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Expression of Inflammation-related Intercellular Adhesion Molecules in Cardiomyocytes *In Vitro* and Modulation by Pro-inflammatory Agents

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Abstract. *Background: Cell-surface adhesion molecules regulate multiple intercellular and intracellular processes and play important roles in inflammation by facilitating leukocyte endothelial transmigration. Whether cardiomyocytes express surface-adhesion molecules related to inflammation and the effect of pro-inflammatory mediators remain unknown. Materials and Methods: In the present study, the expression of different cell-adhesion molecules (CD11a, CD11b, CD31, CD62P, CD162, F11 receptor and mucosal vascular addressin cell adhesion molecule 1 (MADCAM1)) and the effect of pro-inflammatory mediators were investigated in an in vitro model of human cardiomyocytes. Cells were supplied as a primary culture of cardiac alpha actin-positive cells from human heart tissue. The cells were incubated for 24 h with 1 U/ml thrombin or 700 ng/ml lipopolysaccharide (LPS) or with a combination of both. The expression of the cell adhesion molecules was measured by flow cytometry. Results. In cultured human cardiomyocytes, 22.8% of cells expressed CD31, 7.1% MADCAM1 and 2.6% F11R. CD11a, CD11b, CD62P and CD162 were expressed by fewer than 2% of the cells at baseline. CD31 expression increased on incubation of cardiomyocytes with thrombin by 26% ($p<0.05$) and with LPS by 26% ($p=0.06$). The combination of thrombin and LPS did not result in increased levels of CD31 ($p>0.10$). The pro-inflammatory agents LPS and thrombin had no effect on the expression of MADCAM1 and F11R. Conclusion: Inflammation-related cell-adhesion molecules CD31, MADCAM1 and F11R were shown to be expressed on the surface of human cardiomyocytes in an in vitro model. Incubation with LPS or thrombin resulted in increased expression of CD31, however, it did not modify the expression of the cell adhesion molecules MADCAM1 and F11R.*

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Coronary artery disease remains the leading cause of mortality in developed countries despite recent progress in primary and secondary prevention and therapies (1). In patients with coronary artery disease, various mediators of inflammation, fibrinolysis and coagulation are up-regulated (2, 3).

Important mediators of inflammation are lipopolysaccharides (LPS) and thrombin. LPS are able to activate the innate immune system, and thrombin plays vital roles within the coagulation cascade. In addition, thrombin can amplify inflammation induced by other stimuli, either through ischemia, indirectly through generation of downstream mediators such as activated protein C, or directly *via* signals through protease activated receptors (4, 5). Mammalian hearts use both innate and adaptive immunity to respond to inflammation. Macrophages, endothelial cells and sparse populations of dendritic cells reside in the heart. Mast cells, a small number of B-cells and regulatory T-cell subsets are found in resting cardiac tissue (2, 3). Inflammation leads to interaction between cardiac and immune cells in the myocardium. Intercellular adhesion molecules (ICAM) are the key elements of interaction between these cells.

The role of ICAM in the myocardium has been studied to some degree. *In vitro* and *in vivo* studies revealed that in the

case of myocardial inflammation within ischemia, CD40 ligand (CD40L or CD154) on platelets is up-regulated, which led to high coagulalory activation (6).

Whether cardiomyocytes express surface adhesion molecules related to inflammation remains unknown. The aim of the current study was to investigate the expression of selected cell-surface adhesion molecules, including CD11a, CD11b, CD31, CD 62P, CD162, F11 receptor and mucosal vascular addressin cell adhesion molecule 1 (MADCAM1) by *in vitro* culture of human cardiomyocytes. In addition, modifying effects of pro-inflammatory mediators such as LPS and thrombin on the expression of these surface-adhesion molecules was tested.

Materials and Methods

Cultivation of human cardiomyocytes, incubation with inflammation reagents and flow cytometry. Human cardiomyocytes from ventricles of adult heart (PromoCell GmbH, Heidelberg, Germany) were prepared as described by PromoCell Cryopreserved cells were thawed at 37˚C and transferred to 75 cm^2 cell culture vessel (Becton, Dickinson and Company, Heidelberg, Germany) containing the pre-warmed medium [20 ml myocyte growth medium (PromoCell GmbH, Heidelberg, Germany) with 1% penicillin/streptomycin and 0.5% amphotericin (Life Technologies Corp., Darmstadt, Germany)]. The cell vessel was placed in an incubator (37˚C, 5% CO_2). The cell medium was replaced after 24 h and then every two days once cells had reached 80-90% confluency (Figure 1).The medium was then aspirated and the cells were washed twice with 20 ml phosphate-buffered saline (PBS; Life Technologies Corp.). After aspirating the PBS, about 10 ml 0.05% trypsin (Life Technologies Corp.) at room temperature was added then 90 seconds later, 10 ml of pre-warmed medium was added. The trypsin-medium solution was then aspirated carefully and transferred to a centrifugation tube and centrifuged for 3 min at 220 ×g. The supernatant was discarded and after adding 10 ml of pre-warmed medium, the cells were pipetted up and down. Sixty milliliters of medium was then added to the cell medium suspension and mixed carefully before adding the cells to a 6-well plate (Becton, Dickinson and Company), at 3 ml per well. After the cells reached confluency, they were cultured for 2 weeks. After this time, experiments with different inflammatory agents were carried out.

The contents of each well were suspended suspended with 1 ml medium containing 1 U/ml thrombin (Merck Millipore, Schwalbach, Germany), 700 ng/ml LPS (Sigma-Aldrich, Munich, Germany) or LPS plus thrombin (1 U/ml thrombin plus 700 ng/ml LPS), or only medium as control. After incubation for 24 h, the cells were washed using PBS and detached with 0.05% trypsin. Eighty microliters of each sample was stained for 30 minutes at room temperature with 10 μl aliquots of mouse antibodies to CD11a, CD11b, CD31, CD62P, CD162, F11R and MADCAM1 (Becton, Dickinson and Company). Corresponding isotypes (Becton, Dickinson and Company) were used as a control. Flow cytometric analysis of cell-surface molecules was carried out using a FACSCalibur and FACSCanto II (Becton, Dickinson and Company) equipped with an argon ion laser tuned at 488 nm. WinMDI 2.9 software, BD FACSDiva and CellQuest software were used for data acquisition and evaluation. The percentage of positively stained cells was defined as the percentage of the population falling above the 99th percentile of an isotype-matched antibody control cell population. The presence of adhesion molecules on cardiomyocytes was defined when more than 2% of the cells were positive for the tested surface molecule.

Statistical analysis. The measurements of flow cytometry were performed in duplicate from the same well (technical replicates) and were repeated with cells from five different experiments (biological replicates). The mean±SD therefore indicate biological replicates. All calculations were performed using SAS release 9.2 (SAS institute Inc., Cary, NC, USA). Student's *t*-test was applied as a parametric test. A value of $p \leq 0.05$ (two-tailed) was considered significant and $0.05 < p < 0.10$ (two-tailed) to indicate tendency.

Results

After the cells reached confluency (Figure 1A), they were cultured for 2 weeks before flow cytometry analysis. The cells were characterized by histochemistry as positive for cardiac alpha actin (Figure 1B). As shown by flow cytometry, 22.8% of cells expressed CD31 and 7.1% expressed MadCam1 at baseline. F11R was only expressed by 2.6% of the cells. CD11a, CD 11b, CD62P and CD162 were expressed by fewer than 2% of all cells and therefore, according to our definition, were not detectable on the surface of cardiomyocytes. Figure 2 illustrates the expression of CD31 compared to the isotype control. It shows that the fluorescence intensity of CD31-positive cells was higher than that of the isotype control ($p<0.05$).

Cardiomyocytes were stimulated with thrombin, LPS, or the combination of both for 24 h, control cells were not stimulated. The expression of cell-adhesion molecules was evaluated 24 hours after incubation by flow cytometry. The surface expression of CD31 (Figure 2) increased significantly by 26% ($p<0.05$) due to stimulation with thrombin and by 26% after stimulation with LPS ($p=0.06$) (Figure 3). However, the expression of CD31 after incubation with the combination of thrombin and LPS was not significantly changed ($p>0.10$).

The cell-surface expression of F11R and MADCAM1 did not change, neither by adding thrombin or LPS, nor by combined treatment with thrombin and LPS (Figures 4 and 5).

Discussion

The pathogenesis of atherosclerosis is multifactorial, but the initiating process considered to be the most essential is the deposition and oxidation of lipids in the vascular wall, with the subsequent generation of a variety of a dysfunctional endothelium (2, 7). Immune cells dominate early atherosclerotic lesions, their effector molecules accelerate progression of the lesions, and activation of inflammation can elicit acute coronary syndromes. The inflammatory process in the atherosclerotic artery may lead to increased

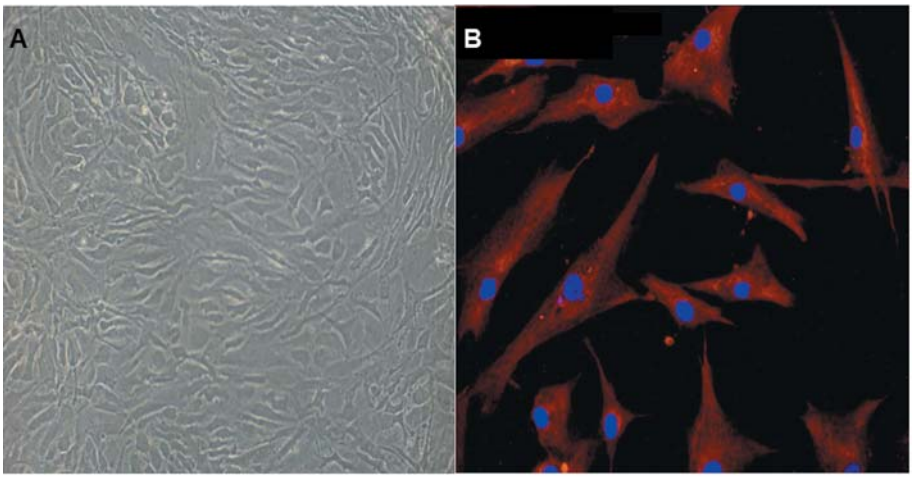

Figure 1. *A: Confluent human cardiomyocytes (×10). B: Staining of cardiomyocytes by cardiac alpha actin (red) (×20).*

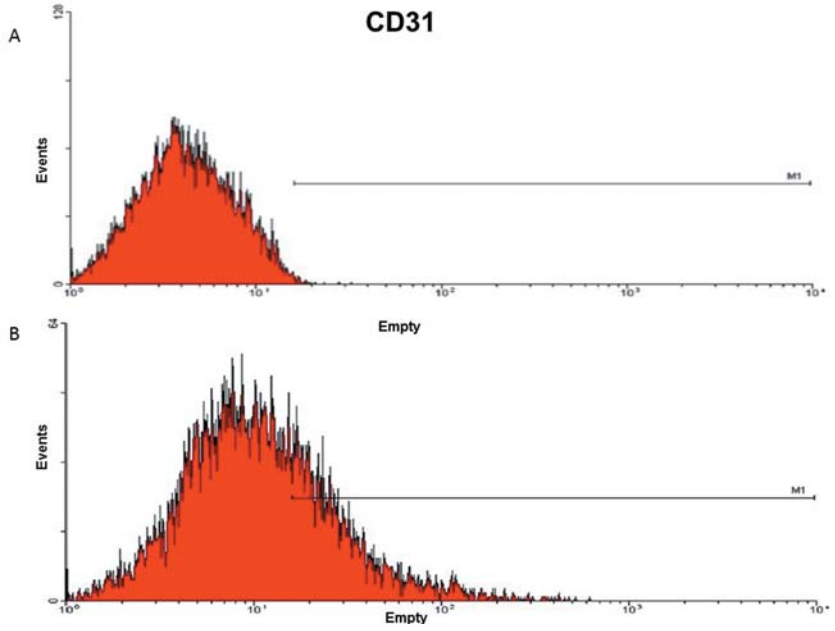

Figure 2. *Histogram of isotype-control (A) and of CD31 (B) of human cardiomyocytes. 22.8% of cultured human cardiomyocytes expressed CD31.*

blood levels of inflammatory cytokines and other acute-phase reactants (C-reactive protein, fibrinogen, interleukin-7, interleukin-8, soluble CD40 ligand, and the C-reactive protein-related protein pentraxin) (4-7). Experimental studies confirm that inflammatory mediators such as LPS and thrombin influence the metabolism and electrophysiological pathway of cardiac cells in cardiovascular disorders (3, 4). Dysfunctional endothelium attracts circulating inflammatory cells and cytokines through ICAMs (immunoglobulin gene family, selectin family and the integrin family) (8, 9). Such inflamed endothelium interacts with platelets and cardiomyocytes, but the pathways involved have been less well characterized.

In the present study, we show, to our knowledge for the first time, that human cardiomyocytes in an *in vitro* model express CD31. CD31 expressed on most leukocyte subtypes, platelets, and on endothelial cells has been described as an ICAM protein which influences intercellular junctions and

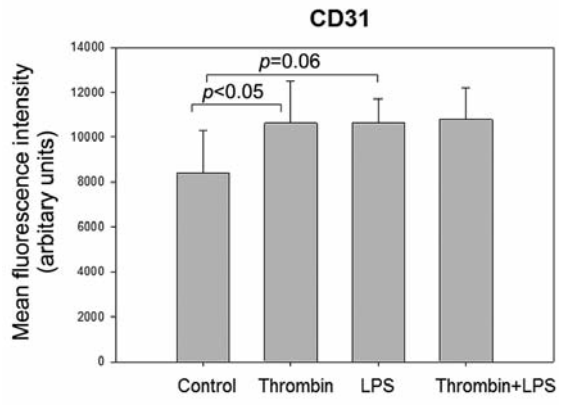

Figure 3. *Mean fluorescence intensity of CD31 before and after stimulation with pro-inflammatory agents. CD31 expression increased by incubating human cardiomyocytes with thrombin and with LPS. The combination of thrombin and LPS did not increase the CD31 level.*

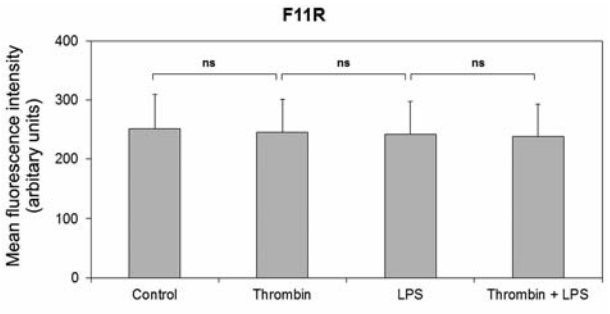

Figure 4. *Mean fluorescence intensity of F11R before and after stimulation of human cardiomyocytes with pro-inflammatory agents. LPS, thrombin, and the combination of thrombin and LPS did not increase the level of F11R compared to the baseline. ns: p>0.05.*

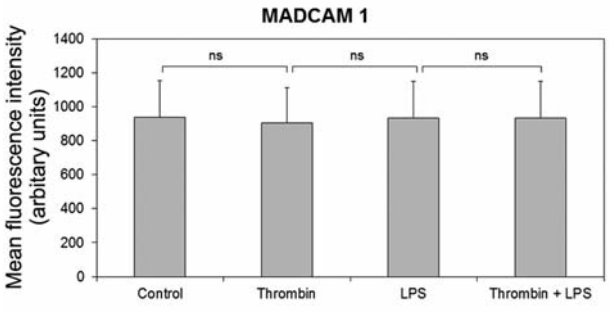

Figure 5. *Mean fluorescence intensity of MADCAM1 before and after stimulation of human cardiomyocytes with pro-inflammatory agents. LPS, thrombin, and the combination of thrombin and LPS did not affect the expression of MADCAM1 by cardiomyocytes. ns: p>0.05.*

platelet function (8, 9). Furthermore, after incubating the cardiomyocytes with pro-inflammatory mediators, CD31 was expressed significantly more compared to baseline. Our findings are in accordance with a previous study on the expression of CD31 on the platelet surface in inflammation such as myocardial infarction (10). A significant decrease in platelet CD31 expression was observed 3 hours after thrombolysis compared with baseline. The current evidence supports the association between certain polymorphisms in the *CD31* gene and elevated incidence of atherosclerosis, coronary artery disease, and myocardial infarction (11-13). Exploring the role of CD31 in cardiomyocytes should be the goal of further studies in families with a positive history of coronary artery disease.

Furthermore, we also revealed for the first time that F11R is expressed on the surface of human cardiomyocytes in an *in vitro* model. F11R is a member of the immunoglobulin superfamily found on the surface of human platelets, and was determined as playing a role in platelet aggregation, secretion, adhesion and spreading (14). However, the role of F11R in the heart is still unknown. Previous studies of the interactions between human platelets and endothelial cells suggested that F11R plays a crucial role in inflammatory thrombosis and atherosclerosis (14, 15). Significantly higher levels of plasma F11R are found in patients with more advanced coronary artery disease (16). It has been reported that pro-inflammatory agents increase the endothelial expression of F11R. However, in the present study, we did notfind that that pro-inflammatory agents modify the expression of F11R.

Finally, we revealed that *in vitro*, cardiomyocytes express MADCAM1. MADCAM1 is also found on the surface of endothelial cells. It induces the interaction between endothelial cells and leucocytes (17). It was demonstrated by (18) Connor *et al*. that stimulating endothelial cells with pro-inflammatory agents elevated the expression of MADCAM1. However, in our study with cardiomyocytes, we showed that adding thrombin, LPS or a combination of these did not significantly change the expression of MADCAM1. The multiple functional roles and associations of CD31, F11R and MADCAM1 in atherosclerosis encourage further investigation into the mechanisms that regulate the expression and function of these molecules in the pathogenesis of atherosclerosis and highlight the potential benefits of targeting ICAM for development of novel anti-atherosclerotic therapies. Conclusion

This is the first study showing the expression of CD31, F11R and MADCAM1 on the surface of human cardiomyocytes in an *in vitro* model. These intriguing findings may trigger further studies to investigate the role of these surface adhesion molecules and the interaction between inflammatory cells and cardiomyocytes. The possible role of

these cell-adhesion molecules in the pathogenesis of coronary artery disease in families with a positive history of coronary artery disease should also be studied.

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References

1 Danesh J, Whincup P, Walker M, Lennon L, Thomson A, Appleby P, Gallimore JR and Pepys MB: Low-grade inflammation and coronary heart disease: prospective study and updated meta-analyses. Br Med J *321*: 199-204, 2000.

2 Briasoulis A, Androulakis E, Christophides T and Tousoulis D: The role of inflammation and cell death in the pathogenesis, progression and treatment of heart failure. Heart Fail Rev, 2016. [Epub ahead of print]

3 Safranow K, Dziedziejko V, Rzeuski R, Czyzycka E, Bukowska H, Wojtarowicz A, Binczak-Kuleta A, Jakubowska K, Olszewska M, Ciechanowicz A, Kornacewicz-Jach Z, Machalinski B, Pawlik A and Chlubek D: Inflammation markers are associated with metabolic syndrome and ventricular arrhythmia in patients with coronary artery disease. Postepy Hig Med Dosw (Online) *70*: 56-66, 2016.

4 Elmas E, Lang S, Dempfle CE, Kaelsch T, Hannak D, Sueselbeck T, Wolpert C, Borggrefe M and Brueckmann M: High plasma levels of tissue inhibitor of metalloproteinase-1 (TIMP1) and interleukin-8 (IL8) characterize patients prone to ventricular fibrillation complicating myocardial infarction. Clin Chem Lab Med *45*: 1360-1365, 2007.

5 Sparkenbaugh EM, Chantrathammachart P, Chandarajoti K, Mackman N, Key NS and Pawlinski R: Thrombin-independent contribution of tissue factor to inflammation and cardiac hypertrophy in a mouse model of sickle cell disease. Blood, 2016. [Epub ahead of print]

6 Kalsch T, Nguyen XD, Elmas E, Grebert N, Suselbeck T, Kluter H, Borggrefe M, and Dempfle CE: Coagulation activation and expression of CD40 ligand on platelets upon *in vitro* lipopolysaccharide-challenge in patients with unstable angina. Int J Cardiol *111*: 217-223, 2006.

7 Karshovska E, Zhao Z, Blanchet X, Schmitt MM, Bidzhekov K, Soehnlein O, von Hundelshausen P, Mattheij NJ, Cosemans JM, Megens RT, Koeppel TA, Schober A, Hackeng TM, Weber C and Koenen RR: Hyperreactivity of junctional adhesion molecule A-deficient platelets accelerates atherosclerosis in hyperlipidemic mice. Circ Res *116*: 587-599, 2015.

8 Krieglstein CF and Granger DN: Adhesion molecules and their role in vascular disease. Am J Hypertens *14*: 44S-54S, 2001.

9 Parker C, 3rd, Vita JA, and Freedman JE: Soluble adhesion molecules and unstable coronary artery disease. Atherosclerosis *156*: 417-424, 2001.

10 Serebruany VL and Gurbel PA: Effect of thrombolytic therapy on platelet expression and plasma concentration of PECAM1 (CD31) in patients with acute myocardial infarction. Arterioscl Throm Vas *19*: 153-158, 1999.

11 Elrayess MA, Webb KE, Bellingan GJ, Whittall RA, Kabir J, Hawe E, Syvanne M, Taskinen MR, Frick MH, Nieminen MS, Kesaniemi YA, Pasternack A, Miller GJ and Humphries SE: R643G polymorphism in *PECAM1* influences transendothelial migration of monocytes and is associated with progression of CHD and CHD events. Atherosclerosis *177*: 127-135, 2004.

12 Elrayess MA, Webb KE, Flavell DM, Syvanne M, Taskinen MR, Frick MH, Nieminen MS, Kesaniemi YA, Pasternack A, Jukema JW, Kastelein JJ, Zwinderman AH and Humphries SE: A novel functional polymorphism in the PECAM1 gene (53G>A) is associated with progression of atherosclerosis in the LOCAT and REGRESS studies. Atherosclerosis *168*: 131-138, 2003.

13 Fang L, Wei H, Chowdhury SH, Gong N, Song J, Heng CK, Sethi S, Koh TH and Chatterjee S: Association of Leu125Val polymorphism of platelet endothelial cell adhesion molecule-1 (*PECAM1*) gene and soluble level of PECAM1 with coronary artery disease in Asian Indians. Indian J Med Res *121*: 92-99, 2005.

14 Fang TJ, Lin CH, Lin YZ, Li RN, Ou TT, Wu CC, Tsai WC and Yen JH: F11R mRNA expression and promoter polymorphisms in patients with rheumatoid arthritis. Int J Rheum Dis, 2015. [Epub ahead of print]

15 Babinska A, Kedees MH, Athar H, Ahmed T, Batuman O, Ehrlich YH, Hussain MM and Kornecki E: F11-receptor (F11R/JAM) mediates platelet adhesion to endothelial cells: role in inflammatory thrombosis. Thromb Haemost *88*: 843-850, 2002.

16 Cavusoglu E, Kornecki E, Sobocka MB, Babinska A, Ehrlich YH, Chopra V, Yanamadala S, Ruwende C, Salifu MO, Clark LT, Eng C, Pinsky DJ and Marmur JD: Association of plasma levels of F11 receptor/junctional adhesion molecule-A (F11R/JAM-A) with human atherosclerosis. J Am Coll Cardiol *50*: 1768-1776, 2007.

17 Sakai Y and Kobayashi M: Lymphocyte 'homing' and chronic inflammation. Pathol Int *65*: 344-354, 2015.

18 Connor EM, Eppihimer MJ, Morise Z, Granger DN and Grisham MB: Expression of mucosal addressin cell adhesion molecule-1 (MADCAM1) in acute and chronic inflammation. J Leukocyte Biol *65*: 349-355, 1999

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