28) An important first step required for cardiac regeneration by circulating stem cells involves adhesion of these cells to cardiac microvascular endothelium (CMVE). In the present study we show, for the first time, that there is a biological and molecular basis for an intercellular interaction between MSCs and CMVE, and that both cell types can be activated for mutual interaction by certain cytokines.

Inflammatory cytokines (TNF-α and IL-1β) enhanced adhesion of MSCs to CMVE in conditions of no flow, in conditions of flow in vitro and the intact animal. These cytokines are released in myocarditis, during acute coronary syndromes and in chronic heart failure.(7; 19) The present observation that these pro-inflammatory cytokines promote homing of stem cells in the heart may suggest that these cytokines have a positive effect on cardiac regeneration. Albeit speculative, the potential beneficial effect of TNF-α on cardiac cellular homeostasis could be an explanation for the disappointing results of recent clinical trials with TNF-α inhibitors in heart failure.(19) Activation of MSCs adhesion does not seem to be a common feature of cytokines in general, since other cytokines, including IL-6, IL-3, SCF and SDF-1 had no effect on MSC adhesion to CMVE.

Our experiments showed that adhesion of MSCs to CMVE was dependent on the flow velocity of the superfusing fluid and rapidly decreased when flow velocities increased. This

relation followed a hyperbolic curve, which bends at levels of shear stress between 1 and 2 dyne/cm^2. Importantly, this range corresponds to the level of stress observed in veins and capillaries. Whether adhesion of MSCs remains still significant at arterial shear stress levels (usually assumed between 6 and 40 dyne/cm^2) remains to be determined.(13) Interestingly, a hyperbolic relation between adhesion and shear stress suggests a role for α4-integrins in mediating cell adhesion(13) and contrasts with the adhesion-shear stress relationship mediated by β2-integrins, which cannot support adhesion when wall shear stress is higher than 0.5 dyne/cm^2.

Consistently, vascular cell adhesion molecule-1 (VCAM-1), which interacts with α4-integrins, appeared to be the dominant adhesion molecule in the cytokine-induced adhesion of MSCs to CMVE. Interestingly, VCAM-1 was inducible in both CMVE and MSCs. Similarly, the α4β1-integrin very late antigen-4 (VLA-4), the most important ligand of VCAM-1, was also expressed in both MSCs and CMVE. VLA-4 expression, however, was not inducible by TNF-α and IL-1β indicating that MSCs adhesion to CMVE is likely controlled by variations in VCAM-1 expression levels. Interestingly, in contrast to neutrophil adhesion to endothelium,(17; 27) adhesion of MSCs to CMVE was less dependent on CD18 - ICAM-1 interactions.

A model for the interactions between stem cells and cardiac endothelium is depicted in figure 7. Regeneration of cardiac tissue by adult bone marrow stem cells will require mobilization of stem cells upon cardiac insult. Stromal cell derived factor-1 (SDF-1) is cited frequently as a candidate to attract stem cells to injured heart(10). Once circulating in the blood stream, adhesion to endothelial cells at the site of interest is the first step in homing of those stem cells to heart. Our in vitro results show that inflammatory cytokines can activate both endothelial cells and mesenchymal stem cells. It has been shown previously that cardiac damage augments endothelial VCAM-1 expression in vivo(16). Our in vivo data show that

pre-treatment of MSC with TNF-α increases homing of those cells to the heart and that MSCs home in infarcted tissue which is consistent with previous reports(3; 30). Whether circulating MSCs express VCAM-1 in conditions of myocardial infarction for homing purposes remains to be elucidated.

In our in vivo model, 1.5 million of stem cells are injected in the vasculature in a few seconds, which might be therapeutically relevant but obviously is far from the physiological situation. The number of circulating stem cells in the intact animal following myocardial damage will only be a very small fraction of the number of cells we injected, and only become biologically significant if sustained over a longer period.

Besides interacting with CMVE, MSCs also adhered to aortic endothelial cells, indicating that interaction of MSCs with endothelial cells may be a general endothelial feature. Adhesion of MSCs to vascular endothelial cells, can be of importance for repair of arterial wall.(2; 6) To what extent, however, this process may be blunted by high shear forces in the arterial vasculature remains to be determined. Furthermore, based on the present study, homing of stem cells will depend on the number of adhesion molecules expressed by the endothelium. Interestingly, a recent report indicates that microvascular endothelial cells have a much higher constitutive VCAM-1 expression in the heart compared with the lungs.(15)

In summary, we demonstrated for the first time that MSCs adhere to CMVE and that adherence properties of both cell types can be modified by pro-inflammatory cytokines (TNF-α and IL-1β). Cytokine induced adhesion is, at least partly, mediated by the VCAM-1 – VLA-4 pathway, with both components of this pathway expressed on both CMVE and MSCs.

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Figure Legends

Figure 1. Molecular characterization of MSCs.

(**A**): RT-PCR of passage 2. Cells were negative for CD34 (haematopoietic stem cell marker) and positive for CD90, CD105 and CD166 (mesenchymal stem cell markers). (**B**): Passage 4 was analyzed with flow cytometry using antibodies against CD34, CD45, CD29 and CD90, antibodies are represented by solid black lines, grey lines represent isotype-controls. Cells were negative for CD34 and CD45 (leukocyte marker) and positive for CD29 and CD90 (mesenchymal stem cell markers).

Figure 2. Adhesion assays.

(**A**): Adhesion (Y-axis: % of 8000 initial plated MSCs) after pre-treatment (24h) of CMVE with different cytokines (10 ng/ml). (**B**): Concentration-dependent increase of MSC adhesion to CMVE after pre-treatment of CMVE with TNF-α 24h prior to adhesion assay. (**C**): Concentration-dependent increase of MSC adhesion after pre-treatment of CMVE with IL-1β 24h before adhesion assay. (**D**): Addition of TNF-α (10 ng/ml) to CMVE, MSCs or both 24h before adhesion assay increased adhesion of MSCs to CMVE. (**E**): Adhesion of MSCs to different cell types with or without pre-treatment with TNF-α (10 ng/ml) for 24h. CMVE= cardiac microvascular endothelium, AE= aortic endothelium. Mean ± SEM, *= $p<0.05$ compared to control.

Figure 3. Effect of shear stress on adhesion.

A flow chamber with a confluent CMVE monolayer on the bottom was perfused for 2h with medium containing 200 MSCs/µl. Adhesion of MSCs to CMVE decreased with increasing levels of shear stress. MSCs or CMVE (full squares or full triangles respectively) were pre-treated with TNF-α for 24h resulting in a significant upward shift of both curves. Mean ± SEM.

Figure 4. In vivo homing of MSCs.

(**A**) & (**B**): Fluorescence microscopy mosaic image of left ventricle 24h after intracavitary injection of 1.5 million control MSCs (A) and TNF-α-treated MSCs (B). Red= DiI-labeled MSCs, Blue= Hoechst-labeled nuclei, Green= RECA-labeled endothelial cells. (**C**): DiI-labeled MSCs counted in 3 low

magnification fields per rat heart (40X), 24h after intracavitary injection of 1.5 million MSCs, 6 animals per group. Mean ± SEM. (**D**): 3D-projection of a Z-stack reconstruction image of a MSC (red, arrowhead) residing in a capillary. Most MSCs were inside capillaries 24h after injection. (**E**): 3D-projection of a Z-stack reconstruction image of a MSC (red, arrowhead) outside a capillary, representative for a minor fraction of MSCs that already crossed endothelial barrier 24h after injection. Red= DiI-labeled MSCs, Blue= Hoechst-labeled nuclei, Green= RECA-labeled endothelial cells. (**F**): Fluorescence microscopy mosaic image of subendocardial myocardium, damaged by ischemia (30 min) – reperfusion injury. Injured myocytes are stained dark green (F-actin staining) and are more losely organised than intact areas. As evident from the images, there was abundant homing of MSCs (DiI, red) in the infarcted region of the subendocardial infarct. Red= DiI-labeled MSCs, Blue= Hoechst-labeled nuclei, Green= Phaloidin-labeled F-actin.

Figure 5. Blocking adhesion with antibodies.

(**A**) CMVE were treated with TNF-α (10 ng/ml) 24h prior to the adhesion assay and with monoclonal antibodies against VCAM-1 and ICAM-1 (10 µg/ml) 1h before adhesion assay. (**B**) MSCs were treated with TNF-α (10 ng/ml) 24h prior to the adhesion assay and incubated with monoclonal antibodies against VCAM-1 and ICAM-1 (10 µg/ml) 1h before adhesion. Mean ± SEM, *= $p<0.05$ compared to control, §= $p<0.05$ compared to TNF-α treated control.

Figure 6. Expression of VCAM-1.

Expression levels of VCAM-1 mRNA compared to control in CMVE and MSCs measured with Real-Time PCR and normalized to GAPDH mRNA expression levels. TNF-α (10 ng/ml) and IL-1β (10 ng/ml) were added to CMVE and MSCs for 7h. *$P<0.05$ compared to control CMVE, §$P<0.05$ compared to control MSCs.

Figure 7. Model of MSC – CMVE interactions.

Upon cardiac insult, inflammatory cytokines play a crucial role in signaling to cardiac endothelial cells and possibly to MSCs. Both cell types express more VCAM-1 after stimulation with these cytokines leading eventually to increased adhesion of MSCs in capillaries.

Tables

Table 1. PCR Primers and Probes Used in the Study

	Sense Primers	Antisense Primers	TaqMan® Probes
CD34	TGTTGCCTCGGAGAATTCTAC	GCACTCCTCGGATTCCTGAAC	
CD45	AAAGAGGAAATGGCTCCTCAG	CTATTTCTGTGCTTGTGGTGG	
CD90 (Thy-1)	CAGTCATCAGCATCACTCTCC	AAGTCCGTGGCTTGGAGGAAG	
CD105 (Endoglin)	CAGGCATCCAACACCATAGAG	AAGTTCATGGCCGATGGTTCC	
CD166 (ALCAM)	CTCGGATGGTACACTGTCAAC	TGGACACCTCTCCATCAACAG	
VCAM-1	TGTGACCTGTCAGCGAAGGA	CCCGTGTACAAGTGGTCCACTTA	CAATCCAAGTGGAGGTCTACTCATTCCCTG
VLA-4	CAAATCTTGGCGACATTGACA	CCTCGCAAGTCATCTTCTTGTG	TGGCTTTGAAGATATTGCTATTGGCGCA
GAPDH	GCCTCGTCTCATAGACAAGATGGT	GAAGGCAGCCCTGGTAACC	CGTCCGATACGGCCAAATCCGTT

Figures

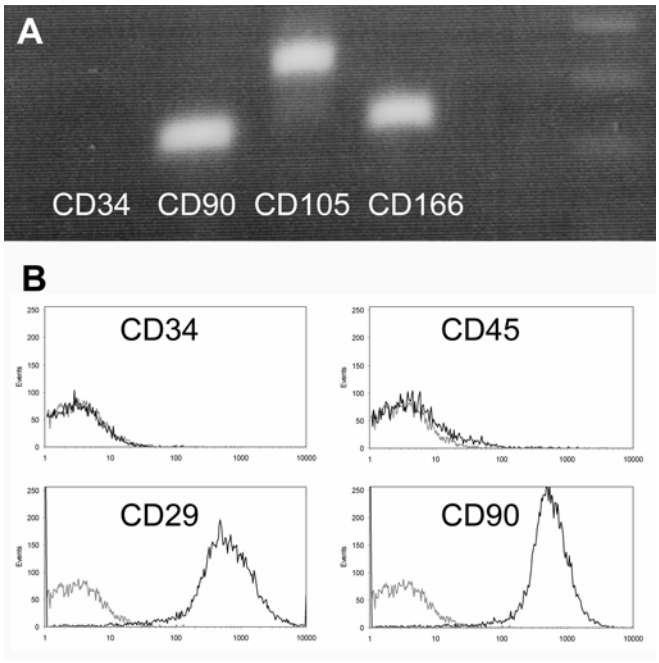

Figure 1

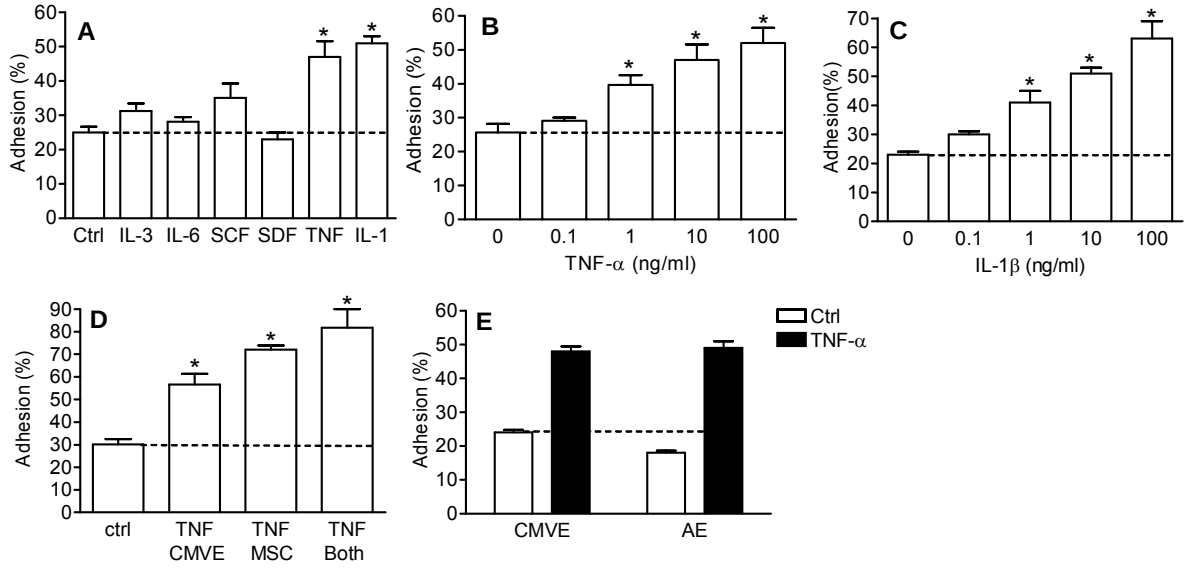
A
Adhesion (%)
Ctrl
IL-3
IL-6
SCF
SDF
TNF
IL-1
B
TNF-α (ng/ml)
C
IL-1β (ng/ml)
D
ctrl
TNF CMVE
TNF MSC
TNF Both
E
Ctrl
TNF-α
CMVE
AE

Figure 2

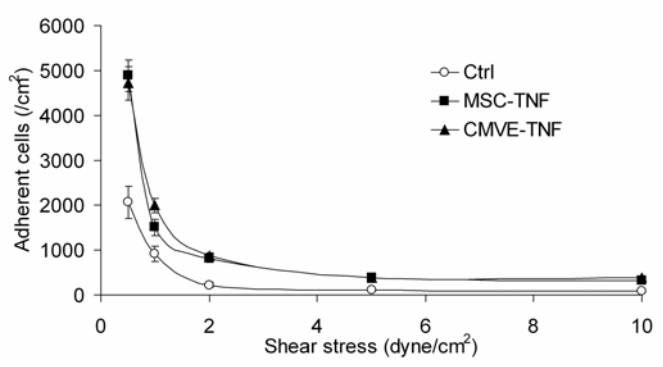
6000
5000
4000
3000
2000
1000
0
Adherent cells (/cm2)
0
2
4
6
8
10
Shear stress (dyne/cm2)
Ctrl
MSC-TNF
CMVE-TNF

Figure 3

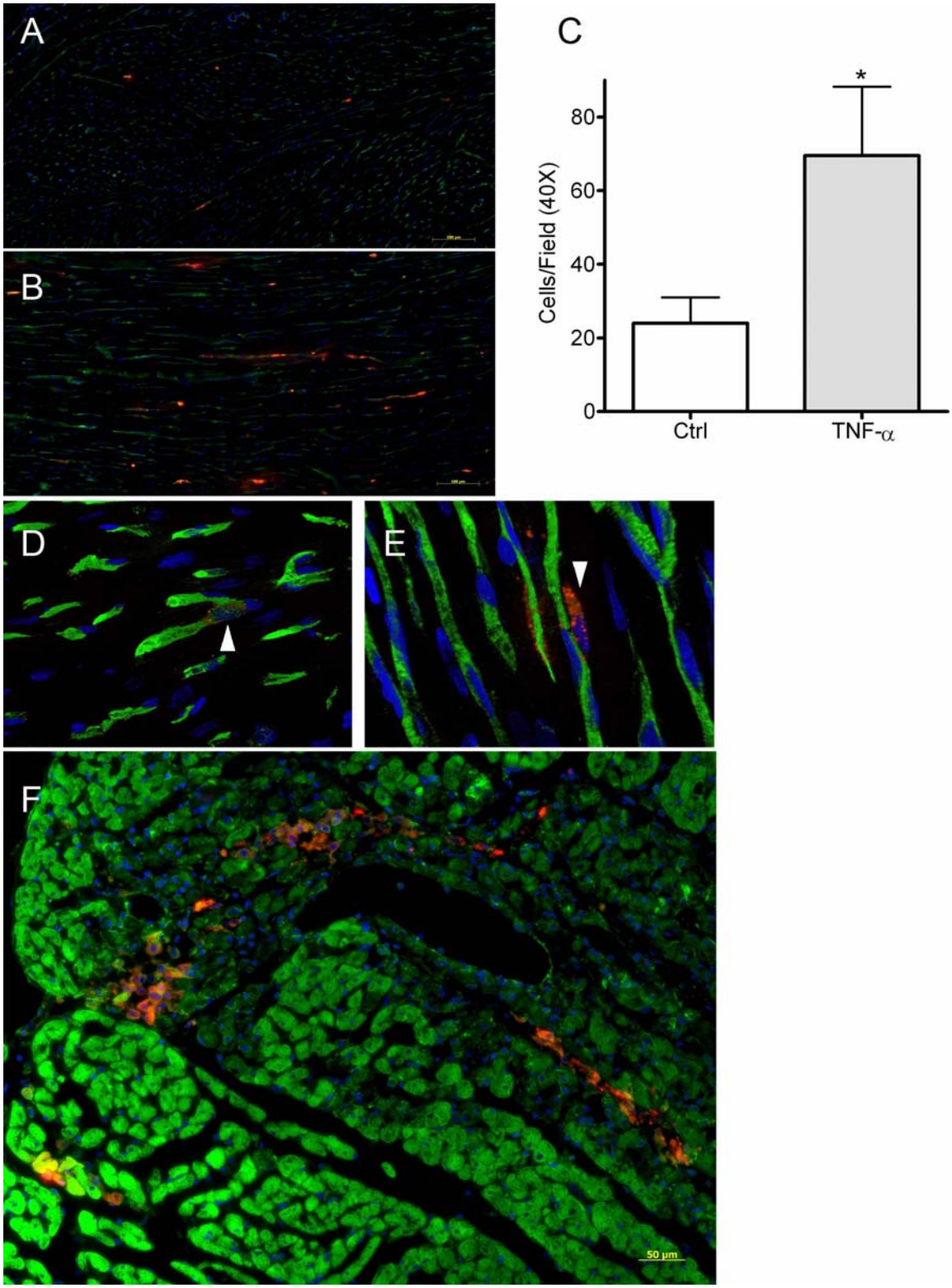

Figure 4

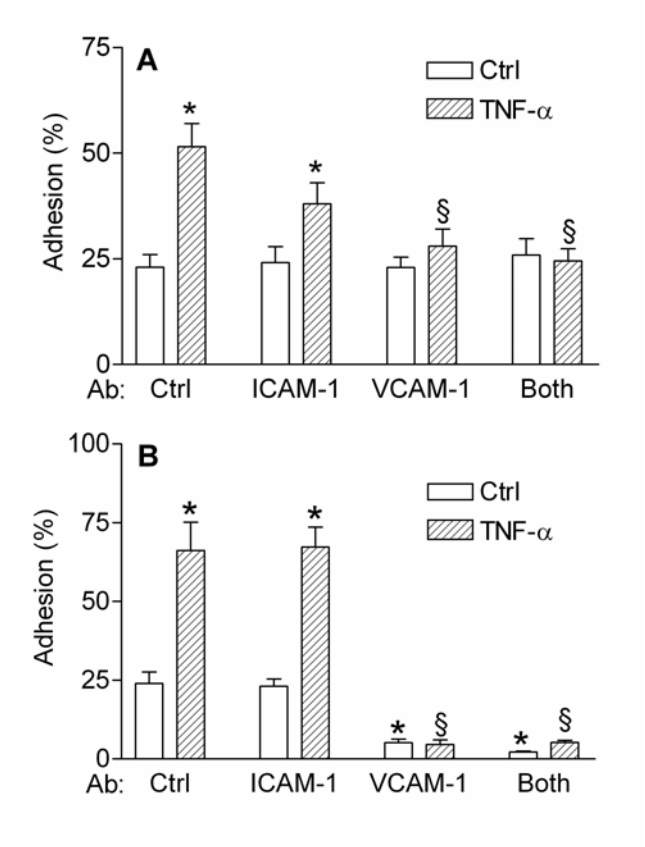
A
Ctrl
TNF-α
Adhesion (%)
75
50
25
0
*
*
§
§
Ab: Ctrl ICAM-1 VCAM-1 Both
B
Ctrl
TNF-α
Adhesion (%)
100
75
50
25
0
*
*
*
§
*
§
Ab: Ctrl ICAM-1 VCAM-1 Both

Figure 5

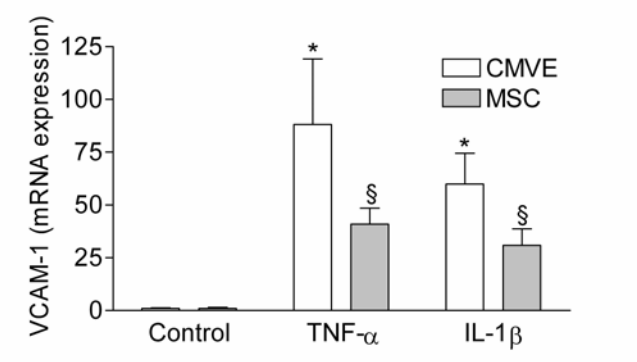
VCAM-1 (mRNA expression)
125
100
75
50
25
0
*
§
*
§
CMVE
MSC
Control
TNF-α
IL-1β

Figure 6

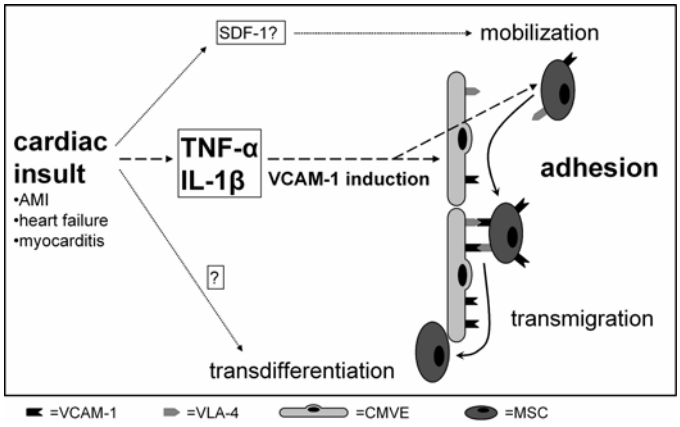
SDF-1?
mobilization
cardiac
insult
•AMI
•heart failure
•myocarditis
TNF-α
IL-1β
VCAM-1 induction
adhesion
?
transmigration
transdifferentiation
=VCAM-1
=VLA-4
=CMVE
=MSC

Figure 7